Inorg. Chem. **2002**, *41*, 3983–3989

Substrate Binding in Catechol Oxidase Activity: Biomimetic Approach

Stéphane Torelli, Catherine Belle,* Sylvain Hamman, and Jean-Louis Pierre

LEDSS, Chimie Biomimétique, UMR CNRS 5616, Université J. Fourier, B.P. 53, 38041 Grenoble Cedex, France

Eric Saint-Aman

Laboratoire d'Electrochimie Organique et de Photochimie Redox, UMR CNRS 5630, Université J. Fourier, B.P. 53, 38041 Grenoble Cedex, France

Received March 15, 2002

A series of dicopper(II) complexes have been investigated as model systems for the catechol oxidase active site enzyme, regarding the binding of catechol substrate in the first step of the catalytic cycle. The $[Cu_2(L_R)(\mu\text{-OH})](ClO_4)_2$ and $[Cu_2(L_R)(H_2O)_2](ClO_4)_3$ complexes are based on the L_R ligands (2,6-bis[{bis(2-pyridylmethyl)amino}-methyl]-4-R-substituted phenol) with $-R = -OCH_3$, $-CH_3$, or $-F$. Binding studies of diphenol substrates were investigated using UV–vis and EPR spectroscopy, electrochemistry, and ^{19}F NMR (fluorinated derivatives). All the complexes are able to bind two *ortho*-diphenol substrates (tetrachlorocatechol and 3,5-di-*tert*-butylcatechol). Two successive fixation steps, respectively fast and slower, were evidenced for the μ-OH complexes (the bis(aqua) complexes are inactive in catalysis) by stopped-flow measurement and ^{19}F NMR. From the μ-OH species, the 1:1 complex/substrate adduct is the catalytically active form. In relation with the substrate specificity observed in the enzyme, different substrate/inhibitor combinations were also examined. These studies enabled us to propose that *ortho*-diphenol binds monodentately one copper(II) center with the concomitant cleavage of the OH bridge. This hydroxo ligand appears to be a key factor to achieve the complete deprotonation of the catechol, leading to a bridging catecholate.

Introduction

Catechol oxidases (CO) are ubiquitous plant enzymes which catalyze the oxidation of a broad range of *ortho*-diphenols to *ortho*-quinones in the presence of oxygen.[1] These enzymes belong to the type 3 copper proteins characterized by a binuclear copper active site which is EPR silent in the native met $Cu^{II}-Cu^{II}$ form, due to a strong antiferromagnetic coupling between the μ-OH-bridged copper(II) atoms.[2,3] The two other known members of the type 3 copper proteins, hemocyanins (Hc) and tyrosinases (Tyr), have also been studied extensively.[4] Hemocyanins bind reversibly molecular oxygen, and tyrosinases catalyze both the hydroxylation of phenols to *ortho*-diphenols and the two-electron oxidation of *ortho*-diphenols to *ortho*-quinones (catecholase activity) by molecular oxygen. To date, the functional differences between Tyr, CO, and Hc could not be rationalized, due to a lack of structural information.

Recently, the crystal structure of catechol oxidase from sweet potatoes (*Ipomoea batatas*) has been reported in a number of forms: the oxidized $Cu^{II}-Cu^{II}$ met form (both metal binding sites involve histidine ligands, and the Cu–Cu distance is 2.87 Å); the reduced deoxy $Cu^{I}-Cu^{I}$ state; the species with the bound inhibitor phenylthiourea.[5] These structural data allow new insights into the catalytic mechanism. However, important questions remain unanswered, in particular concerning the coordination mode of the substrate: does it consist on a bridging catecholate between the two copper(II) centers[4] rather than a one metal-centered coordination?[6]

* To whom correspondence should be addressed. E-mail: Catherine.Belle@ujf-grenoble.fr.

(1) Trémolières, J. B.; Bieth, J. B. *Phytochemistry* **1984**, *23*, 501−505.
(2) Rompel, A.; Fischer, H.; Meiwes, D.; Buldt Karentzopoulos, K.; Dillinger, R.; Tuczek, F.; Witzel, H.; Krebs, B. *J. Biol. Inorg. Chem.* **1999**, *4*, 56−63.
(3) Gerdemann, C.; Eicken, C.; Krebs, B. *Acc. Chem. Res.* **2002**, *35* (3), 183−191.
(4) Solomon, E. I.; Sudaram, U. M.; Mackonkin, T. E. *Chem. Rev.* **1996**, *96*, 2563−2605.
(5) Klabunde, T.; Eicken, C.; Sacchettini, J. C.; Krebs, B. *Nat. Struct. Biol.* **1998**, *5*, 1084−1090.
(6) Eicken, C.; Krebs, B.; Sacchettini, J. C. *Curr. Opin. Struct. Biol.* **1999**, *9*, 677−683.

