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Tyrosinase and catecholase-like activities of a dinuclear Cu(II) complex

Arnab Chatterjee^a, Hare Ram Yadav^b, Angshuman Roy Choudhury^b, Anzar Ali^c, Yogesh Singh^c and Rajarshi Ghosh^{a,*}

^aDepartment of Chemistry, The University of Burdwan, Burdwan 713 104, India ^bDepartment of Chemical Sciences, Indian Institute of Science Education & Research, Mohali, Sector 81, S.A.S. Nagar, Knowledge City, Manauli PO, Mohali 140 306, India ^cDepartment of Physical Sciences, Indian Institute of Science Education & Research, Mohali, Sector 81, S.A.S. Nagar, Knowledge City, Manauli PO, Mohali 140 306, India

Abstract

A dinuclear Cu(II) complex was synthesized and crystallographically characterized. The compound was found to have antiferromagnetic interaction in between the Cu(II) centres in the molecule. It had weak intermolecular ferromagnetic interaction. The compound was found to be tyrosinase and catecholase active. In case of tyrosinase activity, the diphenol formed was isolated using thin layer chromatography (TLC) and characterized through ¹H NMR as well as mass spectrometry. The *o*-quinone derivative formed in this reaction was characterized using GC-MS. The latter activity was monitored spectrophotometrically and the product *o*-quinone derivative was isolated (in MeOH and MeCN) column chromatographically and characterized using melting point determination. These were followed by Michealis-Menten kinetics with turnover numbers 4.95×10^3 and 1.58×10^3 h⁻¹ in MeOH and MeCN, respectively.

Keyword: Copper(II), Schiff base, catecholase activity, tyrosinase activity *Corresponding author, E-mail: rghosh@chem.buruniv.ac.in

1. Introduction

Modelling molecular oxygen binding/activating copper proteins to mimic a number of important biochemical oxidation reactions is an important area of upsurge research interest [1,2]. In biological systems copper proteins are excellent catalysts which cross the kinetic barriers associated with spin functionality of molecular oxygen. Tyrosinase, catechol oxidase and hemocyanin, each of which contain magnetically coupled dinuclear copper active sites, are type 3 copper proteins [3]. Orthohydroxylation of phenols and their subsequent oxidation to *o*-quinones which finally converts to melanin pigment in plants is catalysed by tyrosinase. Catecholase in plant kingdom only converts 1,2-diphenols to o-quinones without showing any monooxygenase activity. Furthermore, oxygen transportation and storage in mollusks and arthopods is carried out by hemocyanin. As soon as the crystal structures of tyrosinase [4] and catecholase [5] appeared in literature, people round the globe started to model those enzymes for conversion of the respective substrates which occurs actually in natural systems [6-8]. Though catecholase has its dicopper active site, a number of monocopper [9] and non-copper [10] complexes appeared which show similar kind of activity. In this present context, we have synthesized and structurally characterized one dinuclear Cu(II) complex with compartmental Schiff base ligand [11] $[Cu_2Cl_3(L)]$ [L-H = 2,6-Bis-[(2-hydroxy-ethylimino)methyl]-4-methyl phenol]. The magnetically characterized compound is found to show tyrosinase and catecholase-like activities at room temperature.

2. Experimental

2.1. Materials and Methods

All reagents were obtained from commercial sources and used as received. Solvents were dried as per standard procedures and distilled before use.

2,6-diformylparacresol was prepared as per reported literature method [12]. The ligand L-H [11] was obtained by condensing a solution of ethanol amine (0.156g, 2.56 mmol) with 2,6-diformylparacresol (0.210g, 1.28 mmol) in ethanol (50 ml) and refluxing the resultant solution over a period of 5 h. Initial yellow coloured solution turned orangish and subsequently deeper in colour over the refluxing period. The obtained solution was carefully evaporated over a rotary evaporator maintaining 50°C temperature of the water bath to get orange coloured product.

It was characterized using IR and Mass spectral measurements. In IR the band at 1650 cm⁻¹ is characteristic of imine bond (Fig. S1; ESI†). Mass spectral data also suggested the formation of our desired ligand (Fig. S2; ESI†). UV-Vis spectrum of L-H was recorded in MeOH solution and peaks at 220, 256 and 450 nm were obtained (Fig. S3; ESI†).

