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Kinetic and Theoretical Comprehension of Diverse Rate Laws and Reactivity Gaps in *Coriolus hirsutus* Laccase-Catalyzed Oxidation of Acido and Cyclometalated Ru^{II} Complexes

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ABSTRACT: The reactivity of the acido Ru^{II} complexes *cis*-[$RuCl_2(LL)_2$], [$RuCO_3(LL)_2$], *cis*-[$RuCO_3$-(bquin)$_2$] (LL = 2,2′-bipyridine (bpy) and 1,10-phenanthroline (phen); bquin = 2,2′-biquinoline) and cyclometalated Ru^{II} derivatives of 2-phenylpyridine and 4-(2-tolyl)pyridine [Ru(*o*-C_6H_4-2-py)(phen)$_2$]PF_6 (**1**), [Ru(*o*-C_6H_3-*p*-R-2-py)(bpy)(MeCN)$_2$]PF_6 (**2**), and [Ru(*o*-C_6H_3-*p*-R-2-py)(phen)(MeCN)$_2$]PF_6 (**3**) (R = H (**a**), Me (**b**)) toward laccase from *Coriolus hirsutus* has been investigated by conventional UV−vis spectroscopy at pH 3−7 and 25 °C. The acido and cyclometalated complexes are readily oxidized into the corresponding Ru^{III} species, but the two types of complexes differ substantially in reactivity and obey different rate laws. The acido complexes are oxidized more slowly and the second-order kinetics, first-order in laccase and Ru^{II}, holds with the rate constants around 5×10^4 M^{-1} s^{-1} at pH 4.5 and 25 °C. The cyclometalated complexes **1**−**3** react much faster and the hyperbolic Michaelis−Menten kinetics holds. However, it is *not* due to formation of an enzyme−substrate complex but rather because of the ping-pong mechanism of catalysis, viz. $E(ox) + Ru^{II} \rightarrow E(red) + Ru^{III}$ (k_1); $E(red) + 1/4O_2 \rightarrow E(ox)$ (k_2), with the rate constants k_1 in the range $(2-9) \times 10^7$ M^{-1} s^{-1} under the same conditions. The huge values of k_1 move the enzymatic oxidation toward a kinetic regime when the dioxygen half-reaction becomes the rate-limiting step. Cyclometalated compounds **1**−**3** can therefore be used for routine estimation of k_2, that is, the rate constant for reoxidation for laccases by dioxygen. The mechanism proposed was confirmed by the direct stopped-flow measurements of the k_2 rate constant (8.1×10^5 M^{-1} s^{-1} at 26 °C) and supported by the theoretical modeling of interaction between the bpy analogue of **1** and *Coriolus hirsutes* laccase using Monte Carlo simulations.

Laccases are copper-containing oxidizing enzymes that use dioxygen as a primary oxidizing agent for degradation of phenols (*1*−*4*). Impressive expansion of principles and rules of green chemistry in everyday life and modern technology (*5*, *6*) increased immensely the applications of laccases, for example, as catalysts for bleaching of industrial dyes, decolorizing pulp and paper effluents (*3*, *7*−*9*), and as components of biofuel cells (*10*). Principle advantages of laccases are their stability and use of O_2 as an essentially free oxidizing agent. The major problem is that laccase itself, though the selectivity of the enzyme is very broad and the reduction potentials vary in ca. 0.4−0.8 V range (versus NHE) (*11*), cannot degrade all constituents of lignin (*12*, *13*). A solution here is its enzyme-catalyzed mediated oxidation (*14*). Particularly challenging is using transition metal complexes as mediators for oxidoreductases (*3*, *14*). From this standpoint, recent studies of laccase-catalyzed oxidation of transition metal ions (*4*) get an extra impulse.

We are particularly interested in advancing technologies that use both laccase from the white-rot fungus *Coriolus hirsute* (*15*−*17*) (EC 1.10.3.2: benzenediol:oxygen oxidoreductase [according to BRENDA] (*18*)) and cyclometalated $Ru^{II/III}$ and $Os^{II/III}$ complexes both resistant to substitution and labile. The transition metal compounds contain a metal−carbon σ-bond, which is stabilized by chelation via a nitrogen donor. They are decent mediators of the electron relays involving several oxidoreductases such as glucose oxidases and peroxidases from different sources and PQQ-dependent dehydrogenases (*19*−*23*). Cyclometalated Ru^{II} complexes are rapidly reacting electron donors for artificial man-made mimics of peroxidases, the so-called Fe^{III}-TAML activators of hydrogen peroxide (*24*). Examples of such complexes **1**−**4** involved in this work are shown in Chart 1. The second-order rate constants for the electron exchange between the complexes and peroxidases, glucose oxidases, or PQQ