10.1021/ic025599d CCC: $22.00
Published on Web 06/27/2002

Dinuclear Cu^{II} complexes have received a great deal of attention owing to their interest in this field of biomimetic chemistry for an improved understanding of the function of the biological sites and as potentially bimetallic catalysts for substrate oxidation.[7−17] With dinuclear complexes, only few structurally characterized complex/substrate adducts are known. The first, described by Karlin et al.,[18] was prepared by the "oxidative addition" reaction from a phenoxo-bridged dicopper(I) complex and tetrachloro-*o*-benzoquinone (tcbq). The complex obtained presents a bridging tetrachlorocatechol (tcc) between the two copper(II) ions with a metal−metal separation of 3.248 Å. The bridging bidentate coordination of 3,5-di-*tert*-butylcatechol (3,5-dtbc), a substrate widely used to test the catecholase activity of copper complexes, with a μ-oxo dicopper(II) complex has been evidenced by Réglier et al. during a UV−vis experiment.[19] Recently,[20,21] different adducts have been isolated and characterized by X-ray crystallography. These studies exemplify various coordination modes of catecholate, i.e., monodentate or bidentate (bridging or not).

In a previous study,[22] we have described the pH-controlled changes of the metal coordination in a dicopper(II) complex of the H-BPMP (2,6-bis[{bis(2-pyridylmethyl)amino}methyl]-4-methylphenol) ligand and have shown that the catecholase activity of this complex was dependent on the copper−copper distance, which was modified according to pH changes. We have also evidenced the drastic influence on the catecholase activity of the substitution in the 4-position of the bridging phenolate by an electron-donating or an electron-withdrawing group, despite the fact that structural properties are only slightly affected by this substitution.[23]

(7) Than, R.; Feldmann, A. A.; Krebs, B. *Coord. Chem. Rev.* **1999**, *182*, 211−241.
(8) Oishi, N.; Nishida, Y.; Ida, K.; Kida, S. *Bull. Chem. Soc. Jpn.* **1980**, *53*, 2847−2850.
(9) Reim, J.; Krebs, B. *J. Chem. Soc., Dalton Trans.* **1997**, 3793−3804.
(10) Monzani, E.; Quinti, L.; Perotti, A.; Casella, L.; Gullotti, M.; Randaccio, L.; Geremia, S.; Nardin, G.; Faleschini, P.; Tabbi, G. *Inorg. Chem.* **1998**, *37*, 553−562.
(11) Manzur, J.; Garcia, A. M.; Vega, A.; Spodine, E. *Polyhedron* **1999**, *18*, 2399−2404.
(12) Malachowski, M. R.; Tomlinson, L. J.; Davidson, M. G.; Hall, M. J. *J. Coord. Chem.* **1992**, *25*, 171−174.
(13) Malachowski, M. R.; Huynh, H. B.; Tomlinson, L. J.; Davidson, M. G.; Kelly, R. S.; Furbee, J. W. *J. Chem. Soc., Dalton Trans.* **1995**, 31−36.
(14) Kao, C. H.; Wei, H. H.; Liu, Y. H.; Lee, G. H.; Wang, Y.; Lee, C. J. *J. Inorg. Biochem.* **2001**, *84*, 171−178.
(15) Gupta, M.; Mathur, P.; Butcher, R. J. *Inorg. Chem.* **2001**, *40*, 878−885.
(16) Gentschev, P.; Moller, N.; Krebs, B. *Inorg. Chim. Acta* **2000**, *300*, 442−452.
(17) Fernandes, C.; Neves, A.; Bortoluzzi, A. J.; Mangrich, A. S.; Rentschler, E.; Szpoganicz, B.; Schwingel, E. *Inorg. Chim. Acta* **2001**, *320*, 12−21.
(18) Karlin, K. D.; Guiltneth, Y.; Nicholson, T.; Zubieta, J. *Inorg. Chem.* **1985**, *24*, 3725−3727.
(19) Réglier, M.; Jorand, C.; Waegell, B. *J. Chem. Soc., Chem. Commun.* **1990**, 1752−1755.
(20) Borzel, H.; Comba, P.; Pritzkow, H. *J. Chem. Soc., Chem. Commun.* **2001**, 97−98.
(21) Ackermann, J.; Meyer, F.; Kaifer, E.; Pritzkow, H. *Chem.−Eur. J.* **2002**, *8*, 247−258.
(22) Torelli, S.; Belle, C.; Gautier Luneau, I.; Pierre, J. L.; Saint Aman, E.; Latour, J. M.; Le Pape, L.; Luneau, D. *Inorg. Chem.* **2000**, *39*, 3526−3536.
(23) Belle, C.; Beguin, C.; Gautier-Luneau, I.; Hamman, S.; Philouze, C.; Pierre, J. L.; Thomas, F.; Torelli, S. *Inorg. Chem.* **2002**, *41*, 479−491.

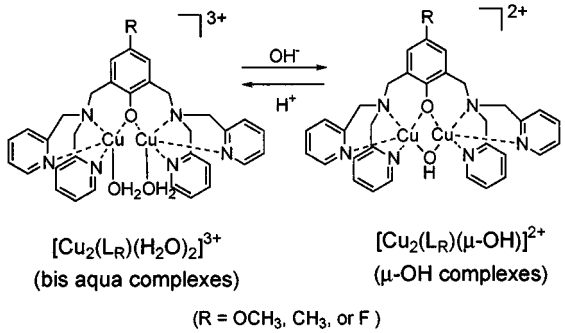

Figure 1. Dicopper(II) complexes used for binding studies.

In this work, we examine the binding properties of different substrates using these previously described dicopper(II) complexes[22,23] (Figure 1), to study the first step of the catecholase activity, i.e. *the coordination of the substrate to the copper centers before the intervening of oxygen in the mechanistic pathway*.

