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Comparison of two different approaches for determination of biomimetic activity of selected copper complexes

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In this article, an evaluation of the catecholase activity of the recently synthesized copper complexes towards dopamine is reported based on a comparison of the performance of two different techniques – UV-Vis spectrophotometry and hydrodynamic amperometry. The results obtained were confronted with those published for natural tyrosinase enzyme (isolated from mushroom 'Agaricus bisporus') and defined by Michaelis–Menten kinetics model. Besides a finding of comparable catalytic activity for the copper catalysts tested, it has to be emphasised that all the measurements have been performed in methanol, in which tyrosinase enzyme loses its catalytic activity.

Keywords: Copper complexes; Tyrosinase; Biomimetic activity; UV-Vis spectrophotometry; Amperometry.

Introduction

In recent years, a variety of biomimetic catalysts that imitate the catechol oxidase activity have been explored in order to get a better understanding of the active site of Cu-containing proteins [1,2]. Moreover, one of the aims for the development of such catalysts — sometimes, also called “artificial enzymes or metallo-enzymes” — is to replace natural enzymes which exhibit a low long-term stability [3,4]. A typical example is enzyme tyrosinase (TYR).

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From literature, it is possible to find out that the TYR enzyme forms a tetramer of 119.5 kDa that is composed of two types of sub-units, namely H sub-units (~43 kDa) and L sub-units (~14 kDa). The H subunit contains a binuclear copper-binding site in the oxy-state, in which three histidine residues coordinate each copper atom [5]. TYR enzyme catalyzes two different kinds of enzymatic reactions: (i) *o*-hydroxylation of monophenols and (ii) oxidation of *o*-diphenols to *o*-quinones (see Fig. 1); both being similar to coordination mode known for haemocyanines. Comparable activity is also expected for biomimetic catalysts.