Yield (Based on 2,6-diformylparacresol): 0.2369g (74.00%). Selected IR bands (KBr pellet, cm⁻¹): 1650, 3380; UV-Vis (λ , nm) 220, 256, 450.

For catecholase activity study, 1×10^{-4} mol dm⁻³ solution of complex (1) (0.0005 g) was treated with 1×10^{-2} mol dm⁻³ (100 equivalents) of 3,5-di-tert-butylcatechol (3,5-DTBC) (0.0222 g) under aerobic conditions.

2.2. Synthesis of 1

1 was prepared by mixing a solution of $CuCl_2.2H_2O$ (0.034g, 0.2 mmol) in methanol to a pre stirred warm solution of L-H (0.025g, 0.1 mmol) in methanol and

stirring the resultant solution for nearly 2 h. The resulting green solution was kept in open air for slow evaporation. After 10-12 days, X-ray quality green crystals of **1** were collected, washed with hexane and were kept under vacuum desiccator.

In IR spectrum of **1** the imine stretch appears at lower value (1635 cm⁻¹) (Fig. S4; ESI[†]) than the free ligand (1650 cm⁻¹) which indicates the coordination of the imine nitrogen to the metal center. UV-Vis spectrum of **1** was recorded in MeOH solution and peaks at 218, 250 and 410 nm were obtained (Fig. S5; ESI[†]). Yield: (Based on metal salt) 0.04580 g (45.76%). Selected IR bands (KBr pellet, cm⁻¹):1635, 3340; UV-Vis (λ , nm) 218, 250, 410.

2.3. Physical measurements

Single crystal X-ray crystallographic analysis of **1** was carried out on a Bruker SMART APEX II CCD diffractometer using Mo-K α radiation ($\lambda = 0.71075$ Å). Diffraction data was collected at 293 K and was identified as P121/c1 space groups. The crystal data and refinement details are listed in Table 1. The structure was solved by direct methods, and the structure solution and refinement were based on $|F|^2$. All calculations were carried out using SHELXL-97 [13] and were refined using SHELSL-97 [14]. All the figures have been generated using ORTEP-32 [15].

IR (KBr discs, 4000-500cm⁻¹) spectra were recorded on Shimadzu IR Prestige spectrophotometer and UV-Vis analysis were done on Shimadzu UV-Vis 2450 spectrophotometer. The ¹H NMR spectral data were collected using CDCl₃ as solvent on a Bruker 400 MH_z spectrometer. Mass spectral data were collected on Thermo Exactive plus spectrometer. The GC-MS measurements were done on Perkin Elmer Clauss 600 with column specification: Elite 5 MS (30 mm × 0.25 mm × 0.25 mm) at maximum temperature -300⁰ temperature. Magnetic susceptibility χ versus

temperature T measurements were performed on powder samples in an applied magnetic field of 1 T, using the VSM option of a Quantum Design PPMS.

3. Results and discussion

3.1. X-ray structure

Thermal ellipsoid plot of **1** is presented in Fig. 1. Bond angles and distances of **1** are given in Table S1 (ESI†). The geometry around each Cu(1) centre in the symmetric doubly chloro phenoxo bridged dinuclear **1** is best described as distorted square pyramid as suggested by its τ value ($\tau = 0.076$) [16] with CuNO₂Cl₂ chromophore. From the bond angle and bond distance values it can be considered that one imine nitrogen (N1), one alkoxo oxygen (O1) and one phenoxo oxygen (O2) from the ligand backbone, and the bridging chloro (Cl2) group form the equatorial plane of the square pyramid. Another chloro group (Cl1) resides at the axial position of the geometry. The equatorial bond distances at Cu(1) range from 2.283(2) to 1.915(7) Å ($\Delta = 0.368$ Å). The axial Cu(1)-Cl(1) distance is 2.535(2) Å. The Cu(2) centre has the same geometry with $\tau = 0.020$ [16].