Experimental Section

General Remarks. The dicopper complexes $[Cu_2(L_R)(\mu\text{-OH})](ClO_4)_2$ and $[Cu_2(L_R)(H_2O)_2](ClO_4)_3$ (R = OCH_3, CH_3, or F) were prepared as described previously.[22,23] All reagents were purchased from commercial sources and used as received. Solvents were purified by standard method before used.

Safety Note. *Although no problems were encountered during the preparation of perchlorate salts, suitable care should be taken when handling such potentially hazardous compounds*.

Spectrometry. UV−vis spectra were obtained using a Perkin-Elmer Lambda 2 spectrophotometer operating in the range 200−900 nm with quartz cells. Temperature was maintained at 25 °C with a temperature control unit. ϵ values are given in M^{-1} cm^{-1}. EPR spectra were recorded on a Bruker ESP 300E spectrometer operating at 9.4 GHz (X-band), with 3 mM solutions. ^{19}F NMR spectra were recorded on a Bruker Advance 300 spectrometer in $[D_6]$acetone, at 25 °C. Chemical shifts were referenced to C_6F_6 as external reference. A typical spectrum consists on recording 3000 scans on the whole spectral bandwidth with a 500 ms relaxation delay. Kinetic measurements were performed with a Kinspec UV (Bio-Logic Co., Claix, France) stopped-flow spectrophotometer equipped with a diode array detector (J & M) and connected to a Tandon microcomputer. The data were treated on line with the commercial Bio-Kine program (Bio-Logic Co., Claix, France). Formation kinetics were carried out under pseudo-first-order conditions at 25 °C.

Catecholase Activities. The catecholase activity has been evaluated in acetone at 25 °C by using the experimental procedure previously described.[23] The reactions of the complexes with 3,5-di-*tert*-butylcatechol (3,5-dtbc), tetrachlorocatechol (tcc), 2,3-dimethylhydroquinone (2,3-dhq), or 4-ethylresorcinol (4-er) were followed by UV−vis spectroscopy. The kinetic parameters were determined for 4.5×10^{-5} M solutions of the complex and 0.225−4.5 mM solutions of the substrate.

Electrochemistry. The electrochemical behavior of millimolar solutions of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ was investigated in 0.1 M tetra-*n*-butylammonium perchlorate (TBAP) in CH_3CN using a EGG 273 potentiostat coupled with a Kipp&Zonen x-y recorder. The experiments were performed in a three-compartment cell, at room temperature, under an argon atmosphere, in a dry glovebox. Potentials are referred to an Ag/10 mM $AgNO_3$ + CH_3CN + 0.1 M TBAP reference electrode (+0.07 V vs ferrocene/ferrocenium

Table 1. Selected Properties of $[Cu_2(L_R)(\mu\text{-OH})]^{2+}$ and $[Cu_2(L_R)(H_2O)_2]^{3+}$ Complexes (R = OCH_3, CH_3, or F)

compd	Cu−Cu distance (Å)[a]	catalytic activities[b]		
		V_{max} (10^{-6} M^{-1} s^{-1})	K_M (mM)	turnover no. (90 min)
$[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$	2.980	2.2	0.25	32
$[Cu_2(L_{CH_3})(\mu\text{-OH})]^{2+}$	2.966	1.1	1.49	16
$[Cu2(L_F)(\mu\text{-OH})]^{2+}$	2.969	0.27	8.8	8
$[Cu_2(L_{OCH_3})(H_2O)_2]^{3+}$	*c*	0		0
$[Cu_2(L_{CH_3})(H_2O)_2]^{3+}$	4.139	0		0
$[Cu_2(L_F)(H_2O)_2]^{3+}$	4.084	0		0

[a] From X-ray studies.[22,23] [b] Determined with 3,5-dtbc as substrate and from a Lineweaver−Burk plot.[22,23] [c] Not determined.

used as an internal reference). The working electrode was a platinum disk of 5 mm diameter for the cyclic voltammetry (CV, 0.1 V s^{-1}) experiments or 2 mm diameter for the rotating disk electrode (RDE, 600 rpm) voltammetry experiments. The working electrode was polished with 1 μm diamond paste prior to each recording.

Results and Discussion

The catecholase activities previously described[23] are summarized in Table 1 where it appears that the affinity of the substrate (revealed by the K_M value) is the major factor tuned by R. Our series of closely related dinuclear complexes offer a unique opportunity to perform mechanistic investigations. Binding experiments with two different *ortho*-diphenols substrates, tetrachlorocatechol (tcc) and 3,5-di-*tert*-butylcatechol (3,5-dtbc), were carried out with the active $[Cu_2(L_{OCH3})(\mu\text{-OH})]^{2+}$ and the inactive $[Cu_2(L_{OCH3})(H_2O)_2]^{2+}$ complexes. During the progressive addition of the substrate, the fixation is evidenced by changes in the UV−vis and in the EPR spectra. As the oxidation of tcc is highly more difficult than that of 3,5-dtbc (the difference in redox potentials is 0.56 V), the former has been used in aerobic experiments and the latter in anaerobic conditions.

tcc Binding Studies. The changes observed in the UV−vis and EPR spectra of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ in acetone upon progressive addition of tcc are depicted in Figure 2.

