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Review on Structural, Spectroscopic and Bio-functional Studies of some Cu complex have Catechol oxidase Activity

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ABSTRACT:

Catecholase is an enzyme that catalyzes the oxidation of catechols to ortho-quinones; a reaction with great importance in different biological processes such as melanin biosynthesis and plant secondary defense mechanisms. This was ascribed to the type-3 active site of the enzyme, which contains a di-copper center that mediates this activity. It contains two Cu ions coordinated by one histidine and two tyrosine residues, providing a conducive environment for the hydroxylation of catechols in both packaged electron-transfer reactions (types-1 & -3 sites). This review provides a concise overview of the mechanistic aspects of catecholase activity within this specific active site. Its electronic configuration of type-3 copper center and interaction with the catechol substrates are important for its high catalytic efficiency, substrate specificity. Newer studies have suggested that the copper ions are necessary to stabilize the transition state and aid in promoting redox chemistry involved with oxidation, respectively. A complete characterization of the catecholase activity provides novel insight into the enzymatic mechanisms as well and has implications for designing biomimetic catalysts or developing future therapeutic targeting copper-dependent enzyme pathways.

Keywords: Catecholase Activity, Transition Metal, Copper Enzyme, Structure–Function Correlation, Turnover Number.

1. INTRODUCTION

Metalloenzymes are those enzymes which have metal(s) in their active sites. The core of these enzymes contains transition metals like Cu^{II}, Ni^{II}, Zn^{II}, Fe^{II} [1]. There may present more than one core in their active sites. Half of the known enzymes are metallo enzymes & most of them contains copper in their active sites. Copper is the essential trace elements in living system. It is key factor in the diverse array of biological redox reaction. Also, copper is easy access in nature, highly active, low toxic and active in very mild conditions. Catecholase activity is a biological process in which type-3 active site [2,3] (Figure 1) of a copper enzyme, named catechol oxidase, catalyzes oxidation of o-diphenolic substrates to the corresponding o-quinones coupled with reduction of molecular dioxygen into water.

Catalytic activity [4,5] of coordination compounds is a fascinating area of research. Catecholase activity is one of such activity. Catechol oxidase are ubiquitous plant enzymes belonging to the oxidoreductase class. They contain a dinuclear copper center in their active site that catalyzes the oxidation of a wide range of o-diphenols (catechols) to the corresponding o-quinones coupled with $2e/2H^+$ reduction of O_2 to H_2O, in a process known as catecholase activity. The structure determination of catecholase oxidase in 1998 has encouraged an extensive investigation on model compounds to understand the structure-property relationship. As the structure contains dicopper moiety, several dicopper complexes derived from nitrogen containing dinucleating ligands have been mainly employed for this purpose. While the metallo-enzyme contains hydroxo-bridged dicopper(II), activity has been observed for dicopper(II) systems having hydroxo or various other bridging moieties. Several monocopper(II) complexes are also known to exhibit the activity. Recently, catecholase activity of complexes of other metal ions, manganese, cobalt for example, has also been observed. Moreover, few structure-property correlations that determined are not sufficiently wide but only applicable for a set of new compounds. Recently, catecholase activity by copper clustures and polymers has been reported. All in all, the problem of modelling the catecholase activity remains still open and therefore catecholase activity of new systems should be explored to get better insight and more straightforward structure-property correlations. It includes presentations of some of the proposed mechanisms (Scheme 2 & 3) along with structural descriptions of the relevant catalysts and an attempt to establish a structure–function correlation. In the summary, this review may provide inspiration for the development of new catalysts and for a more effective approach to draw structure–activity correlations.

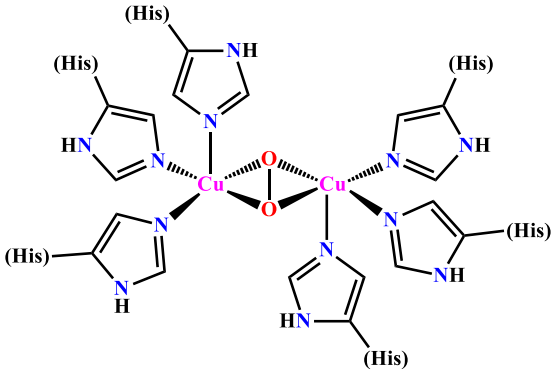
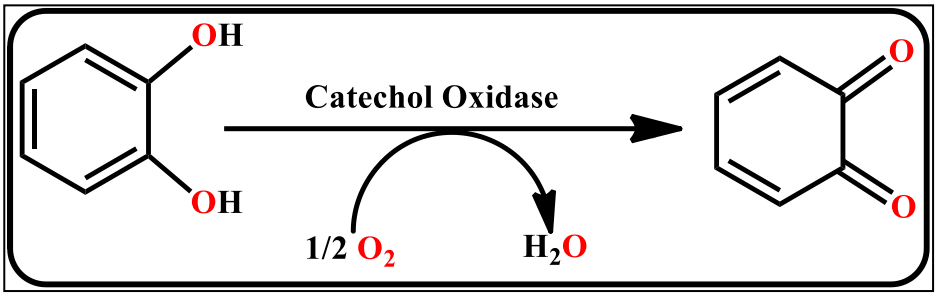


Figure 1. Schematic representations of selected active type-3sites (oxyhemocyanin [3,4]) of copper proteins.