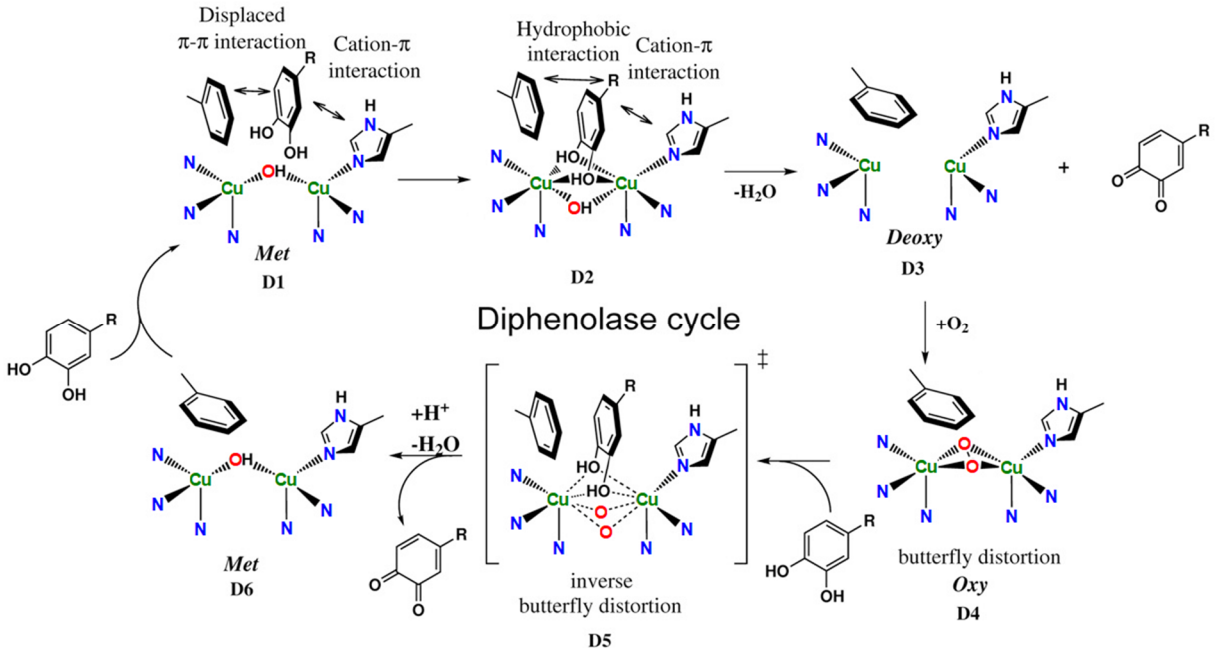

Fig. 1 The diphenolase cycle of mushroom tyrosinase

A number of methods for studying the enzyme kinetics has already been employed; among them, UV-Vis spectrophotometry [6], radiometry [7], mass spectrometry with electrospray ionization and ion trap [8], manometry [9], or selected electrochemical techniques [10]. Some of them were also examined to compare biomimetic activity of various copper-based catalysts, when the most frequently used approach was that employing UV-Vis spectrophotometry.

Several contrasts between the studies on catalyst kinetics and investigations of enzyme kinetics should be mentioned. Whereas the enzyme kinetics is commonly measured in aqueous media of *ca.* pH 7, kinetics of a catalyst is usually ascertained in non-aqueous media (methanol or acetonitrile) due to the lipophilic character of synthetic catalysts. This high contrast makes a comparison of the catecholase activity of synthetic and natural enzymes rather difficult. Yet another contrast is in a substrate used for kinetic studies. Whereas most of naturally present and biologically active catechol derivatives (e.g. neurotransmitters like