The LMCT band at 440 nm is shifted up to 450 nm, and ϵ is raised from 560 to 1050 M^{-1} cm^{-1} (1 mol equiv of tcc added) and then to 2100 M^{-1} cm^{-1} (2 mol equiv of tcc added). The d−d transitions are also affected, especially during the addition of the second 1 mol equiv of substrate (Figure 2A). These features indicate that a first substrate binding occurs, followed by a second one. Further addition of tcc does not induce additional changes on the spectrum.

The OH-bridged starting complex is EPR-silent, in accordance with the strong interaction between the Cu^{II} centers.[23] Upon addition of 1 mol equiv of tcc, the spectrum reveals a $\Delta M_s = \pm 2$ signal at 150 mT and a $\Delta M_s = \pm 1$ signal from 200 to 440 mT (Figure 2B). These EPR features, similar to that obtained with the corresponding free $[Cu_2(L_{OCH3})(H_2O)_2]^{3+}$ complex,[23] indicate two copper(II) ions in a moderate interaction, thus suggesting the cleavage of the hydroxo bridge after binding of one tcc. Minor changes are then observed upon addition of the second 1 mol equiv of tcc. Addition of tcc does not induce noticeable change in the EPR spectrum of the bis(aqua) complex. The binding of 2 mol equiv of substrate on the bis(aqua) complex could be

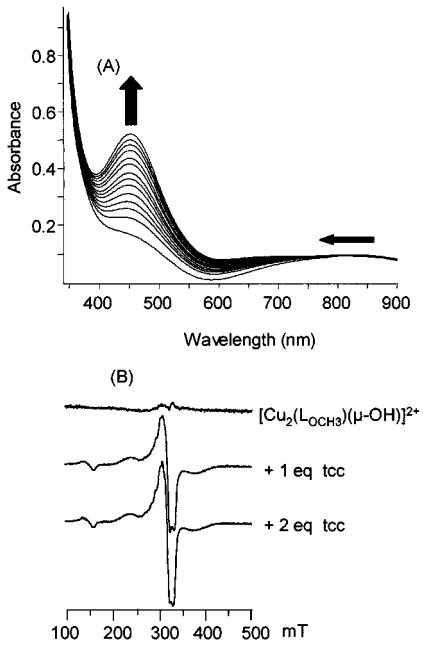

Figure 2. Changes in (A) the UV−vis spectrum (25 °C in acetone) and in (B) the EPR spectrum (77 K in acetone) of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ upon addition of tcc (from 0 to 2 equiv).

revealed only by UV−vis studies (see Figures S1 and S2 in the Supporting Information).

Because the fixation of tcc is fast, experiments have been run using stopped-flow spectrophotometer equipment. Two successive steps have been evidenced when the complex is mixed with 2 mol equiv of tcc. The fixation of the first substrate is still too fast to allow the determination of a kinetic constant, but the slower fixation of the second substrate can be measured by monitoring the absorbance changes at 460 nm with time leading to $k_{obs} = 1.3$ s^{-1} (see Figure S3 in the Supporting Information). While two successive steps have been evidenced for the fixation of two tcc's to $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$, the reaction of 2 tcc's on the $[Cu_2(L_{OCH_3})(H_2O)_2]^{3+}$ complex is too fast to be distinguished.

3,5-dtbc Binding Studies (Anaerobic Conditions) Using Paramagnetic ^{19}F NMR. As observed for tcc, the UV−vis measurements show that all the complexes are able to form 2:1 substrate/complex adduct with 3,5-dtbc in anaerobic conditions.

As previously reported, modifications in the coordination sphere around bridged dicopper(II) centers are easily evidenced by changes in fluorine chemicals shifts on ^{19}F NMR spectra, when the fluorine-labeled derivatives are available.[23,24] In Figure 3 is depicted the evolution of the ^{19}F NMR spectra of the $[Cu_2(L_F)(\mu\text{-OH})]^{2+}$ and $[Cu_2(L_F)(H_2O)_2]^{3+}$ complexes, upon progressive addition of 3,5-dtbc in $[D_6]$-acetone. For $[Cu_2(L_F)(\mu\text{-OH})]^{2+}$ (Figure 3A), the resulting spectra exhibit well-resolved resonances. The equilibrium processes are slow compared to the ^{19}F NMR time scale, leading to the observation of a mixture of two species simultaneously present in the medium. Spectra in Figure 3A show that two products are successively formed upon

(24) Torelli, S.; Belle, C.; Gautier-Luneau, I.; Hamman, S.; Pierre, J. L. *Inorg. Chim. Acta* **2002**, *333*, 144−147.

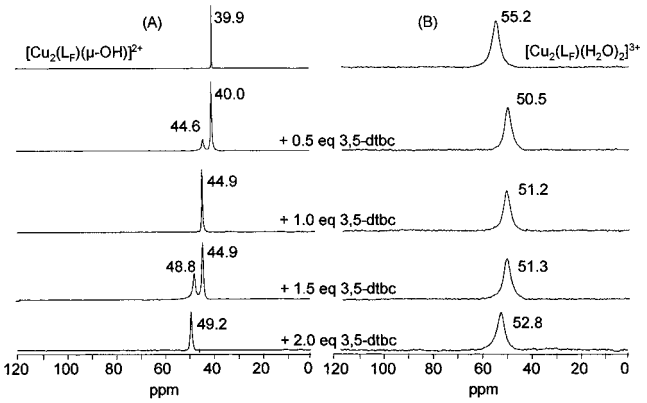

Figure 3. ^{19}F NMR (ppm vs C_6F_6) in [D_6]acetone for (A) $[Cu_2(L_F)(\mu\text{-OH})]^{2+}$ and (B) $[Cu_2(L_F)(H_2O)_2]^{3+}$ and upon progressive addition of 3,5-dtbc (from 0 to 2 equiv).