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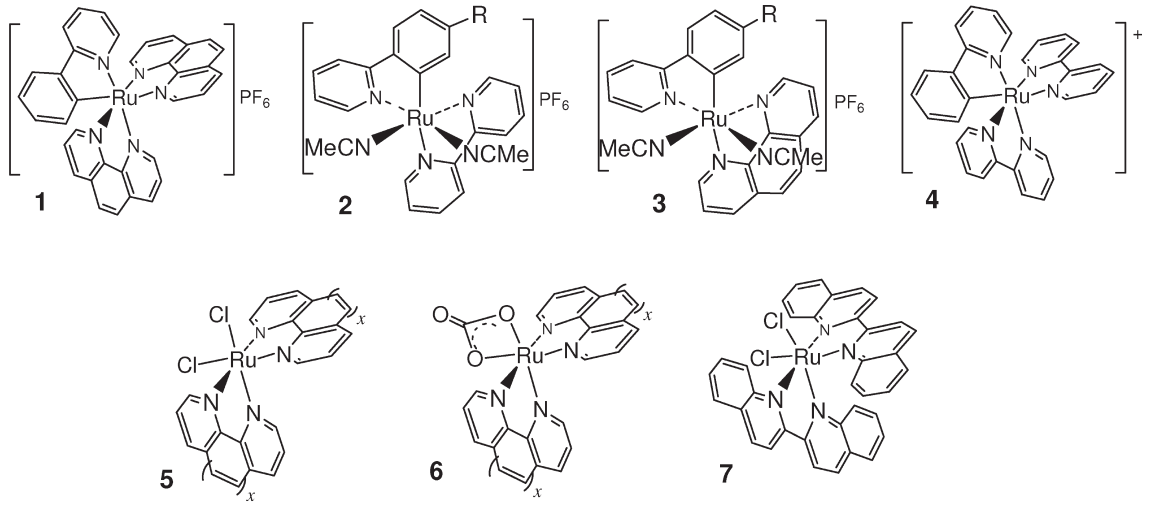
Chart 1: Cyclometalated (**1**−**4**) and Acido (**5**−**7**) Ruthenium(II) Complexes Involved in This Study



R = H (a), CH_3 (b); x = 0 (c), 1 (d)

dehydrogenases reach the 10^7−10^8 M^{-1} s^{-1} level. Here, two directions of research interest meet, and this work reports on the results of kinetic studies of *Coriolus hirsutus* laccase-catalyzed oxidation of cyclometalated Ru^{II} complexes **1**−**3**. It is shown that the cyclometalated fragment of complexes **1**−**3**, that is, the σ-C−Ru bond stabilized by chelation, brings about a superior reactivity that is considerably higher compared to that of traditional acido Ru^{II} complexes with the diimine ligands 2,2′-bipyridine and 1,10-phenanthroline such as *cis*-$[RuCl_2(bpy)_2]$,[1] *cis*-$[RuCl_2(phen)_2]$, or $[RuCO_3(bpy)_2]$ (**5**−**7**, Chart 1). High reactivity of **1**−**3** changes the rate law and drives the oxidation into a kinetic regime where the reoxidation of reduced laccase by dioxygen contributes to the overall rate. Thus, complexes **1**−**3** can be used as reactive reagents for estimating the rate constants for reactions of laccases with O_2. The mechanistic conclusions made in this work are strengthened by direct measurements of kinetics of reoxidation of reduced laccase by dioxygen using the stopped flow-technique and a theoretical analysis of mechanistic patterns of oxidation of a cyclometalated Ru^{II} complex by laccase using the functional docking Monte Carlo simulation.

METHODS

Materials. Acido Ru^{II} complexes **5**−**7** were prepared as described elsewhere (*25*−*27*). Orthometalated complexes **1**, **2**, and **3** were synthesized as previously reported (*19*, *28*). Laccase from *C. hirsutus* was obtained as described previously (*16*). 4-Hydroxyphenylacetic acid (purum) was from Fluka, Milwaukee, WI. Air Liquide Danmark A/S supplied mixtures 10% and 15% oxygen in nitrogen (mol/mol) and argon (Alphagaz Ar1). The catechol oxidase activity of laccase was determined as previously reported (*16*), and it equals 3.38×10^{-6} mol s^{-1} mg^{-1}. All chemicals used as buffers and substrates were commercial products of at least reagent grade, unless otherwise indicated.

Purification of Laccase for Stopped-Flow Studies. Prior to transient state kinetics the laccase was further purified by gel filtration on a 1000 mL XK50 column loaded with sephadex G-75 material (GE Healtcare Life Sciences). Column material was equilibrated with 20 mM sodium phosphate and 150 mM sodium chloride pH 7.0. Column eluates were monitored at 280 nm. Fractions were collected, pooled, and ultrafiltrated before being transferred to 20 mM Tris-acetate buffer (pH 8.0) by diafiltration (four times) to remove chloride ions which inhibit reoxidation of laccase by O_2. The laccase was stored at −80 °C.