3.2. Magnetism

Magnetic susceptibility χ versus temperature T measurements were performed on powder samples in an applied magnetic field of H = 1 T, using the VSM option of a Quantum Design PPMS.

The magnetic susceptibility χ (T) vs T data are shown in the main figure below (Fig. 2). There are 4 Cu's per formula unit and the χ data are shown per Cu. The χ (T) shows paramagnetic behaviour at high temperatures before passing over a maximum around 60 K and then decreasing at lower temperatures. Below 20 K a sharp rise in

observed which continues to the lowest temperature. This behaviour is consistent with expectation for localized Cu spins which interact antiferromagnetically and form magnetic dimers below about 65 K. Our system is indeed built up of Cu...Cu dimers which are isolated from each other and are expected to be interacting very weakly with each other.

Below the Cu...Cu dimerization temperature, χ should go to zero as T goes to zero. However, the sharp upturn at low temperatures points to the presence of unpaired Cu spins which can be treated as impurity spins. From C, the effective moment μ_{eff} can be estimated using the expression $C = N_A \mu_{eff}^2/3k_BT$, where N_A is the Avogadro's number and k_B is the Boltzmann constant. This gives $\mu_{eff} = 1.75(2) \mu_B$ which is close to the value expected for $S = \frac{1}{2}$ magnetic moments is $\mu_{eff} = 1.73 \mu_B$ assuming a *g*-factor of g = 2. This fit is shown as the red curve through the data in inset to Fig. 2.

We have thus tried fitting our χ data using a model of non-interacting S = $\frac{1}{2}$ dimers. The magnetic susceptibility χ (T) for a collection of non-interacting S = $\frac{1}{2}$ dimers with intra-dimer coupling J_1 can be written as

$$\chi_D(T) = \left(\frac{2N_A\mu_B^2g^2}{k_BT}\right) \left[\frac{3}{3+e^{\frac{J_1}{2T}}}\right]$$

where N_A is the Avogadro's number, μ_B is the Bohr magneton, g is the electronic gfactor, and k_B is the Boltzmann constant.

Assuming that the interactions between dimers acts as a mean-field, the susceptibility of interacting dimers can then be written as

$$\chi_{Dimer}(T) = \left(\frac{\chi_0}{1 + \lambda \chi_0}\right)$$

Where λ is the exchange coupling which can be given in terms of the inter-dimer exchange J₂ as $\lambda = J_2/3C$, where C is the Curie constant for a S = ½. In addition to the contribution from dimers, there could be other core (T independent) and paramagnetic impurity (giving the large Curie tail at low T) contributions. The experimental $\chi(T)$ data were therefore fit by the expression

$$\chi(T) = \chi_0 + \frac{C_{imp}}{T - \theta_{imp}} + \chi_{int}$$

where, χ_0 is a T independent term. C_{imp} is the Curie constant of impurity spins, and θ_{imp} is the Weiss temperature giving interactions between these impurity spins. The fit, shown in Fig. 5 as the red curve through the data in the main panel gave the values $\chi_0 = 2.8(3) \times 10^{-4} \text{ cm}^3/\text{Cu}$ mol, $C_{imp} = 0.003(1) \text{ cm}^3/\text{Cu}$ mol K, $\theta_{imp} = -0.21(3)$ K, $J_1 = -98(5)$ K and $J_2 = 0.75(6)$ K. The error factor came out to be R = 1.7 x 10⁻⁵. The small values of C_{imp} and θ_{imp} indicate that paramagnetic impurities, if present, are in small numbers and are almost non-interacting. The value $J_1 = -98$ K shows the presence of strong anti-ferromagnetic interactions within Cu...Cu dimers and the small value of $J_2 = -0.75$ K suggests weak ferromagnetic interactions between dimers. If the T independent part χ_0 and θ_{imp} are subtracted from the total χ then one can obtain the magnetic contribution χ_{Dimer} from only the Cu...Cu dimers. This contribution is shown in the inset of the above figure. One can see that the dimer susceptibility goes to zero as T goes to zero as expected for antiferromagnetic dimers.