2. CATECHOL OXIDASE ACTIVITY

2.1. Defination.

The ability of copper containing metallo-enzymes to reversibly bind and activate molecular oxygen plays vital role in a number of biological catalysis [6,7]. Therefore, many research groups have been inspired to study the structural, spectroscopic and catalytic properties of the metallo-enzymes as well as of the model compounds. A well-known representative of this family of metallo-enzyme is catechol oxidase. It is a type-3 copper enzyme usually encountered in plant tissues and in some insects and crustaceans. It catalyses the conversion of large number of *o*-diphenols (such as cafferic acid and its derivatives) into the respective *o*-benzoquinones in a process known as catechol oxidase activity or catecholase activity (Scheme 1). The later highly reactive compound subsequently auto-polymerizes, resulting in the formation of melanin, a dark pigment thought to protect a damaged tissue from pathogens [7a].



Scheme 1. Oxidation catalyzed by catechol oxidase.

2.2 Structure of Catechol Oxidase.

In 1998, Krebs and co-authors [7e] 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. 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.

In the native *met* state, the 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 catechol oxidases from *Lycopus europaeus* and *Ipomoea batatas*, confirming the presence of four N/O donor atoms and a Cu^{II}–Cu^{II} distance of 2.9 Å in solution for both enzymes [8,9]. The apical positions are occupied by the His 109 and His 240 residues for CuA and CuB, respectively (Figure 2, left). EPR data reveal a strong antiferromagnetic coupling between the copper ions, in agreement with a solvent molecule bridging two metal centers, as found in the crystal structure.

Upon reduction of the copper(II) ions to the +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 [7e]. 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.
In the adduct of catechol oxidase with the inhibitor phenylthiourea, the later binds to catechol oxidase by replacing the hydroxo bridge present in the *met* form increasing the distance between the two metal centers to 4.2 Å.

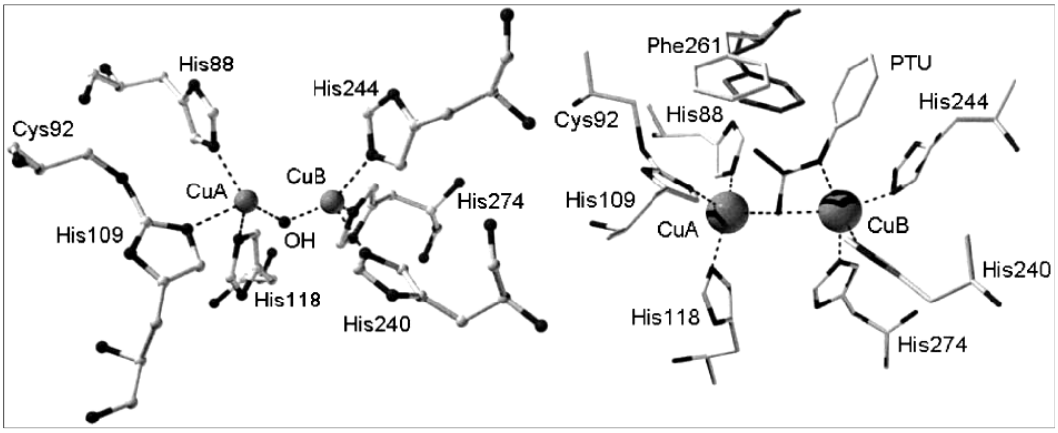


Figure 2. Left: coordination sphere of the dinuclear copper(II) center of catechol oxidase in the *met* state. Right: crystal structure of the inhibitor complex of catechol oxidase with phenylthiourea. Phe 261 is shown additionally in the orientation of the native Catechol oxidase (in dark) to show the rotation of Phe 261 in the inhibitor complex (in light color) [10].

It is evident that catechol in some way has to be coordinated with one or both copper centers during catalysis. But the coordination mode and also the mechanism of activity are complicated basically due to several possible modes of coordination, which are also function of the oxidation state(s) of the metal centers. The possible binding modes of catechol with one or two copper centers are demonstrated in Chart 1 [7a].