Table 2. Electrochemical Data[a,b] (V vs Ag/Ag^+) for $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ in the Presence of tcc and 3,5-dtbc (1 mol equiv)

	oxidn	redn
$[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$	0.60 (1), 0.96 (1)	−0.95 (1)
+ tcc	0.14 (1), 0.50 (1), 1.34 (2)	−0.42 (1)
+ 3,5-dtbc	0.51 (2), 0.80 (2)	−0.54 (1)

[a] Determined from RDE experiments in CH_3CN + 0.1 M TBAP. [b] In parentheses is indicated the number of exchanged electron.

addition of substrate (1 and 2 mol equiv), characterized by chemical shifts at 44.9 and 49.2 ppm, respectively. The ^{19}F NMR chemical shifts observed for each species are slightly modified due to the presence of other compounds in solution. With the bis(aqua) complex $[Cu_2(L_F)(H_2O)_2]^{3+}$, a different behavior is observed (Figure 3B). From the relatively broad signal of the starting material, addition of 1 and 2 mol equiv of 3,5-dtbc leads to the formation of different adducts characterized by a shifted signal. The peak broadening could be due to exchange processes faster than the ^{19}F NMR time scale in addition to the paramagnetic properties of the species in the solution.

These observations underline the interest of ^{19}F NMR as a probe in mechanistic studies to complete UV−vis and EPR investigations. Under the same conditions, the exchange is slow compared to the ^1H NMR time scale. Different binding processes of 3,5-dtbc have been evidenced herein. Both $[Cu_2(L_F)(\mu\text{-OH})]^{2+}$ and $[Cu_2(L_F)(H_2O)_2]^{3+}$ species can bind one or two substrates, but *different adducts* are formed. From the hydroxo-bridged species, the substrate binding occurs in two *successive* steps; on the contrary, from the bis(aqua) complex, no successive steps can be clearly evidenced.

Electrochemical Investigations. The influence of added tcc or 3,5-dtbc on the electrochemical behavior of the dicopper(II) complexes of the H-BPMP-type ligand was studied by cyclic voltammetry (CV) and rotating disk electrode (RDE) voltammetry in CH_3CN electrolyte under anaerobic atmosphere using $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ (Table 2) and $[Cu_2(L_{OCH_3})(H_2O)_2]^{3+}$ as examples. In the absence of substrate, as previously established, the electrochemical behavior of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ (Figure 4) and $[Cu_2(L_{OCH_3})(H_2O)_2]^{3+}$ is characterized by a well-defined one-electron reversible redox wave at $E_{1/2} = 0.60$ and 0.50 V, respectively,

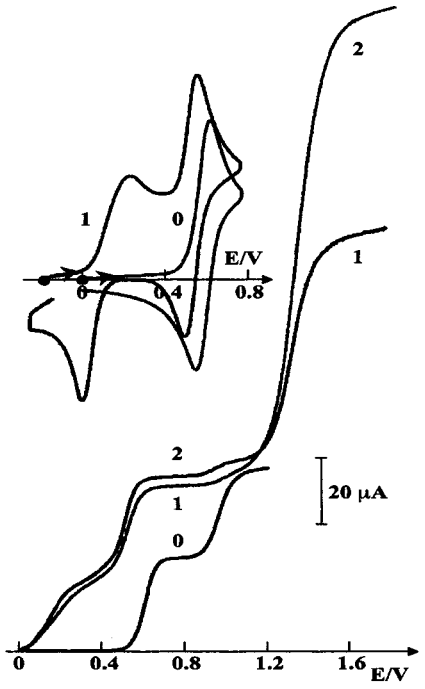

Figure 4. Voltammetric curves of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ (2 mM) in CH_3CN + TBAP 0.1 M at a Pt disk electrode (RDE (2 mm diameter), 600 rpm). Insert: Voltammetric curves (5 mm diameter) at 0.1 V s^{-1} (tcc/$[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ = 0, 1, and 2; *E* vs Ag/10 mM $AgNO_3$ + 0.1 M TBAP + CH_3CN).

attributed to the oxidation of the phenolate bridge. In the case of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$, this signal is followed at higher potential ($E_{1/2} = 0.96$ V from RDE experiment) by the irreversible one-electron oxidation of the OH bridge. In the negative region of potentials, a reversible one-electron redox wave is observed on the voltammetric curve at $E_{1/2} = -0.95$ and −0.35 V, respectively, attributable to the $Cu^{II}Cu^{II}/Cu^{II}Cu^{I}$ redox couple, the mixed-valence $Cu^{II}Cu^{I}$ species being not stable at the electrolysis time scale.

The electrochemical behavior of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ is deeply affected by the addition of tcc or 3,5-dtbc in the electrolytic solution (Table 2) despite that, as judged from RDE experiments, no change in the redox state of the metal center occurs, the copper centers remaining at the +2 redox state.