Methods. Electronic spectra were obtained on a Shimadzu U-160A spectrophotometer. Electrochemical measurements were performed on a PC-interfaced potentiostat-galvanostat IPC-4 (Institute of Physical Chemistry, RAS, Moscow, Russia). A three-electrode scheme was used with a BAS working glassy carbon electrode, saturated calomel or Ag/AgCl reference electrodes, and auxiliary Pt electrode. Before measurements the working electrode was polished with a diamond paste and rinsed with ethanol and distilled water. Reaction rates at 25 °C were measured spectrophotometrically using Hitachi 150-20 and Hitachi 557 spectrophotometers. Stock solutions of Ru-complexes were prepared in acetonitrile as solvent. Stock solution of laccase (3.8×10^{-7} M) was prepared in 50 mM phosphate buffer (pH 6.5). The stock solution of laccase was added to 100 mM acetate buffer (pH 4.5) to achieve a final concentration of 3.8×10^{-9} M. The reactions were initiated by addition of the stock solutions of Ru complexes to solutions of laccase in the acetate buffer to achieve a final concentration of complexes in the range 1×10^{-5} to 3×10^{-4} M. The reaction progress was monitored by an absorbance decrease at a wavelength corresponding to a maximum absorbance of Ru^{II}. The initial rates were calculated from dependences of absorbance versus time. All steady-state measurements were done in triplicate.

Stopped-Flow Measurements. The reaction of reduced laccase with dioxygen was studied at various temperatures using a DX17MV stopped-flow spectrometer from Applied Photophysics, Surrey, UK. The dead time of the instrument was 1.5 ms. The concentration of dissolved dioxygen was controlled by purging the buffer with various gas mixtures of dioxygen and nitrogen and atmospheric air. Concentrations of dioxygen dissolved in the buffer at different temperatures were calculated based on data from the literature (*29*). The data are based on pure

[1]Abbreviations: bpy, 2,2′-bipyridine; phen, 1,10-phenanthroline; bquin, 2,2′-biquinoline; ABTS, [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)]; HRP, horseradish peroxidase; PQQ, pyrroloquinoline quinone.

Table 1: Second-Order Rate Constants for the Oxidation of Acido Ru^{II} Complexes Catalyzed by *Coriolus hirsutus* Laccase Obtained at the Optimal pH at 25 °C

complex	pH	$k_{Ox}/M^{-1}\ s^{-1}$	HRP pH 6.7, from ref *39*	reduction potential/V vs Ag/AgCl
cis-$[RuCl_2(bpy)_2]$ (**5c**)	4.5	$(5.0 \pm 0.3) \times 10^4$	$(1.54 \pm 0.05) \times 10^4$	0.42
$[RuCO_3(bpy)_2]$ (**6c**)	4.5	$(5.0 \pm 0.4) \times 10^4$	$(1.26 \pm 0.01) \times 10^4$	0.42
	5.75	$(15 \pm 0.8) \times 10^4$		0.38
cis-$[RuCl_2(phen)_2]$ (**5d**)	4.5	$(4.6 \pm 0.2) \times 10^4$	$(15 \pm 4) \times 10^4$	0.39
$[RuCO_3(phen)_2]$ (**6d**)	4.5	$(5.1 \pm 0.3) \times 10^4$		0.37
cis-$[RuCl_2(bquin)_2]$ (**7**)	5.0	$(14 \pm 1.1) \times 10^4$		0.63

water, and no correction was made concerning the effect of ionic strength on the solubility of dioxygen in 0.10 M buffer. Oxygen concentrations determined by oxygen electrode measurements were, within experimental error, the same in pure water and in our buffers in accordance with data from Millero et al. (*30*). The buffer employed was made from sodium acetate (0.10 M) adjusted to pH 5.0 with acetic acid. The temperature of the reaction chamber was controlled by a closed circulating water bath. To obtain varying concentrations of dioxygen in the buffer, a specially designed mixing chamber was used to purge the buffer with atmospheric air or gas containing either 10 or 15% dioxygen in nitrogen (mol/mol). Because of the problem with diffusion of dioxygen through different types of polymers such as polyethylene and PVC, the mixing chamber was designed in glass to avoid contamination with O_2 from the atmosphere and was fitted directly to one of the inlets of the stopped-flow apparatus. The mixing chamber was an 80 mL glass canister equipped with a thermostatic collar connected to a water bath. The gas was purged through the buffer by a glass filter and to prevent contamination by atmospheric air. The tonometer was equipped with an air lock filled with buffer. In a second glass syringe, fitted directly to the stopped-flow apparatus, 1950 μL of buffer and 100 μL of reducing substrate (4-hydroxyphenylacetic acid; 175.0 μM) were purged with argon for at least 10 min before 40 μL of laccase (ca. 7 μM) was added with a Hamilton syringe. The concentration of the laccase stock solution was based on its estimated molar absorbance of 6100 $M^{-1}\ cm^{-1}$ (*31*). Before the experiments were run, the solutions were kept for 3 min for temperature equilibration. During stopped-flow experiments equal volumes (75 μL) of buffer with known O_2 concentration and reduced enzyme solution were mixed giving a final laccase concentration of ca. 3.5 μM. The absorbance was monitored at 610 nm and 18−20 replicates were carried out for each measurement.