dopamine or serotonin) can be used in combination with TYR enzyme, the studies on the kinetic activity of biomimetic catalysts utilize the only compound, 3,5-di-*tert*-butylcatechol, as the most frequently used model substrate [11]. The main reason for this difference is the formation of more-or-less defined products of oxidation of the afore-mentioned phenols (for details, see below).

In this article, we report on comparison of the catechol oxidase activity of two recently synthesized copper complexes (Fig. 2) towards dopamine as a substrate. Moreover, as shown by the title itself, certain part of discussion is focused on comparison of two different methods for the determination of biomimetic activity, when UV-Vis spectrophotometry and amperometry have been the techniques of choice. From practical point of view, the highlights from such comparative study can be utilized in nowadays attractive field of the development of biomimetic sensors.

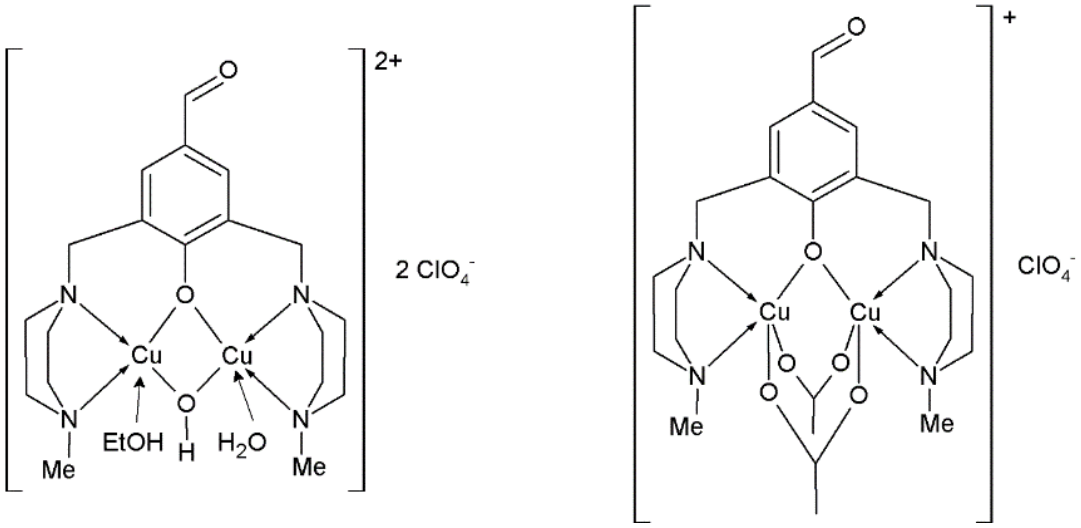

Fig. 2 Structures of copper complexes **1** and **2**
Left: $[Cu_2L(OH)(H_2O)(EtOH)]\ (ClO_4)_2$ and right: $[Cu_2L(OAc)_2(H_2O)]\ (ClO_4)$