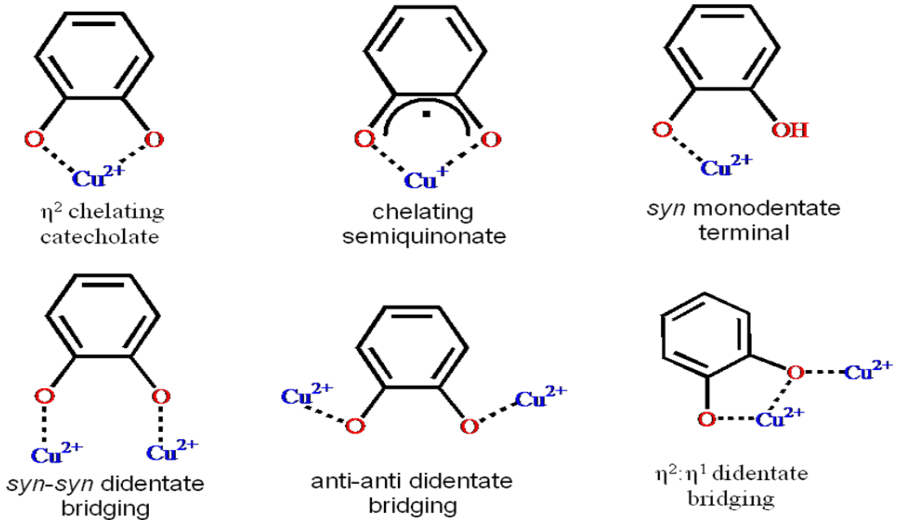


Chart 1. Possible binding modes of the catechol substrate to copper center(s).

3. THE PROTOCOL OF STUDY

The catecholase activities of various model complexes have been analyzed by UV–vis absorption spectroscopy. The experiment is conducted via two steps: the first step is spectral investigation to confirm the catalytic behavior and the second step is kinetic study for determining the rate constant to understand the catalytic efficiency. DTBC is used commonly as substrate for this experiment because of advantages like low quinone-catechol reduction potential, stability of the product DTBQ, etc. DTBQ shows maximum absorbance at 400 nm which makes it easily identifiable. DTBQ is soluble in common solvents such as MeCN, MeOH, DMF, and DMSO. The molar extinction coefficient value is quite high. Besides, the presence of two bulky tertiary butyl groups on the ring prevents over oxidation and ring opening [11]. The general protocol of the experiment is briefly described below.

3.1. Spectral investigation

A particular concentration of the complex (preferably in the order of 104 M) is prepared in an appropriate solvent (acetonitrile/DMF/DMSO/methanol) and is treated with 100 times more concentrated solution of the substrate in the same solvent. The mixture is subjected to UV–vis spectra at a particular temperature (preferably room temperature) under aerobic condition for 60–90 min (saturation time). The activity can be monitored by appearance and gradual increase of intensity of the corresponding quinone band at 400 nm, indicating formation of quinone with time. However, depending upon the solvent and the complex used, the quinone absorption maxima may show a slight red shift and even sometimes a blue shift. The spectral change recorded can easily be compared with the blank experiment (obtained for the substrate without addition of complex) where no change is found even after 3 h of observation. A representative figure is shown in Figure 3.

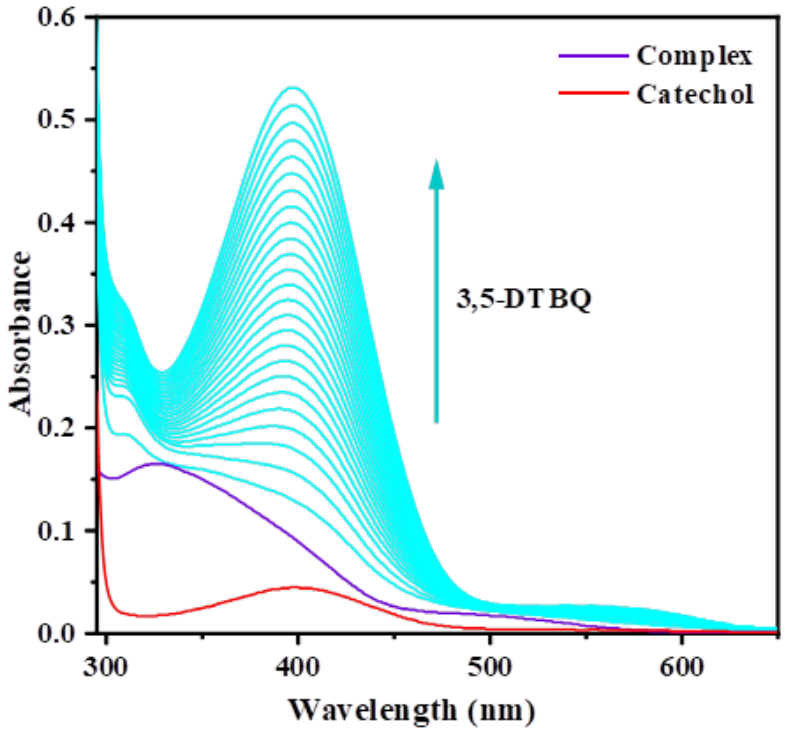


Figure 3. A representative look of the time dependent UV-vis spectral scan of the complex DTBC mixture.

3.2. Kinetic study

Kinetic study was done by time dependent UV–vis spectral scan. The study is performed with mixture of the same concentration of the complex solution and 10- to 100-fold concentration of the substrate solution under the same experimental condition as followed in the previous step. The rate constant versus concentration plot is determined from the optical density versus time plot by initial method (a representative figure is shown in Figure 4).