Computational Details. Monte Carlo reactive docking calculations were performed as described elsewhere (*32*) using the HARLEM program (*33*). Effective interactions between laccase and complex **4** included electrostatic interactions, steric interactions, and the term corresponding to the electron-transfer rate between Ru and T1 Cu centers computed using PATHWAYS model (*34*). The nonadiabatic model for estimation of relative rates of the electron transfer between ruthenium(II) and laccase copper was applied, according to which the rates are proportional to H_{DA}^2, that is, the electronic coupling element computed by PATHWAYS, because reorganization energies and absolute rates of electron transfer were not estimated. The Monte Carlo simulations with the effective energy functional, which comprise the intermolecular interaction energy and the electron transfer rate (*32*), probe those configurations that contribute to the bimolecular electron transfer. The model of 3D structure of *Coriolus hirsutus* laccase was built from X-ray data (*35*) and was assumed rigid in calculations. Electrostatic interactions between **4** and laccase were computed using the "Charges in Field" model as a sum over atomic charges of **4** multiplied by values of the electric field at atomic positions of the complex created by laccase atoms and computed using the Poisson−Boltzmann routine. Atomic charges of complex **4** atoms were derived from DFT (BLYP-3 functional, 6-31G* basis set) calculations of the reduced and oxidized forms of the compound (formal charges +1e and +2e, respectively) using the Merz−Kollman charge-fitting scheme (*36*) and GAUSSIAN-98 (*37*). Finite-difference solutions of Poisson−Boltzmann equation for laccase electrostatic filed used 241 × 241 × 241 grid, 100 mM ionic strength. Steric interactions between laccase and **4** were computed using the exclusion volume of laccase expanded on a 241 × 241 × 241 grid. PATHWAYS calculations of donor/acceptor electronic interactions used a decay constant for intermolecular nonbonded constant of $\beta_{NB} = 0.9\ Å^{-1}$ as was adopted elsewhere (*32*). Other PATHWAYS model parameters are as used by Beratan et al. (*34*). The AMBER-94 charges were used as introduced by Conell et al. (*38*).

RESULTS

*Kinetics of Laccase-Catalyzed $Ru^{II} \rightarrow Ru^{III}$ Oxidation of the Acido Complexes **5**−**7***. Kinetic features of the laccase-catalyzed oxidation of acido ruthenium(II) complexes **5**−**7** (eq 1), which are summarized in Table 1, have been found to have much in common with the HRP-catalyzed oxidation of these complexes by H_2O_2 (*39*). In particular, the oxidation rate is directly proportional to laccase and Ru^{II} concentrations in the ranges of (1−100) × 10^{-9} M and (2−10) × 10^{-4} M, respectively. The corresponding second-order rate constants (Table 1) are around 5 × 10^4 $M^{-1}\ s^{-1}$. Curiously, similar rate constants have been measured for the HRP-catalyzed oxidation of these compounds (*39*).

$$4Ru^{II} + O_2 + 4H^+ \rightarrow 4Ru^{III} + 2H_2O \quad (1)$$

As in the HRP case, the first-order kinetics in Ru^{II} does not support a mechanistic model that involves the formation of a kinetically meaningful intermediate between an acido Ru^{II} complex and laccase. This is however not surprising for the enzyme of a broad substrate specificity, which oxidizes unnatural targets, that is, transition metal complexes. It should be mentioned that complexes **5**−**7** listed in Table 1 undergo hydrolysis in an aqueous solution to form cationic aqua species such as $[Ru(bpy)(H_2O)_2]^{2+}$ (*39*, *40*). The products of hydrolysis with easy-to-substitute aqua ligands could have a stronger tendency to bind to donor amino acid residues of laccase. Experimental support for the formation of hydrolyzed species is presented in Figure 1 where the reduction potentials of $[Ru(CO_3)(bpy)_2]$ measured by

the cyclic voltammetry are plotted against pH. There is a linear dependence with a slope of −52 ± 2 mV indicating that the compound becomes more reducing with an increase in pH and one proton is involved in the process (*41*). Nevertheless, there is no kinetic evidence for formation of the enzyme−substrate Michaelis complex. The data in Figure 1 agree with a higher rate constant for the oxidation of **6c** at pH 5.75 than at pH 4.5 as a result of increasing the reaction driving force that increases the rate of intermolecular electron transfer (*42*).

The pH profile for the catalytic activity of laccase in the oxidation of *cis*-[$RuCl_2(bpy)_2$] shown in Figure 2 is typical of oxidations by this family of enzymes (*1*) and can be compared with that observed for hexacyanoferrate(II) (*43*). The activity remains practically constant in the pH range 3−5 but declines sharply at pH > 5.5. The enzyme is practically inactive at pH 7.