Materials and methods

Chemicals and reagents

Dopamine, methanol (MeOH; purity: 99.99%), and lithium perchlorate (99.99%) were purchased from Sigma-Aldrich (Prague, Czech Republic). All the solvents were used without any purification.

The copper complexes studied were as follows: $[Cu_2L(OH)(H_2O)(EtOH)]\ (ClO_4)_2$ and $[Cu_2L(OAc)_2(H_2O)]\ (ClO_4)$ were synthesized according to refs. [11,12]; in this report being denoted as complex **1** and complex **2**.

A deionized water characterised by the resistivity of 18.3 MΩ cm was prepared in a Millipore Milli-Q® purification system (Merck; Darmstadt, Germany).

Methods and instrumentation for UV-Vis spectrophotometry

All the measurements of UV-Vis spectra were carried out with a spectrophotometer UV2450 (Shimadzu; Kyoto, Japan) using 1 cm quartz cuvette (Fisher Scientific; Pardubice, Czech Republic) in the range of wavelengths from 800 to 200 nm at scanning rate of 0.5 nm s^{-1}.

Measurements of kinetics were performed in 3 mL MeOH containing 0.1 mol L^{-1} $LiClO_4$, and 150 μmol L^{-1} of the respective biomimetic complex, and appropriate amount of dopamine within the concentration range from 100 to 1600 μmol L^{-1}. At first, UV-Vis spectrophotometry of the biomimetic complex solution was only measured and then, the spectrum obtained was corrected for the blank absorbance signals. To determine the inevitable parameters, namely Michaelis constant (K_m^{app}) and the apparent maximum reaction velocity (V_{max}^{app}), absorbance values at 465 nm for dopamine were used.

Methods and instrumentation for amperometry

Each measurement intended for hydrodynamic amperometry was performed in a batch arrangement where a conventional voltammetric glass cell was used. Traditional three-electrode cell, consisting of the glassy carbon electrode (GCE, (working), saturated calomel electrode (SCE, reference) with 0.1 mol L^{-1} $LiClO_4$ in MeOH as salt bridge, and a platinum sheet (auxiliary), were connected together to the AUTOLAB PGSTAT101 potentiostat/galvanostat (Metrohm; Prague, Czech Republic) operated through NOVA 1.11 software.

Each experiment was performed in a conventional glass cell (International Chemistry; Matsudo-Shi, Japan) containing 500 μmol L^{-1} synthetised dinuclear copper complex in a non-deaerated pure MeOH with 0.1 mol L^{-1} $LiClO_4$ at detection potential of 0.1 V and a stirring speed of 400 rpm. If not stated otherwise, all important changes in the working conditions are shown in the legends of each figure concerning the amperometric experiments.

Results and discussion

Although the kinetics of tyrosinase (TYR), as well as biomimetic copper complexes, have been mostly studied by spectrophotometric techniques [10], the application of an enzyme or a synthetic catalyst in biosensing usually utilises electrochemical approaches. Here, we are trying to compare these two techniques, UV-Vis spectra vs. amperometry, allowing us to reveal also their practical aspects on biomimetic activity of two recently synthesized copper complexes **1** and **2** towards dopamine.