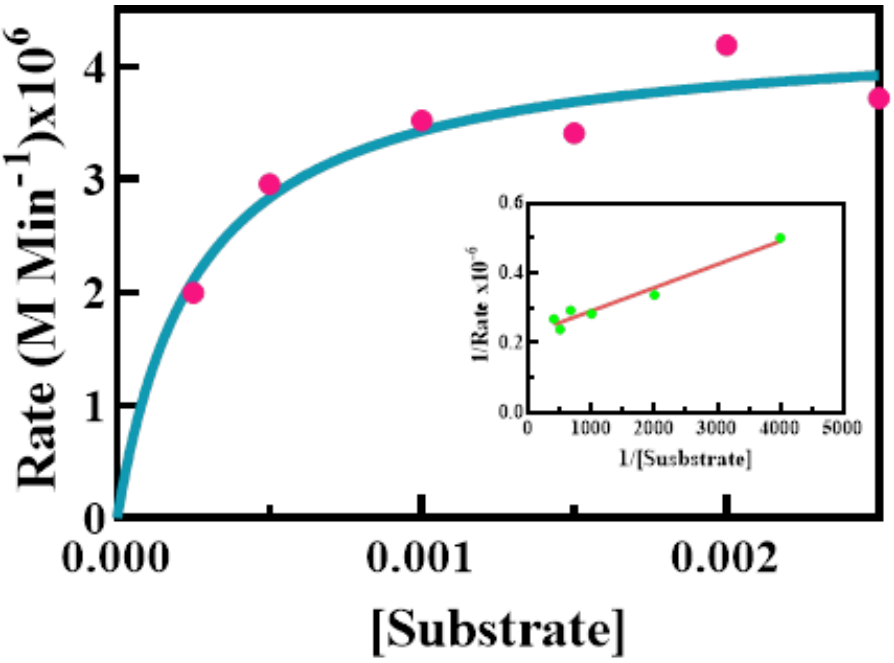


Figure 4. A representative look of the initial rate versus [substrate] (like, DTBC) plot with Lineweaver-Burk plot (1/rate vs. 1/[substrate]) in the inset.

The kinetic parameters like V_{max} (maximum velocity), k_M (Michaelis–Menten constant) and k_{cat} (turnover number) have been determined from Lineweaver–Burk plot on the basis of Michalelis–Menten approach following equation (1).

$$\frac{1}{V} = \frac{k_M}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

Liberation of hydrogen peroxide or water as the byproduct has been found in course of catecholic oxidation as shown in equations (2) and (3), respectively.

$$DTBC + O_2 \longrightarrow DTBQ + H_2O_2 \quad (2)$$

$$DTBC + 0.5\ O_2 \longrightarrow DTBQ + H_2O \quad (3)$$

The stoichiometry between DTBQ and the consumed oxygen can be studied by the method proposed by Ackermann et al. [12].

Formation of H_2O_2 can be detected by I_3^- assay where in acidic medium the extracted aqueous layer of the reaction mixture is able to oxidize KI solution to iodine. In other words, it confirms the presence of H_2O_2 as per the following reaction (Equation 4).

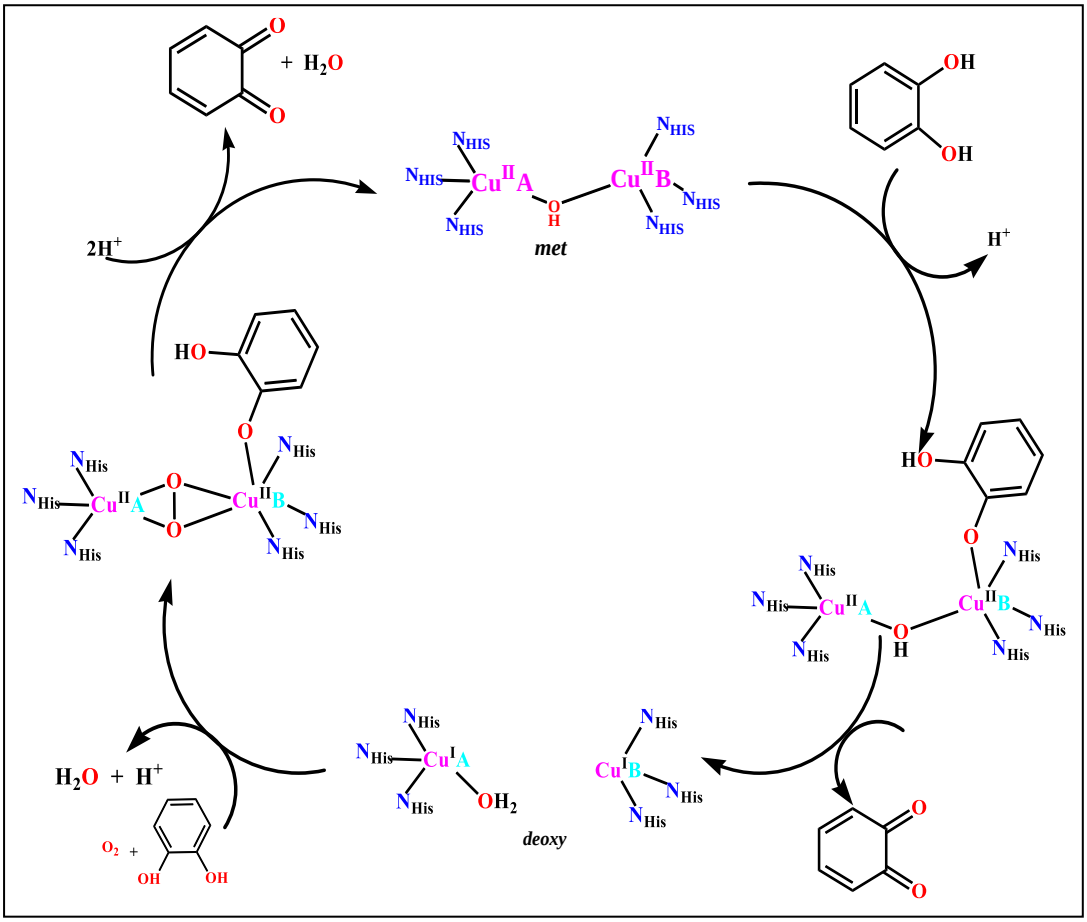
$$H_2O_2 + 3I^- + 2H^+ \longrightarrow 2H_2O + I_3^- \quad (4)$$