*Kinetics of Laccase-Catalyzed $Ru^{II} \rightarrow Ru^{III}$ Oxidation of the Cyclometalated Complexes **1**−**3***. The laccase-catalyzed formation of cyclometalated Ru^{III} species via eq 1 was confirmed by the EPR measurements by the example of complex **2a** (Figure 1S, Supporting Information). Cyclometalated compounds such as **1**−**3** are commonly more reactive toward enzymes as compared to related acido complexes such as **5**−**7** in both oxidizing or reducing enzymatic half-reactions (*19*, *39*, *40*). The data reported here show that this relation holds for the *Coriolus hirsutus* laccase.

The reactivity of **1**−**3** is noticeably higher than that of the acido Ru^{II} complexes under the same conditions (Figure 3; Tables 1 and 2). Additionally, there is a rate law changeover — the oxidation of the cyclometalated Ru^{II} species follows a hyperbolic dependence on Ru^{II} concentration formally corresponding to the Michaelis−Menten equation. The second-order kinetics as for the acido Ru^{II} complexes **5**−**7** does not hold. There are two *principal* explanations of this kinetic changeover. First, it could be due to the formation of reactive intermediate between laccase and a cyclometalated complex followed by the rate-limiting intramolecular electron transfer from Ru^{II} to a T1 copper of the enzyme (*44*) (mechanism 1 in Scheme 1). Alternatively, the hyperbolic kinetics may arise from a change of the rate-limiting step. Laccase is a two-substrate oxidoreductase and a slower step may be the reoxidation of reduced laccase by dioxygen (a ping-pong mechanism 2 in Scheme 1).

Mechanisms 1 and 2 (Scheme 1) lead to eqs 2 and 3, respectively. Both mechanisms predict the leveling off of the reaction rate at high concentrations of Ru^{II}.

$$v = \frac{k_2[Ru^{II}][E]_t}{\frac{k_{-1}+k_2}{k_1} + [Ru^{II}]} \quad (2)$$

$$v = \frac{k_2[O_2][Ru^{II}][E]_t}{\frac{4k_2[O_2]}{k_1} + [Ru^{II}]} \quad (3)$$

Fitting the data in Figure 3 to eqs 2 and 3 allows the calculation of parameters of both equations. These are k_2 and $(k_{-1}+k_2)/k_1$ for eq 2 and $4k_1[O_2]$ and k_2 for eq 3. The numerical values of the rate constants are shown in Table 2. An equation similar to 3 has been previously put forward for the horseradish HPR-catalyzed oxidation of complexes **1**−**3** by hydrogen peroxide (*19*). Using complexes such as **1** allowed the reliable estimation of the rate constant for the oxidation of the resting state of HRP into its compound I by H_2O_2. Similarly, the rate constant for the reoxidation of reduced laccase by O_2 can be estimated. Below

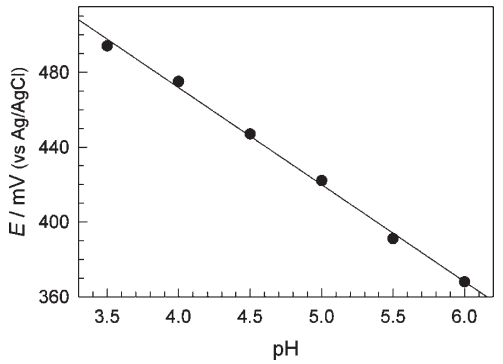


FIGURE 1: Reduction potential of [$Ru(CO_3)(bpy)_2$] (**6c**) measured by cyclic voltammetry in 0.01 M phosphate at 25 °C.

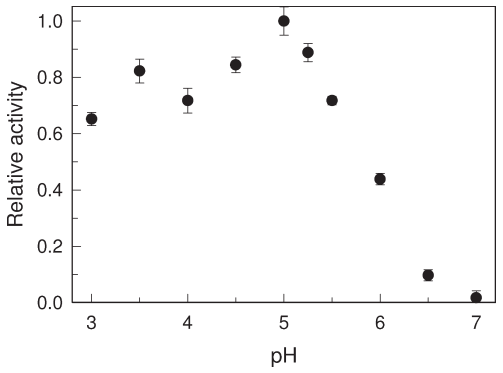


FIGURE 2: Normalized second-order rate constants for the oxidation of *cis*-[$RuCl_2(bpy)_2$] (**5c**) by laccase in citrate-phosphate buffer (0.01 M) at 25 °C.

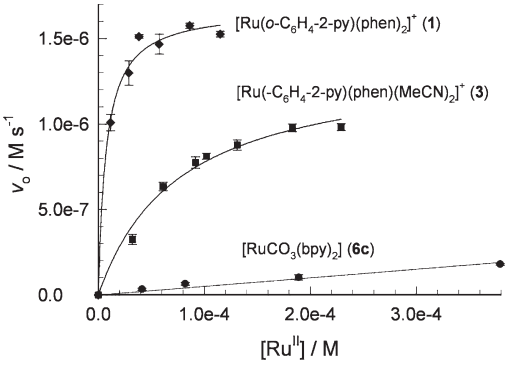


FIGURE 3: Initial rates of laccase-catalyzed oxidation of orthometalated Ru^{II} complexes. Data for [$Ru(CO_3)(bpy)_2$] (**6c**) are included for comparison. Conditions: [laccase] 4.2×10^{-9} M, citrate buffer (0.01 M), 25 °C.

we present evidence in favor of mechanism 2 that includes (i) direct single turnover measurements of the rate constants k_2 for the oxidation of reduced laccases by O_2 and (ii) computational docking analysis of laccase from *Coriolus hirsutus* using the Monte Carlo routine, which showed that this enzyme does have energetically favorable binding sites for a cyclometalated complex neither in the vicinity of crucial for electron transfer copper T1 site nor within the entire protein globule. This theoretical modeling became possible due to the recently reported X-ray structural characterization of the enzyme (*35*).