Already at the beginning, it is worth of mentioning that the oxidation of dopamine is not associated with formation of the only product. Initially, dopamine is oxidized (either by enzyme or copper catalyst) to dopamine-quinone, which undergoes a nucleophilic reaction (cyclization) and forms a leukodopamine-chrome (5,6-dihydroxyindole) [13]. Therefore, it is important to determine the wavelength of maximum absorbance of dopamine-quinone product. The UV-Vis spectra (Fig. 3A) of 150 μmol L^{-1} dopamine (black line) in the presence of 150 μmol L^{-1} of the corresponding binuclear copper(II) complex were recorded every 10 min (red). The wavelength of 460 nm was set to measure kinetic curves for 100–1600 μmol L^{-1} dopamine (Fig. 3B) interpolated by a quadratic function. For coefficients of determination (R^2) higher than 0.998, the *b*-values of the respective quadratic equations characterizing the change in absorbance (A) over time (t), and subsequently the reaction velocity V_0, were used for construction of Michaelis–Menten saturation curve for the catalytic reaction monitored (Fig. 3C).

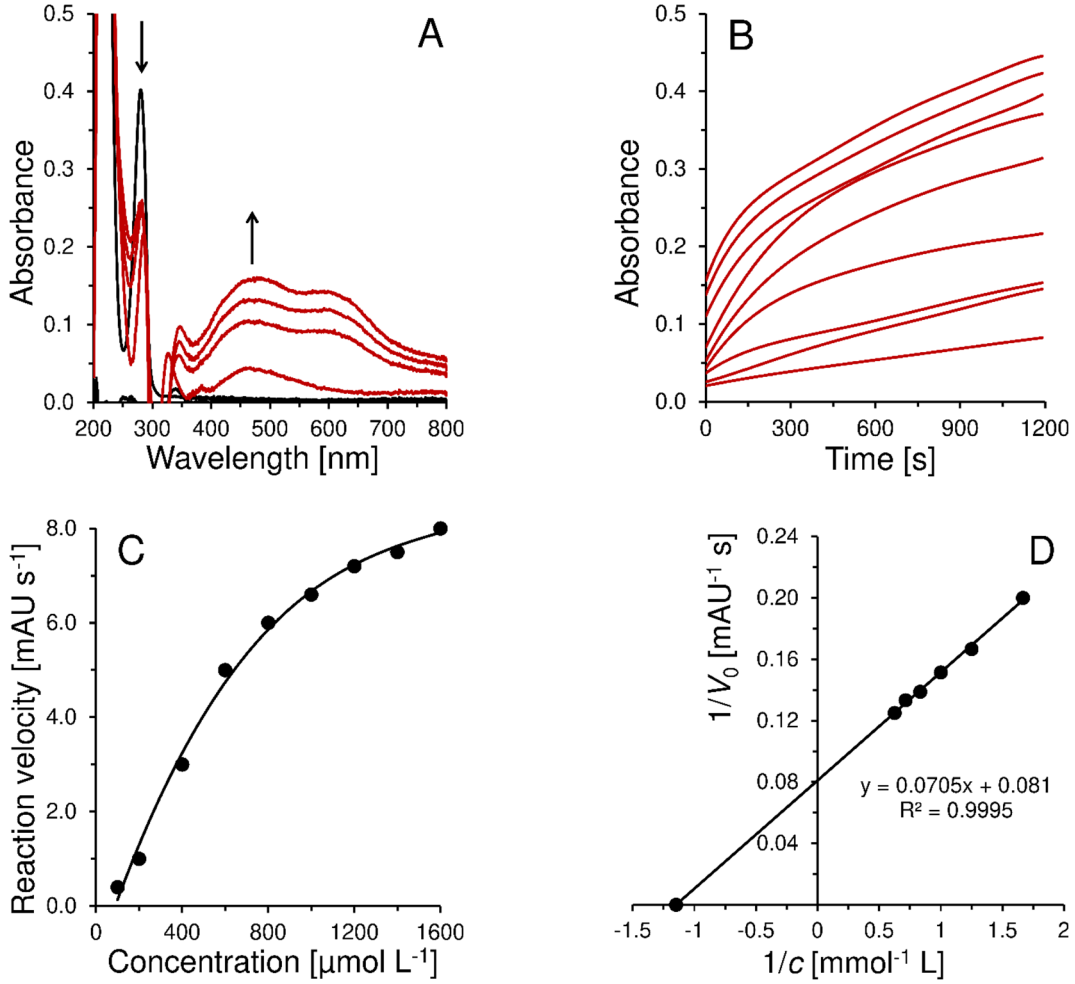

Fig. 3 Spectrophotometric kinetic study of the catecholase activity of complex **1**, $[Cu_2L(OH)(H_2O)(EtOH)](ClO_4)_2$, towards the oxidation of dopamine

The appropriate Lineweaver–Burk plot is shown in Fig. 3D, from which key parameters Michaelis constant K_m^{app} = 0.870 mmol L^{-1} dopamine and the apparent maximum reaction velocity V_{max}^{app} = 12.35 mAU s^{-1} could be obtained. Just for completeness, a lower K_m^{app} value indicates a higher affinity of enzyme or, in our case, the copper complex towards the substrate.

Moreover, an analogical kinetic study as that in the previous case was also carried out amperometrically (Fig. 4) because the product of the catalytic oxidation of dopamine (i.e. dopamine-quinone) is electrochemically reducible (see potentials applied at 0 (A), −0.1 (B), and −0.2 V (C). Kinetic curves obtained (D) were linearized in order to calculate K_m^{app} value, as illustrated in Fig. 4E.