Addition of tcc (Figure 4) causes the emergence of new electrochemical signals at $E_{1/2} = 0.14$, 0.50, and 1.34 V, at the expense of the original ones. The responses at 0.14 and 0.50 V appears reversible and correspond to one-electron processes when the signal at 1.34 V corresponds to an irreversible two-electron transfer. The two first waves reach their maximal intensity after addition of 1 mol equiv in tcc and remain unmodified upon further addition in tcc. This contrasts with the behavior of the signal at 1.34 V. Its intensity increases linearly with the amount of tcc added. Moreover, the electrochemical wave due to the irreversible oxidation of the OH bridge at $E_{1/2} = 0.96$ V is no longer observed on the voltammetric curve when 1 mol equiv in tcc has been added. In the negative region of potentials, the metal-centered one-electron redox wave is shifted toward the positive potential from −0.95 up to −0.42 V and reaches its full development at a complex/tcc molar ratio equal to 1.

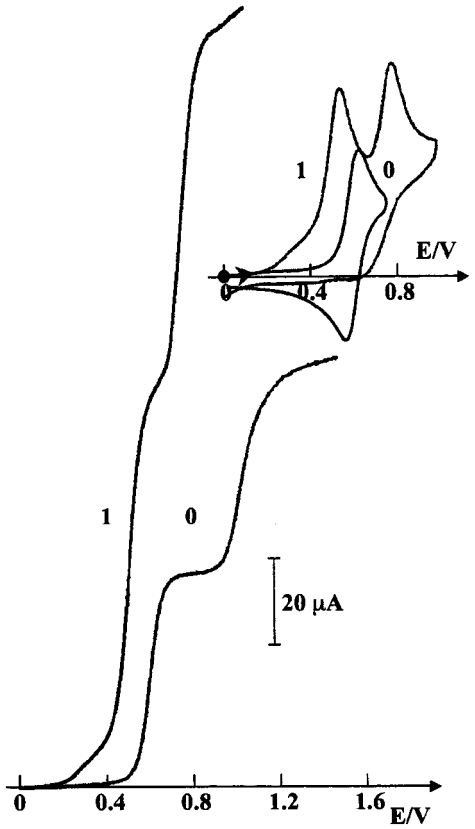

Figure 5. Voltammetric curves of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ (1 mM) in CH_3CN + TBAP 0.1 M at a Pt disk electrode (RDE (2 mm diameter), 600 rpm). Insert: Voltammetric curves (5 mm diameter) at 0.1 V s^{-1} (3,5-dtbc/$[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ = 0 and 1; *E* vs Ag/10 mM $AgNO_3$ + 0.1 M TBAP + CH_3CN).

The modifications in the electrochemical behavior of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ (Figure 5) due to the presence of 3,5-dtbc are different from those observed in the presence of tcc. Addition of 3,5-dtbc causes the emergence of only two new electrochemical signals at $E_{1/2} = 0.51$ and 0.80 V, respectively. They appear irreversible and correspond to two-electron processes. The wave at 0.51 V reach its maximal intensity after addition of 1 mol equiv in 3,5-dtbc when the signal at 0.80 V grows linearly with the amount of 3,5-dtbc added. As with tcc, the electrochemical wave due to the irreversible oxidation of the OH bridge progressively vanishes. The cathodic wave is shifted toward the more positive potential from $E_{1/2} = -0.95$ V up to -0.54 V, its full development being reached at a complex/3,5-dtbc molar ratio equal to 1.

The strong differences observed in the electrochemical response of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ with and without substrate suggest deep modifications in the coordination sphere around the metal center, the interaction with the substrate strongly affecting the electronic density around the metal center.

First, since the electrochemical response of the OH bridge vanishes in the presence of substrate, complexation causes the cleavage of the OH bridge.

On the other hand, the signals observed at 1.34 V in the presence of tcc or at 0.80 V in the presence of 3,5-dtbc are likely to be due to the electron transfer undergone by the bound substrate. It can be noticed that its interaction with the metal center in $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ induces only a slight modification in its redox potential, the latter being located at 1.34 V for free tcc and 0.78 V for free 3,5-dtbc. Consequently, as observed, the substrate-based electrochemical response of $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ in the presence of substrate increases continuously with the amount of substrate added. However, as noticed above, the complex-based electrochemical signals at 0.14 and 0.50 V with tcc, or 0.51 V with 3,5-dtbc, reach full development at a complex/substrate molar ratio equal to 1, revealing that the electroactivity of the complex is affected only by the first coordinated substrate. Interaction between the metal center with an additional substrate does not induce a significant perturbation in the complex-based electrochemical response. Two different modes of coordination are thus involved in the association process between $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ and tcc or 3,5-dtbc.

The main conclusion which can be drawn from these results is that $[Cu_2(L_{OCH_3})(\mu\text{-OH})]^{2+}$ is made more easily oxidizable (by 0.46 or 0.09 V) and reducible (by 0.53 or 0.41 V) in the presence of tcc or 3,5-dtbc, respectively. Its stability domain is decreased from 1.55 V to 0.56 or 1.05 V in the presence of tcc and 3,5-dtbc, respectively. The electrochemical reactivity of the substrate−complex species compared to the free complex is thus improved toward further electron transfer.