Stopped-Flow Kinetic Measurements of the Half-Reaction between Reduced Coriolus hirsutus Laccase and Dioxygen. The oxidation of reduced laccase and dioxygen

Table 2: Kinetic Parameters of eqs 2 and 3 for the Oxidation of Cyclometalated Ru^{II} Complexes **1**−**3** by *Coriolus hirsutus* Laccase Obtained at pH 4.5 (0.05 M acetate, 2% MeCN) and Their Reduction Potentials Measured by CVA under the Same Conditions

	eq 2		eq 3		
complex	k_2/s^{-1}	$(k_{-1} + k_2)/k_1/M$	$k_2[O_2]/s^{-1}$	$k_1/M^{-1}\ s^{-1}$	E/V vs Ag/AgCl
1	620 ± 60	$(3.1 \pm 0.5) \times 10^{-5}$	620 ± 60	$(8.0 \pm 2.0) \times 10^{7}$	0.24
3a	500 ± 40	$(7.3 \pm 0.1) \times 10^{-5}$	500 ± 40	$(2.7 \pm 0.3) \times 10^{7}$	0.26
2a	294 ± 9	$(1.3 \pm 0.2) \times 10^{-5}$	294 ± 9	$(9.1 \pm 1.7) \times 10^{7}$	0.29
2b	650 ± 100	$(6.5 \pm 2.6) \times 10^{-5}$	650 ± 100	$(4.0 \pm 2.2) \times 10^{7}$	0.26
3b	455 ± 60	$(8.3 \pm 1.7) \times 10^{-5}$	455 ± 60	$(2.2 \pm 0.7) \times 10^{7}$	0.23

Scheme 1: Plausible Mechanisms of the Laccase-Catalyzed Oxidation of Cyclometalated Ruthenium(II) Complexes That Lead to the Michaelis−Menten Type Dependence in Figure 3

$$E(ox) + Ru^{II} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \{E(ox),Ru^{II}\} \qquad E(ox) + Ru^{II} \xrightarrow{k_1} E(red) + Ru^{III}$$

$$\{E(ox),Ru^{II}\} \xrightarrow{k_2} E(red) + Ru^{III} \qquad E(red) + {}^1/_4O_2 \xrightarrow{k_2} E(ox)$$

$$E(red) + O_2 \xrightarrow{fast} E(ox) \qquad \textbf{1} \qquad \textbf{2}$$

was found to follow a single exponential behavior (Figure 4) consistent with a pseudo-first-order reaction with O_2 in a large excess. The observed pseudo-first-order rate constant k_{obs} for the reoxidation of laccase was measured at various concentrations of dioxygen.

Figure 5 shows that k_{obs} is directly proportional to the dioxygen concentration as in eq 4 at 10, 26, and 38 °C. This observation agrees with the previous studies of reoxidation of reduced laccases by O_2 (*45*, *46*).

$$k_{obs} = k_{O_2}[O_2] \tag{4}$$

The second-order rate constants k_{O_2} equal $(4.8 \pm 0.2) \times 10^5$, $(8.1 \pm 0.8) \times 10^5$, and $(1.2 \pm 0.1) \times 10^6\ M^{-1}\ s^{-1}$ at 10, 26, and 38 °C, respectively. The data obtained at 26 and 38 °C may be affected by a slight diffusion of dioxygen through the hose from the syringes to the reaction cell of the stopped-flow system. The values of k_{O_2} obtained in this work are similar to those reported previously for laccases from various species, ceruloplasmin, and ascorbate oxidase (*45*, *47*−*49*). The activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of 21 ± 2 kJ mol^{-1} and -62 ± 8 J $mol^{-1}\ K^{-1}$, respectively, were calculated by using the Eyring equation (Figure 2S, Supporting Information).