Generated I_3^- finds absorption maxima at ~353 nm with ε value 26000 $M^{-1}cm^{-1}$. With increase of acidity, the I_3^- band shows a gradual increase which can be monitored by UV-vis spectroscopy [13]. Hence, one electron reduction to water and two electron reductions to hydrogen peroxide, either is possible in course of the catalytic cycle. For most of the model

complexes, instead of water, hydrogen peroxide is formed as the byproduct. In fact, this process may provide a better alternative for production of hydrogen peroxide in industry than the old method using costly palladium catalyst and anthraquinone source [14].

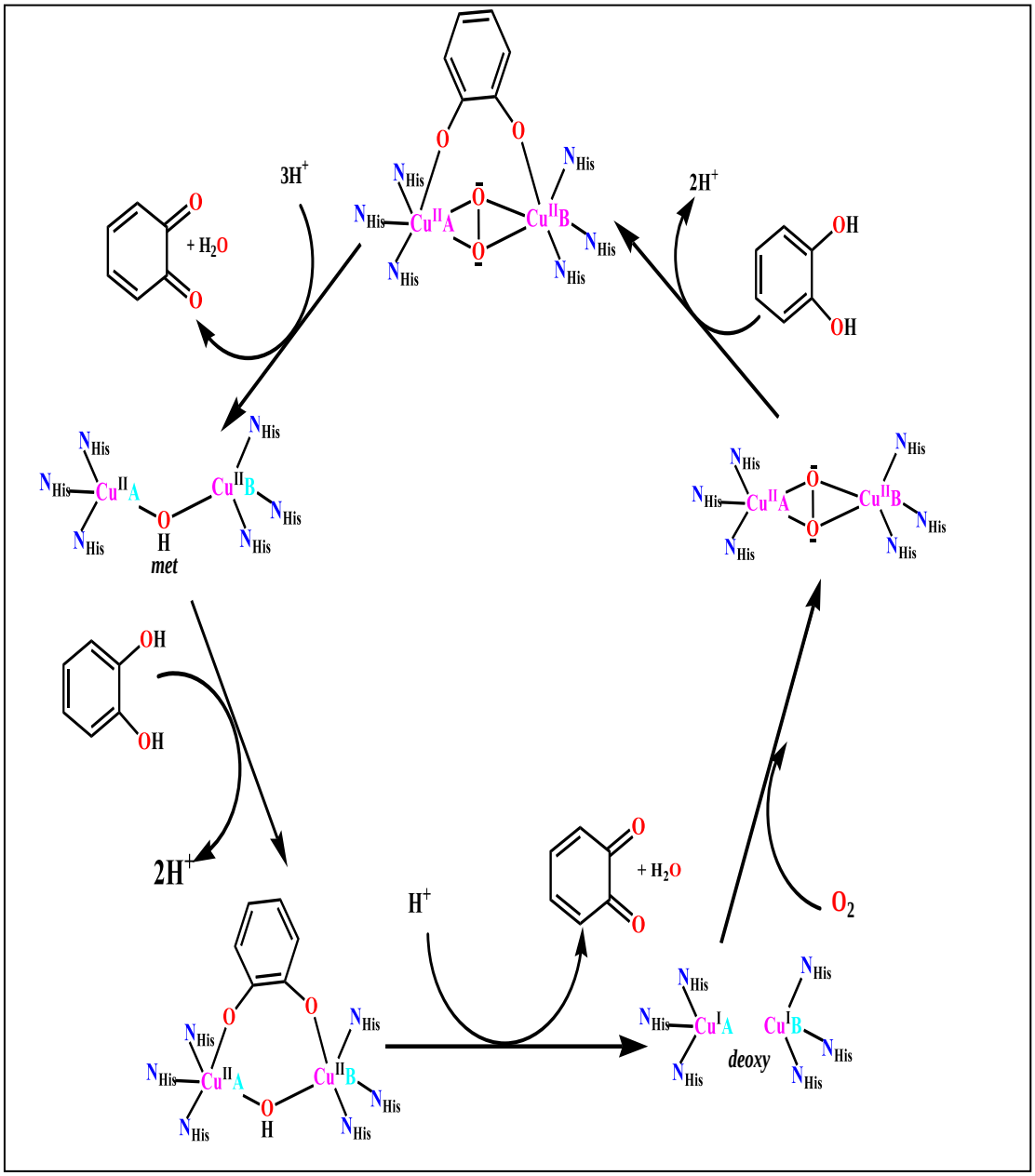
3.3. Reaction Mechanism of Catechol Oxidase Activity.