On the contrary, the electrochemical behavior of $[Cu_2(L_{OCH_3})(H_2O)_2]^{3+}$ is weakly affected by the addition of tcc or 3,5-dtbc in the electrolytic solution. In particular, no significant changes are observed in the characteristic potentials of the anodic and cathodic responses centered on the complex. The CV curves recorded in the presence of substrate display the superimposition of the electrochemical responses of the complex and of the added substrate, at potentials corresponding to the free forms of both species. However, the interaction between the complex and the substrate is revealed on the CV curves by the irreversibility of the complex-centered electrochemical response induced by the presence of the substrate.

Relevance to the Enzyme. (a) Substrate Specificity. A particular point of interest is the substrate specificity observed in the enzyme itself: only *ortho*-diphenols are oxidized into quinone.[25]

Comparative binding experiments with different diphenol substrates (meta and para) were carried out with the catalytically active $[Cu_2(L_{CH_3})(\mu\text{-OH})]^{2+}$ complex. Interactions with 2,3-dimethylhydroquinone (2,3-dhq) and 4-ethylresorcinol (4-er) have been pointed out by changes on the UV−vis and/or on EPR data (Table 3). In addition, binding studies with phenylthiourea (ptu), a known inhibitor of the enzyme,[2] are reported. For each of the *meta*- and *para*-diphenol substrates, no oxidation occurs, whereas the binding on the dicopper(II) complex is evidenced in UV−vis spectra by the

(25) Rompel, A.; Fischer, H.; Meiwes, D.; Buldt Karentzopoulos, K.; Magrini, A.; Eicken, C.; Gerdemann, C.; Krebs, B. *FEBS Lett.* **1999**, *445*, 103−110.

Table 3. Binding Studies of Substrates (1 mol equiv) on the $[Cu_2(L_{CH_3})(\mu\text{-OH})]^{2+}$ Complex

	UV−vis[a] λ_{max} (nm) (ϵ (M^{-1} cm^{-1}))		
compd	LMCT	d−d	EPR[b] (g)
Initial Complex[22] $[Cu_2(L_{CH_3})(\mu\text{-OH})]^{2+}$	410 (480)	785 (280)	silent
Substrates			
ortho-diphenols			
+ tcc	440 (1080)	780 (280)	4.3, 2.1
+ 3,5-dtbc[c]	465 (1800)	*d*	4.3, 2.1
para-diphenol			
+ 2,3-dhq	470 (810)	790 (290)	silent
meta-diphenol			
+ 4-er	475 (1345)	750 (280)	silent
inhibitor			
+ 1 ptu	375 (3000) 475 (1200)	785 (400)	silent

[a] Acetone solution (1 mM at 25 °C). [b] Spectral data given at 77 K and recorded in frozen solution of acetone. [c] Determined in anaerobic conditions. [d] Poorly defined.

development of an LMCT absorption near 450 nm, after addition of 1 mol equiv of substrate, together with spectral changes near 700 nm.

The observed modifications in the EPR spectrum (77 K, acetone) have been also reported in Table 3. After addition of 1 mol equiv of 2,3-dhq (or 4-er) the spectrum remains silent, indicating no important changes around the copper centers, in particular for magnetic properties.

As a bidentate coordination to one copper atom or a bridging bidentate coordination on the two copper centers is most unlikely with *meta*- and *para*- diphenols, the results argue for a monodentate coordination of one phenolate in the case of 2,3-dhq and 4-er.

The binding of phenylthiourea (ptu) on the $[Cu_2(L_{CH_3})(\mu\text{-OH})]^{2+}$ complex has been evidenced by UV−vis changes (Table 3). After addition of 1 mol equiv of ptu, the d−d transitions remain unchanged, and the EPR spectrum is still silent. These behaviors indicate no important changes around the copper centers which are in strong interaction. When the catalytic 3,5-dtbc oxidation is estimated in the presence of ptu, a strong inhibition effect is observed, as in the enzyme.[2] Attempts to characterize the adduct between ptu and a μ-OH dicopper complex by single-crystal X-ray structure studies have unfortunately been unsuccessful so far.

(b) Anaerobic Oxidation. Interestingly, on the catechol oxidase enzyme, a stoichiometric amount of the quinone is produced immediately after the addition of catechol even in absence of dioxygen.[6] It has to be emphasized that with 3,5-dtbc and all the μ-OH complexes used herein, *the formation of quinone is not observed, in the absence of oxygen*. When oxygen is introduced after the fixation of *1 equiv* of the substrate under anaerobic conditions, 1 equiv of quinone is obtained, easily monitored by the evening of the transition at 410 nm (ϵ = 1900 M^{-1} cm^{-1}, 3,5-di-*tert*-butyl quinone). When oxygen is introduced after the fixation of *2 equiv* of the substrate under anaerobic conditions, the formation of the quinone is observed: a first 1 equiv is liberated instantaneously, while a second one is released after several hours.