Computational Search for Optimal Binding and Reactivity Sites for Cyclometalated Ru^{II} Complex on the Surface of Laccase. A theoretical analysis of possible interactions between cyclometalated Ru^{II} complexes and *Coriolus hirsutus* laccase was performed with a goal to find computational support for either mechanism 1 or 2 in Scheme 1. The complex $[Ru^{II}(o\text{-}C_6H_4\text{-}2\text{-}py)(bpy)_2]^+$ (**4**), which is a 2,2'-bipyridine analogue of **1**, was used in calculations for simplicity as the reactivity of **1** and **4** toward oxidoreductases is very close (*19*) and because **4** was used in our previous work for theoretical modeling of reactions of its Δ and Λ enantiomers with glucose oxidase from *A. niger* (*23*). Reduced (Ru^{II}) and oxidized (Ru^{III}) states of **4** are positively charged (+1 and +2 formal charges, respectively). Note that neutral acido complexes **5**−**7** are hydrolyzed in water and react with laccase as cationic species $[Ru(LL)_2(H_2O)_2]^{2+}$ (*40*). All complexes **1**−**7** are thus positively charged in H_2O. Cyclometalated $Ru^{II/III}$ complex **4** could be approximated by a compact sphere without surface functional groups. Consequently, the binding between **4** and laccase is presumably driven by nonspecific electrostatic and/or steric interactions. The electrostatic potential around the laccase molecule was computed using a numerical solution of the Poisson−Boltzmann equation. The computed potential was further employed for estimating the electrostatic interactions between laccase and complex **4**. Atomic charges of the ionogenic residues of laccase at pH 4.5 were set using the standard pK_a values of amino acids (*50*). Standard AMBER-94 force field atomic charges were applied for the amino acids. The calculated electrostatic properties of the laccase surface are shown in Figure 6A. Areas with a negative potential,

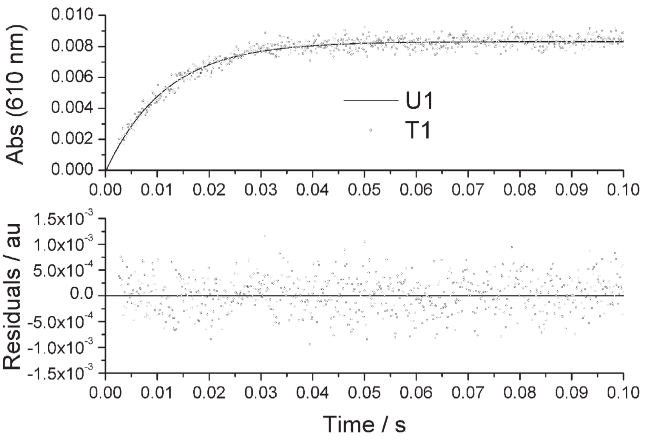


FIGURE 4: Upper part shows transient state kinetics obtained on mixing reduced laccase (ca. 3.5 μM) with dioxygen (171 μM) at 10 °C, pH 5.0, $I = 0.10$. The first-order rate constant, k_{obs}, was obtained by fitting the experimental data to a single exponential function (solid line). Lower part shows a residual plot between the fitted single exponential function and the measured data.

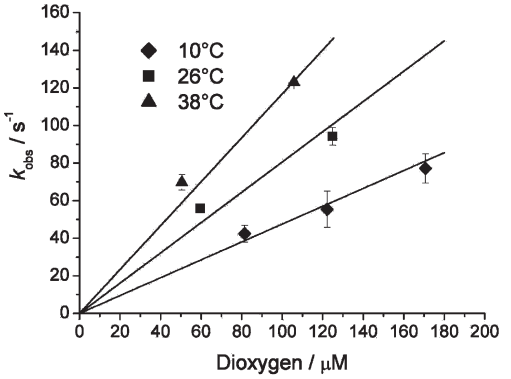


FIGURE 5: The pseudo-first-order rate constants k_{obs} plotted against the concentration of dioxygen at 10 (♦), 26 (■), and 38 (▲) °C. Conditions are as in the legend to Figure 4 except for dioxygen concentration and temperature.