Catechol oxidase catalyzes the oxidation of catechols to the respective quinones through a four-electron reduction of dioxygen to water. Krebs and coworkers proposed a mechanism for the catalytic process [7f], based on biochemical and spectroscopic as well as structural data, which is depicted in Scheme 2 [2,7f]. The catalytic cycle starts with the *met* form which is the resting form of the enzyme. One mono-deprotonated catechol moiety becomes coordinated to the $Cu^{II}B$ center of the *met* form in the first step, followed by the oxidation of the catechol to quinone, reduction of both the copper centers from +II to +I state and rupture of the hydroxide bridge, the latest is converted to water and becomes coordinated with the $Cu^{I}A$ center. The next step is characterized by the following three processes: (i) both the copper centers are oxidized to +II state with Cu···Cu separation 3.8 Å; (ii) another mono-deprotonated catecholate binds with the $Cu^{II}B$ center; (iii) dioxygen is reduced to peroxide and becomes coordinated to both the copper (II) centers in μ-η^2:η^2 binding mode. The final step consists of the following processes: (i) oxidation of the coordinated catechol to quinone; (ii) reduction of peroxide to water and hydroxide; (iii) regeneration of the hydroxo bridged *met* form.



Scheme 2. Catalytic cycle of catechol oxidase activity, as proposed by Krebs and coworkers [7f].

A very similar catalytic mechanism has been proposed by Solomon *et al* (Scheme 3) [2]. The main difference between the two mechanistic proposal involves the binding mode of the substrate to the dicopper(II) core; whereas a monodentate asymmetric coordination of the substrate was proposed by Krebs and co-workers [7f], a simultaneous coordination of the substrate to both copper centers in the bidentate bridging fashion was suggested by Solomon *et al*.



Scheme 3. Catalytic cycle of catechol oxidase activity, as proposed by Solomon and coworkers [2].

One of the pioneering mechanistic studies on catechol oxidation catalyzed by model copper(II) complexes was presented by Lintvedt and Thuruya [15]. They have used 3,5-di-*tert*-butylcatechol (3,5-DTBCH$_2$) as substrate and studied the reaction of 3,5-DTBCH$_2$ with dioxygen catalyzed by bis(1-phenyl-1,3,5-hexanetrionato) dicopper(II) complex. Their observation was: the reaction is first order with respect to the substrate and 2nd order with

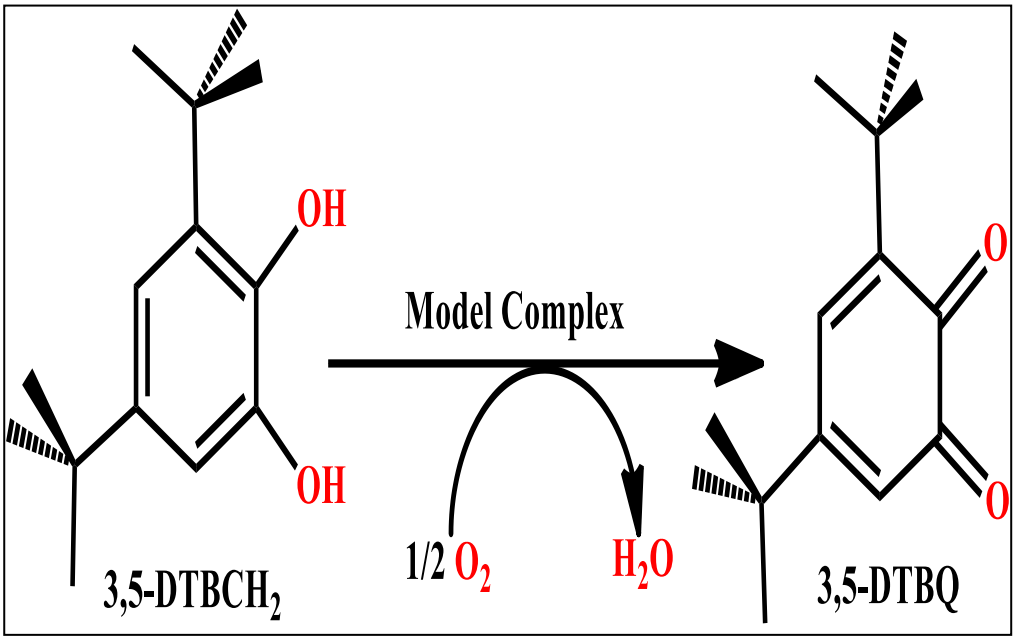
respect to the Cu^{II}_2 complex, thus suggesting the active reaction intermediate involved in the rate-determining step is a dicopper-catecholate adduct. Although much more complicated mechanism may be involved, the results show that treatment on the basis of Michaelis-Menten model, developed for enzyme kinetics, is sufficient for the kinetic descriptions. However, detailed mechanistic studies are unfortunately quite scarce [7b].