3.3. Catecholase activity

The substrate 3,5-DTBC, having two bulky t-butyl substituents on the ring, resulting in lower quinone-catechol reduction potential, was chosen for catecholase

activity study. The oxidised stable product o-quinone shows a maximum absorption at 401 nm in methanol [17]. The electronic bands of 1 were found to be at 250 and 410 nm while 3,5-DTBC shows a single band at around 282 nm. Treating 100 equivalents of 3,5-DTBC with complex 1 in methanol (MeOH), under aerobic conditions, the repetitive UV-Vis spectrum was recorded. Transformation of colourless solution to deep brown with respect to time indicated the conversion of 3,5-DTBC to 3,5-di-tertbutyl-o-quinone (3,5-DTBQ) which was further corroborated by the continuous increase in quinone band at 401 nm in methanol (Fig. 3). The same reaction was done by scaling up the reactants and the desired 3,5-DTBQ was isolated column chromatographically using 10% mixture of ethyl acetate and hexane as eluent. The isolated o-quinone was identified by comparing its melting point to that reported in literature [18]. The aerial oxygen, that oxidises 3,5-DTBC to 3,5-DTBQ in this process, is converted to H₂O₂. H₂O₂ thus liberated was identified and characterized spectrophotometrically [19] (Fig. S6; ESI⁺). Time dependent change in absorbance at wavelength of 401 nm, which is the characteristic absorbance of 3,5-DTBQ in methanol, was observed for 30 minutes to find the rate of reaction between 1 and 3,5-DTBC. The difference in absorbance, in the mentioned wavelength, was plotted against time to obtain the rate for that particular catalyst to substrate concentration ratio (Fig. 4). A first order reaction rate was observed with a rate of 3.3×10^{-3} min⁻¹.

The catecholase activity of **1** was similarly studied in acetonitrile (MeCN) and dichloromethane (DCM). In DCM the catecholase activity was not found to be satisfactory (Fig. S7; ESI†). In MeCN, 3,5-DTBQ shows maximum absorption at 401 nm (Fig. 5). 3,5- DTBQ obtained in acetonitrile media was purified column chromatographically with 67.8% yield. Here again, formation of 3,5-DTBQ was characterized by determining its melting point (~110°C) which agreed well with that

reported in literature [18]. The reaction kinetics was studied in acetonitrile, as described previously with methanol, at a wavelength of 401 nm for catalysis. The difference in absorbance ΔA at this particular wavelength, was plotted against time, to obtain the initial rate of the reaction (Fig. 6). A first-order catalytic reaction was observed, where the initial rates was found to be 7.22×10^{-4} min⁻¹ in MeCN.

3.4. Enzyme kinetics study: catecholase activity

1 was subjected to kinetic experiments spectrophotometrically with the substrate 3,5-DTBC using MeOH and MeCN as solvents. The reaction setup was thermostated at 20°C. 0.04 ml of 1×10^{-4} (M) solution of the complex was added to 2 ml of 3,5-DTBC of a particular concentration (concentration variation was 1×10^{-3} (M) to 1×10^{-2} (M) to achieve a final concentration of the complex as 10^{-4} (M). Conversion of 3,5-DTBC to 3,5- DTBQ was monitored spectrophotometrically at a wavelength of 401 nm in both MeOH and MeCN. Rate of each concentration of the substrate was determined using initial rate method.

The data of rate versus concentration of the substrate were analyzed on the basis of Michealis-Menten approach (Fig. 7) to get the Lineweaver-Burk plot in MeOH. From the later plot various kinetic parameters like V_{max} , K_M , K_{cat} were obtained. The Lineweaver-Burk plot in methanol solution is presented (Fig. 8).

Similar plots in MeCN are presented in Figs. 9-10. The kinetic parameters are listed under Table S2 (ESI[†]). The turnover numbers (K_{cat}) are 4.95×10³ and 1.58×10³ h⁻¹ in MeOH and MeCN, respectively which are better than some reported Cu(II) complexes [20].