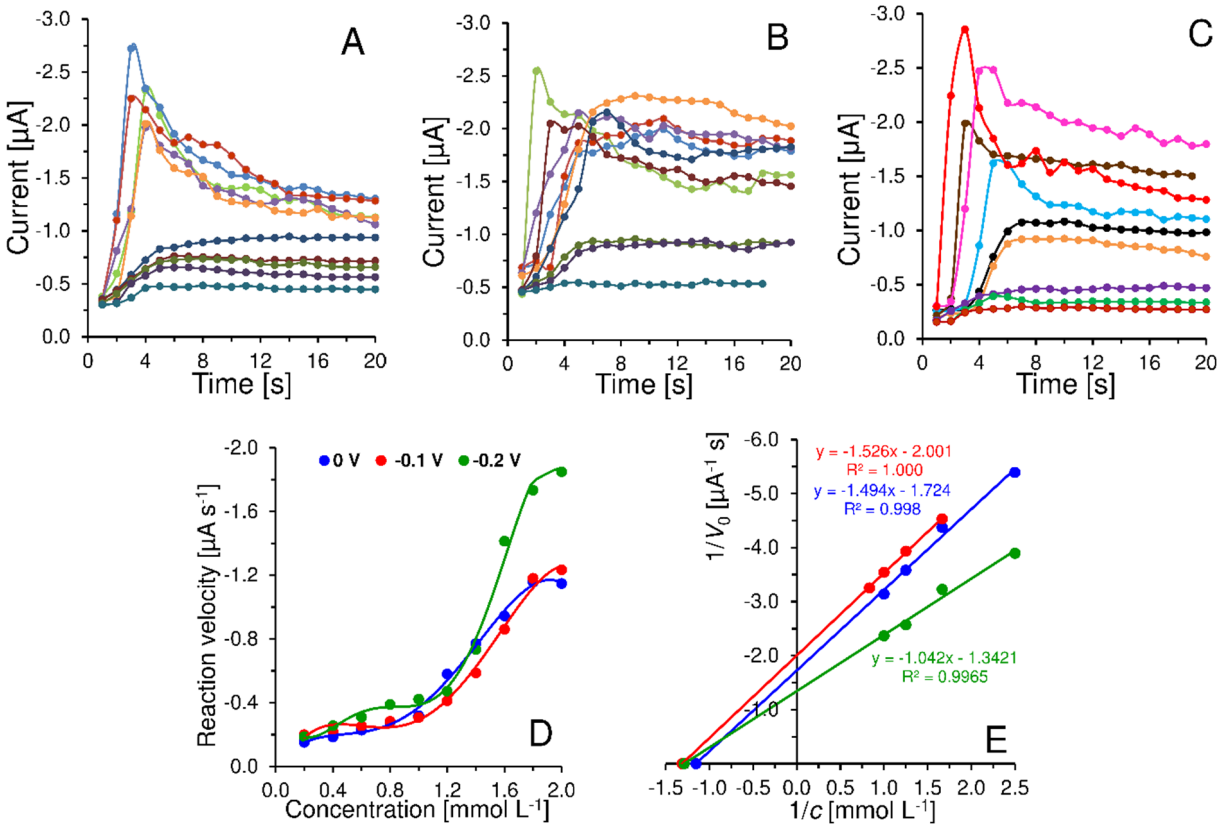

Fig. 4 Amperometric kinetic study of the catecholase activity of complex **1**, $[Cu_2L(OH)(H_2O)(EtOH)]$ $(ClO_4)_2$, towards the oxidation of dopamine

The K_m^{app} values for 0.867, 0.763, and 0.776 mmol L^{-1} dopamine were calculated together with the apparent maximum reaction velocity V_{max}^{app} of 0.745, 0.580, and 0.500 µA s^{-1} for 0, −0.1, and −0.2 V, respectively. All these findings suggest us that detection potential does not have any significant effect on the catecholase activity of complex **1** in amperometric measurements.

Furthermore, it is clear that the redox centrum of complex **1** is not affected by the electrode reaction; therefore, after catalytic oxidation of dopamine the Cu(I) atoms (remaining as the reduced form in complex) are oxidized to Cu(II) just by dissolved oxygen as in the case of TYR enzyme.

When we compare both instrumental approaches — i.e., UV-Vis spectrometry *vs.* amperometry — or rather obtained K_m^{app} values, it can be stated that both techniques offer almost the same results. In contrast to this, the second parameter (V_{max}^{app}) is hardly comparable due to the above-mentioned follow-up reaction of dopa-quinone. Anyway, it can be useful for comparison e.g. within a series of compounds in terms of examination of their catalytic activity.

Additionally, such results are comparable with K_m^{app}=0.840 mmol L^{-1} dopamine from literature (BRENDA database [10] for TYR enzyme (isolated from *Agaricus bisporus*) in 0.1 mol L^{-1} phosphate buffer (pH 7) and obtained spectrophotometrically.

Due to good agreement with the results of both discussed techniques and the respective methods, measurement of the catalytic activity of complex **2** was performed only with amperometry and a value of K_m^{app} = 1.54 mmol L^{-1} dopamine thus obtained. It reflects the influence of the complex coordination on catalytic activity. Moreover, copper(II) acetate hydrate was also tested as a catalyst, but as expected, the K_m^{app} of 8.23 mmol L^{-1} dopamine indicates a worse catalytic activity in this case.

Last but not least, although the obtained results on the catalytic activity of complexes studied and of TYR enzyme are comparable, both of them cannot be studied in the same media because of instability of TYR enzyme in non-aqueous media and vice versa, the copper complex-based catalysts are not stable in phosphate buffer – typical aqueous solution.

Conclusions

In this study, the results obtained have shown that the recently synthetized and studied dinuclear copper complexes provide in pure methanol a similar catalytic (catecholase) activity as that for natural tyrosinase enzyme in aqueous solutions of phosphate buffer (pH 7.0). In this media, the copper complexes are not stable due to the formation of a precipitate, where the N-Cu coordination bond is probably not stable enough.

Although herein-compared instrumental methods based on UV-Vis spectrophotometry and amperometry employ completely different principles, the results obtained have been consistent. Regarding the latter approach, it also offers an additional information on the electrochemical behavior of the compounds studied, which can be helpful in the future – in construction and applicability of new types of electrodes and sensors.

Acknowledgements

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