Conclusion

We have previously shown that the redox potentials of the dicopper complexes are not decisively related to the observed activities, since the nonactive bis(aqua) complexes are more easily reduced or oxidized than the corresponding μ-hydroxo species.[23] However in the presence of substrate, the opposite is observed: the μ-hydroxo complex is more easily oxidized or reduced than the corresponding bis(aqua) complex. The difference in reactivity between the μ-OH and the bis(aqua) complexes, which are both able to bind 2 equiv of substrate, is also revealed in the tcc binding process itself. For μ-OH species, two successive steps are evidenced for the fixation of the substrate: the first one is very fast; the second is slower. For the bis(aqua) complexes, the binding processes are too fast to be distinguished. The active species involved in the catalytic cycle is undoubtedly the 1:1 substrate/complex monoadduct. This assumption is further confirmed by the absence of noticeable modifications in the redox activity of the monoadduct upon further addition of substrate. Casella et al.[26] reached a similar conclusion for another series of dicopper complexes: the 1:1 adduct (and not the 2:1 adduct) is involved in the catalytic cycle.

Substrate specificity studies have shown that a monodentate coordination of 1 mol equiv of diphenol substrates does not allow the oxidation of *meta*- or *para*-diphenol substrates (for which a bidentate coordination is not conceivable).

EPR studies have shown that the μ-OH bridge was cleaved in the mono as well as in the bis adducts of *ortho*-diphenol substrates. ^{19}F NMR studies have clearly revealed that from the μ-OH complex two different adducts are generated, while from the bis(aqua) compound they are undistinguishable. It has to be emphasized that the larger copper−copper distance in the bis(aqua) complexes is unfavorable to a bridging bidentate coordination of a catecholate substrate as pointed out by others.[8,9]

All together, these observations argue for a bridging bidentate coordination of one *ortho*-diphenol substrate on one μ-OH complex, with concomitant cleavage of the hydroxo bridge. Moreover, the electrochemical studies and taking into account the proton balance allow one to propose a more discerning mechanism (Figure 6). It does not involve (in the first step) a true bridging bidentate coordination but a monodentate coordination associated with an interaction between the second phenol group of the catechol substrate and the hydroxyl group bound to the second copper center (arising from cleavage of the initial hydroxo bridge). Then, the proton transfer can occur, followed by the displacement of a water molecule and the bridging coordination of the catecholate. This hypothesis is in accordance with the overall results (unreactivity of *meta*- and *para*-diphenol substrates, unreactivity of the bis(aqua) complexes, *ortho*-diphenol substrates binding with concomitant cleavage of the hydroxo bridge). The mechanism depicted in Figure 6 is related to the two proposals for the enzymatic catalytic cycle (substrate

(26) Monzani, E.; Battaini, G.; Perotti, A.; Casella, L.; Gullotti, M.; Santagostini, L.; Nardin, G.; Randaccio, L.; Geremia, S.; Zanello, P.; Opromolla, G. *Inorg. Chem.* **1999**, *38*, 5359−5369.

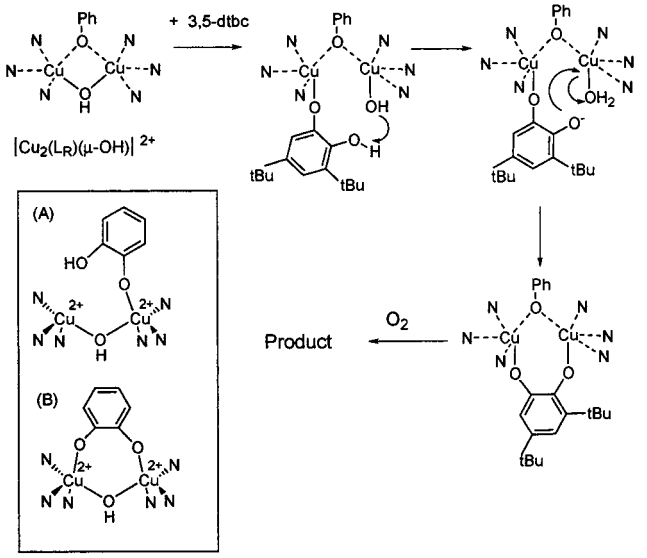

Figure 6. Proposed mechanism for the interaction between the series of dinuclear copper(II) μ-OH complexes and the 3,5-dtbc substrate. Insert: (A) intermediate proposed by Krebs;[6] (B) intermediate proposed by Solomon.[4]

fixation step): the first adduct corresponds to the adduct proposed by Krebs (Figure 6A),[6] and the following one, to the adduct proposed by Solomon (Figure 6B).[4] The complete deprotonation of the monodentate bound catechol leads to a bridging catecholate prior to the electron transfer.

This study sheds new light concerning the binding of the first *ortho*-diphenol substrate. Our proposed mechanism reconciles the two previously described intermediates implied in the enzymatic catalysis, demonstrates the importance of the hydroxo bridge cleavage during the fixation of the catecholate, and underlines the role of the resulting pendant hydroxo ligand.

Acknowledgment. The authors are grateful to Dr. Alain Deronzier for his interest in this work, to André Jeunet for his assistance in the EPR spectroscopic studies, and to Dr. Fabrice Thomas for stopped-flow measurements.

Supporting Information Available: A demonstration of the evolution of UV−vis (Figure S1) and EPR spectra (Figure S2) of $[Cu_2(L_{OCH_3})(H_2O)_2]^{3+}$ upon progressive addition of tcc and stopped-flow measurements for the second 1 equiv of tcc fixation on the $[Cu_2(L_{OCH_3})(\mu\text{-}OH)]^{2+}$ complex (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

IC025599D