3.5. Tyrosinase activity

For investigating tyrosinase activity 0.824 g of 2,4-DTBP was required [6a]. For catalyzing this amount of phenol derivative stoichiometrically 1.0 g of title compound (1) was required. As the yield of the reaction was low (45.76%) it is difficult to prepare this amount of **1**. So the in situ generated title compound was used for its tyrosinase activity. For the tyrosianse activity study L-H (0.545 g, 2.18 m.mole) was dissolved in methanol and CuCl₂.2H₂O (0.745 g, 4.36 m.mole) was added portion wise with gentle stirring. On addition, green coloured solution generated which indicated the formation of the complex in situ as examined from the mass spectral data of the reaction mixture (Fig. S8; ESI⁺). Once the formation of the complex in the solution was confirmed, excess of 2,4-ditertiarybutylphenol 0.824g (4 m.mole) was added to the solution with stirring. The reaction mixture was bubbled with oxygen gas for nearly 48 h. The resultant solution was evaporated under low pressure to get the resultant crude product. The obtained crude was expected to contain the desired product along with other impurities like excess metal complex, excess starting materials, etc. To get a clear picture we performed TLC study with the crude mixture using 10% mixture of ethyl acetate/hexane in solvent chamber. TLC study indicated the presence of unreacted metal complex which was present at the base of the TLC plate and also the presence of 3,5-DTBC (Fig. S9; ESI⁺). The crude was also subjected to column chromatography using 60% mixture of ethyl acetate/hexane where excess unreacted complex was trapped in the column. Although TLC apparently indicated the absence of 3,5-DTBQ, appearance of peak in purified reaction mixture at 401 nm indicated the formation of quinone derivative which may be indicative of the poor yield of the quinone as compared to 3,5-DTBC during the reaction. Besides absorption spectrum, formation of quinone was verified by GC-MS spectra (Fig. S10;

ESI[†]). High resolution mass spectrometric measurements were done which also indicated formation of 3,5-DTBC and 3,5-DTBQ (Figs. S11-S12; ESI[†]). NMR experiments on the product confirmed formation of 3,5-DTBC. H¹ NMR (CDCl3, 400 MHz): δ H =1.32 (s, 9H), 1.45 (s, 9H), 5.21 (s, 1H), 7.107 (d, 1H, J=2.4 Hz), 7.39 (d, 1H, J= 2.4 Hz) (Fig. S13; ESI[†]). In most of the literature [6] the characterization of catechol and quinone, which were the product of tyrosinase activity, were done at low temperature. Interestingly, in our case, the same products were generated and characterized at room temperature using different spectroscopic methods. The quinone formed in this reaction was produced in such a low yield (which is quite common in reported literature) [6a,6b] that it had to be characterized gas chromatographically.

3.6. Catalytic cycle and DFT calculations

A catalytic cycle for the better understanding of tyrosinase activity of **1** is presented in Scheme 1 [21]. The initial active form of the complex is expected to be the form **A** which comes directly from **1** by the opening of phenoxo as well as chloro bridges from both Cu(II) atoms. Structures of all the active form of **1** which are believed to be involved in the catalytic cycle were characterized mass spectrometrically (Figs. S14-S17; ESI[†]).

To get a better understanding of the tyrosinase activity study, we have calculated the absolute energies using TDDFT model at the B3LYP level using Gaussian 5.0 software package and thereby the difference of energies among the various forms that were mass spectrometrically determined and believed to be involved in the catalytic cycle (Scheme 1). Form **A**, which directly comes from **1** by opening of the chloro and the phenoxo bridges is unstable by 459.75 a.u. of energy

compared to **1**. This is probably due to the reduced coordination number as well as the oxidation state of both the copper atoms. Transformation of form **A** to **B** was found to be stable by -149.96 a.u. of energy which can be accountable considering both the increased coordination number as well as the oxidation states of the copper atoms. It was found that coordination of 2,4-ditertiarybutylphenol (2,4-DTBP) with the form **B** lowers the energy of the succeeding form **C** by -620.2 a.u. of energy. Form **D** was found to be having slightly more energy (2.84 a.u. of energy) compared to Form **C**. Completion of the catalytic cycle through transformation of form **D** to form **A** is found to be occurring by consuming around 767.32 a.u. of energy. Inspite of this energy absorption for the completion of the cycle, the formation of the desired product 3,5-DTBQ in this step is expected to be the driving force behind the completion of the catalytic cycle.