4. MODEL SYSTEMS OF CATECHOL OXIDASE ACTIVITY:

The most widely used substrate for catecholase activity is 3,5-di-*tert*-butylcatechol (Scheme 4). The three key points behind that are (i) its low reduction potential so that it can be readily oxidized to respective quinone, (ii) the bulky substituents prevent further reaction such as ring opening, and (iii) the oxidation product 3,5-di-*tert*-butyl quinone is considerably stable and exhibits a strong absorption band at *ca*. 400 nm and therefore activities and reaction rates can be determined by monitoring the electronic absorption spectroscopy at around 400 nm.

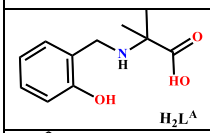
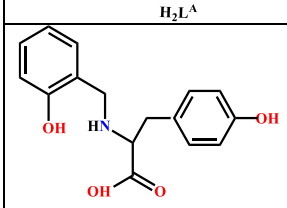
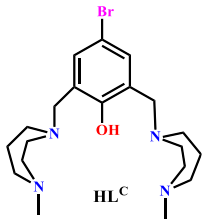
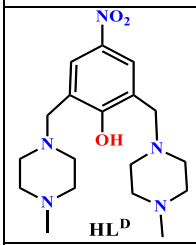
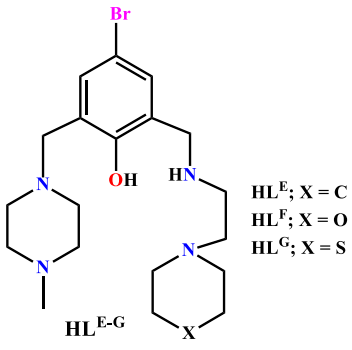
Many model studies of catechol oxidase have been made with dicopper(II) complexes having various types of bridging moieties. Although the native enzyme contains hydroxo bridge, systems having hydroxo bridge [7a–c] as well as other bridges including heterobridges [7a–c] have been found as active catalyst. Interestingly, few monocopper(II) and rare examples of copper(II) clusters and polymers are also known to show catecholase activity. Recently, catecholase activity of complexes of other metal ions, manganese, cobalt for example, has also been observed [16,17].

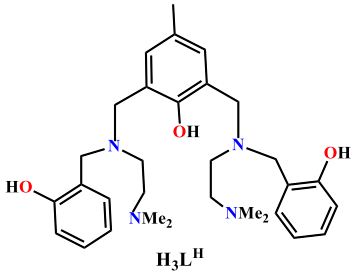
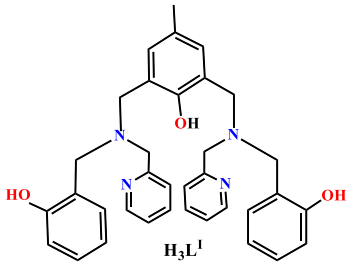
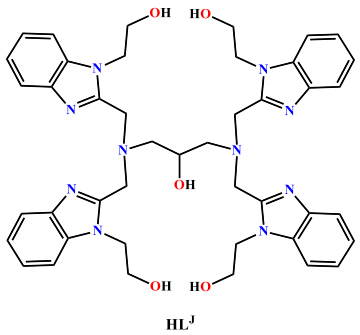
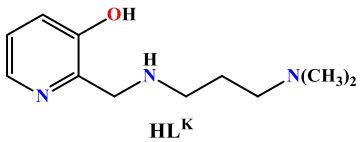
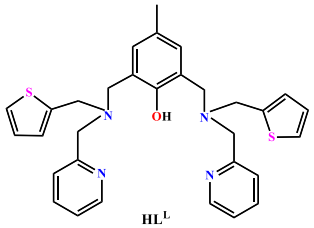
Some representative examples of systems showing catecholase activity is listed in Table 1.



Scheme 4. Conversion of 3,5-di-*tert*-butylcatechol (3,5-DTBCH_2) to 3,5-di-*tert*-butylquinone (3,5-DTBQ) catalyzed by model complex.

Table 1. Some representative examples of ligands, model complexes (for catechol oxidase activity) and their K_{cat} values.