4. Conclusions

Synthesis and X-Ray structural characterization of a dinuclear Cu(II) complex have been reported. The compound is tyrosinase as well as catecholase active. Tyrosinase activity is rare in literature [6, 22]. The catalytically produced 1,2-diphenol was characterized through ¹H NMR spectroscopy and mass spectrometry while the quinone formed was characterized using GC-MS. The mechanistic aspect of similar type of activity reported by Karlin *et.al* [22] was determined characterizing most of the species spectrophotometrically. In our case, all the species in the catalytic cycle were determined mass spectrometrically without isolating them individually from the reaction mixture. Our proposed mechanism is supported by TDDFT calculations. That is why the species we get in the catalytic cycle are to some extent different from that reported by Karlin *et.al* [22].

Though a good number of reports are there for the catecholase active dinuclear Cu(II) complexes, the turnover number of our compound is better than few reported ones [20].

5. Supplementary data

CCDC 1556831 contains the supplementary crystallographic data for **1**. This data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk

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Empirical formula	C13 H18 Cl3 Cu2 N2 O4
Formula weight	499.7976
Т (К)	293
Wavelength (_A)	0.71075
Crystal system	monoclinic
Space group	P 1 21/c 1
Unit cell dimensions	
a (Å)	7.6957(9)
b (Å)	16.2734(19)
c (Å)	14.2917(19)
α (°)	90
β (°)	99.208(6)
γ(°)	90
$V(Å^3)$	1766.8(4)
Z	4
D _{calc} (mg m ⁻³)	1.879
Absorption coefficient (mm ⁻¹)	2.881
F(000)	1004
Crystal size (mm ³)	0.4 imes 0.1 imes 0.1
Theta range for data collection (_)	3.099- 27.483
Index ranges	$-9 \le h \le 9, -21 \le k \le 21,$
	$-18 \le l \le 18$
Reflections collected	18189
Independent reflections	4025
Completeness to theta	
Absorption correction	multi-scan
T _{max} and T _{min}	1.000 and 0.530
Refinement method	
Data/restraints/parameters	4025/6/222
Goodness-of-fit (GOF) on F ²	0.898
Final R indices $[1 > 2s(1)]$	$R_1 = 0.0823, wR_2 = 0.1912$
R indices (all data)	$R_1 = 0.1255, wR_2 = 0.1636$
Largest difference in peak and hole (e _A_3)	0.89, -0.78



Fig. 1 An ORTEP of $[Cu_2Cl_3(L)]$ (1) with atom numbering scheme and 50% probability ellipsoids for all non hydrogen atoms



Fig. 2 Magnetic susceptibility χ (T) versus T plot



Fig. 3. Change in spectral pattern of complex **1** after reaction with 3,5-DTBC, observing the reaction for 6 h in MeOH



Fig. 4. A plot of the difference in absorbance (A) vs. time to evaluate the rate of catalysis of 3,5-DTBC by **1** in MeOH



Fig. 5. Change in spectral pattern of complex **1** after reaction with 3,5-DTBC, observing the reaction for 6 h in MeCN



Fig. 6. A plot of the difference in absorbance (A) vs. time to evaluate the rate of catalysis of 3,5-DTBC by **1** in MeCN



Fig. 7. Plot of rate versus [substrate] (3,5-DTBC) in presence of 1 in MeOH



Fig. 8. Lineweaver-Burk plot of 1 in MeOH



Fig. 9. Plot of rate vs.[substrate] (3,5-DTBC) in presence of 1 in MeCN



Fig. 10. Lineweaver-Burk plot of 1 in MeCN





Graphical abstract

A dinuclear antiferromagnetically coupled Cu(II) complex was synthesized and characterized crystallographically. The compound was found to had tyrosinase-like activity resulting in conversion of 2,4-di-tert-butylphenol to 3,5-di-tert-butylcatechol to 3,5-di-tert-butylquinone. 3,5-di-tert-butylcatechol and 3,5-di-tert-butylquinone were characterized using analytical and spectroscopic methods. The compound was also found to give catecholase-like activity in methanol and acetonitrile.