Ligand	**Formula of the complex**	K_{cat} **(h^{-1})**	**Binding pattern**	**Reference**
 H_2L^A	$[Cu_2(L^A)_2(H_2O)_2]$	564	μ-phenoxo	18
 H_2L^B	$[Cu_2(L^B)_2(H_2O)]\cdot 2H_2O$	265	μ-phenoxo	
 HL^C	$[Cu_2L^C(\mu\text{-}OAc)_2](BF_4)\cdot H_2O$	93	μ-phenoxo bis($\mu_{1,3}$-carboxylate)	19
 HL^D	$[Cu_2L^D(\mu\text{-}OAc)_2](ClO_4)$	155	μ-phenoxo bis($\mu_{1,3}$-carboxylate)	
 HL^{E-G}; HL^E; X = C, HL^F; X = O, HL^G; X = S	$[Cu_2(L^E)(\mu\text{-}OAc)_2](BF_4)\cdot 0.25\ Et_2O$	10.7	μ-phenoxo-bis(μ-carboxylate)	7c
	$[Cu_2(L^F)(\mu\text{-}OAc)_2](BF_4)$	28.9	μ-phenoxo-bis(μ-carboxylate)	
	$[Cu_2(L^G)(\mu\text{-}OAc)_2](BF_4)\cdot 0.25\ MeOH\cdot 0.75H_2O$	40.0	μ-phenoxo-bis(μ-carboxylate)	

 H_3L^H	$[Cu_2(L^H)(N_3)_2]$	Catech-olase inactive	μ-phenoxo $\mu_{1,1}$-azide	20
 H_3L^I	$[Cu_2(HL^I)(O_2CPh)(H_2O)](PhCO_2)$	25.8	μ-phenoxo $\mu_{1,3}$-carboxylate	
 HL^J	$[Cu_2(L^J)(\mu\text{-}OAc)](ClO_4)_2{\cdot}CH_3CN$	3.94	μ-alkoxo-$\mu_{1,3}$-carboxylate	21
 HL^K	$[Cu_2(L^K)_2(CH_3CN)_2](PF_6)_2$	5.13	μ-phenoxo	
 HL^L	$[Cu_2(L^L)(\mu\text{-}Cl)(Cl)_2)]{\cdot}CH_3OH$	Turn over number less than 1 after 30 min	μ-phenoxo-μ-chloride	22
	$[Cu_2(L^L)(\mu\text{-}Br)(Br)_2)]$		μ-phenoxo-μ-bromide	

5. INFLUENCING FACTORS FOR CATECHOL OXIDASE ACTIVITY.

A few structure-activity correlations have also been proposed. But, those are applicable for only a few compounds. Those correlations, that have limitations as well, are mentioned below:

(i) Copper-copper distance. Copper-copper distance in the *met* form of catechol oxidase is very short (2.9 Å only) and comparing the value to that reported by Karlin and coworkers for the *o*-catecholate bridged dicopper complex (3.25 Å), it has been proposed that optimal copper-copper distance for catecholase activity should lie in the range 2.9–3.25 Å [7a]. In fact, on studying a few similar complexes, it has been observed that those having Cu···Cu distance, closest to that in the *met* form, show best activity among the series.

On the other hand, a number of good catalysts are known in which this distance lie at *ca*. 4 Å [22e] and even much larger such as 7.8 Å [7b]. Again, there is example of poor activity in spite of Cu···Cu distance, 2.85 Å, less than even 2.9 Å [22b].

(ii) Coordination geometry. In the crystal structure of the *met* form of the enzyme, two copper(II) centers are hydroxo-bridged and each is trigonal pyramidal. In model systems, some authors observed and proposed better activity if the coordination geometry is trigonal bipyramidal [22c], while some others observed better activity for square pyramidal [22a] or even square planar [22g,h] cases.

(iii) Exogenous bridging ligand. Nature of exogenous bridging ligand has a very crucial role on catalytic activity. The small bridging ligand (for e.g. OH^-) binding the two copper centers in bidentate fashion can promote short metal-metal distance, one of the required criteria of catecholase activity. Again OH^- can facilitate deprotonation of catechol by abstracting proton from it with subsequent release of water. The most important factor is lability and inertness of the ligands. From literature survey it can be stated that bridging ligands such as hydroxide [4c,18□20], alkoxide or phenoxide, imidazolate and carboxylates [4c] can be easily displaced by the incoming catecholate and promotes catalytic activity. A series of diacetate bridged dicopper(II) complexes, reported by Krebs and coworkers, act as effective catalysts due to the lability of acetate groups. On the other hand, compounds having chloride, bromide, and azido as the exogenous ligand are not in general active catalysts, revealing that these ligands are coordinated in stronger way and difficult to be substituted by the catecholate moiety [19].

On the other hand, although it has been proposed that activity should be better for small exogenous ligands, hydroxo for example [22e,f], some hydroxo bridged dicopper(II) systems are known which show very poor activity.

6. CONCLUSIONS:

The investigations carried out by several research groups on the structure and function of catechol oxidase is a perfect example of the essential strategy adopted by the chemists of the 21st century. Indeed, such studies inevitably bring in distinct but complementary disciplines of contemporary chemistry, i.e. biochemistry, synthetic and inorganic chemistry, and spectroscopy. Thus, while early studies on the catechol oxidation by copper complexes only reported some catalytic activities, the model systems described nowadays are able to address various aspects of the catalytic mechanism, such as the binding mode of the substrate to the metal centers and subsequent stoichiometric reaction between the catalytic core and the substrate, the structure and reactivity of intermediate copper-dioxygen species, the reduction mode (two- or four-electron) of dioxygen etc.

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