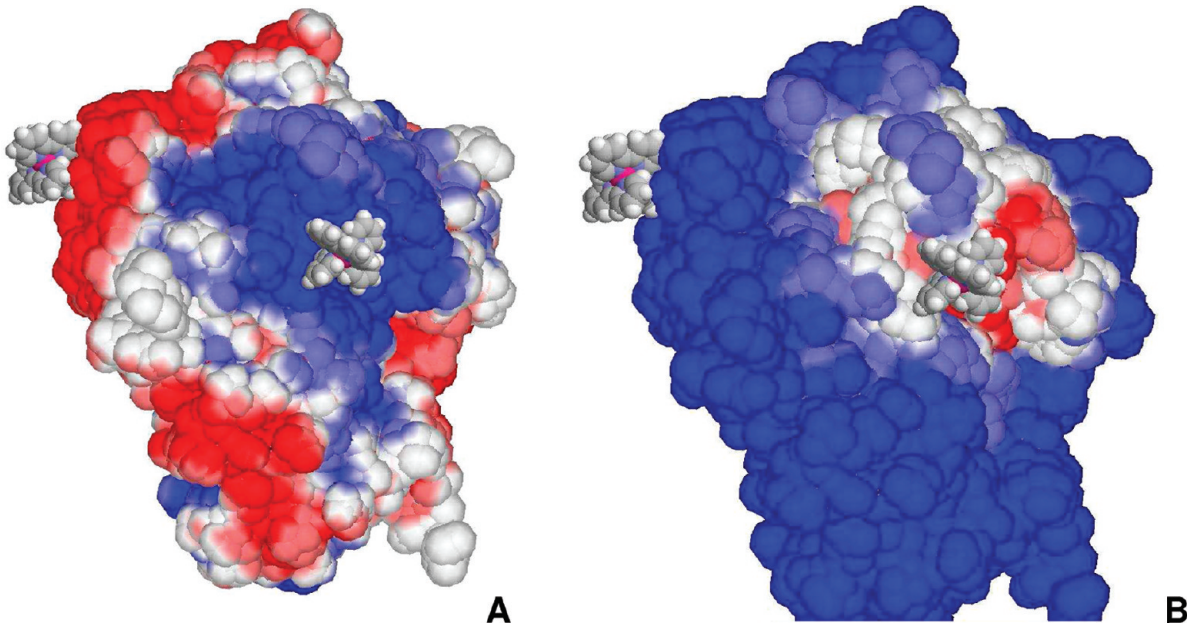


FIGURE 6: (A) Calculated electrostatic properties of the laccase surface. Areas with a negative (excess negative charge) and positive potential (excess positive charge) are in red and blue, respectively. (B) Kinetic (electron transfer) properties of the enzyme surface calculated using the PATHWAYS method. Red and blue colors indicate the surface areas of the highest and the lowest reactivity, respectively. The corresponding positions of cyclometalated complex **4** were evaluated by the Monte Carlo simulations as shown in Figure 7.

that is, of excess negative charge, are in red. Areas with a positive potential, that is, of excess positive charge, are in blue. Complex **4** is positively charged (1+), and therefore the electrostatic model predicts its preferential interactions with the "red" areas of the surface. Figure 6B shows the "electron transfer" properties of the enzyme surface calculated using the PATHWAYS method (*34*), which was also used by us previously for predicting the reactivity of glucose oxidase (*23*). Here, red and blue colors indicate the surface areas of the highest and the lowest reactivity, respectively. It is interesting to note that Figure 6A,B reveals "antagonistic" features of the laccase surface with respect to the reactivity of **4**—the best electrostatic binding site is the worst in terms of the reactivity and vice versa. As could be expected, the site of the highest reactivity is localized at the proximity of crevices close to the type 1 copper center of laccase (T1), that is, the metal center that accepts the first electron from an electron donor in the rate limiting step (*2*, *4*). Note that the electrostatic data indicates that these crevices have a positive electrostatic potential ($>2kT/e$). Therefore, these zones are thermodynamically unfavorable for the binding of positively charged cyclometalated Ru^{II} complex **4**.

More exact "extreme" locations of **4** on the laccase surface, viz. electrostatically and kinetically (closest to T1) favorable, were found by using the docking Monte Carlo simulations (*32*). These allow more accurate separations between Ru^{II} of **4** and copper atoms of laccase to be defined. The distances shown in Figure 7 indicate that the optimal "electrostatic site" is separated from the closest copper atom by ca. 32 Å. Note that this copper is not the T1 site but T3 copper coordinated by the histidine residues 111, 400, and 452. The highest estimated rate constant for the oxidation of Ru^{II} that corresponds to a such separation between Ru^{II} and Cu^{II} can be approximated as $10^{13} \times \exp(-1.4 \times 32) \sim 10^{-5}\ s^{-1}$ (*51*). This value is by 7 orders of magnitude lower than the average catalytic rate constant for enzymatic processes that follow the Michaelis−Menten kinetics (*52*). Therefore, this binding site for the Ru^{II} complex on the laccase surface is irrelevant in terms of reactivity. On the contrary, the closet position of **4** on the laccase surface to the T1 site and therefore the most favorable for the electron transfer is characterized by the $Ru^{II}\cdots Cu^{II}$(T1) separation of just 14 Å. Note that this is the region of a positive electrostatic potential where the interaction between **4** and laccase is repulsive.

Provided that mechanism 1 in Scheme 1 is valid, the minimal concentration of Ru^{II} complex required for the rate law change-over observed in the experiment can be estimated. The rate of reduction of laccase T1 center will stop to increase with the concentration of Ru^{II} when the electron transfer reactive site on the laccase surface becomes fully populated. The population of the site by Ru^{II} was estimated as follows. Given a diameter of complex **4** of about 12 Å, the exclusion volume of the molecule equals ca. 2×10^{-24} L. At a bulk concentration of Ru^{II} of ca. 10^{-4} M, the concentration at the electron transfer reactive site should be about 10 times smaller due to the repulsive positive electrostatic potential of $+2kT/e$ and the population of the reactive region can be estimated as $\{10^{-4}\ \text{mol L}^{-1} \times 10^{-1} \times 2 \times 10^{-24}\ \text{L} \times 6 \times 10^{23}\ \text{mol}^{-1} \sim 1 \times 10^{-4} \ll 1\}$. Thus, the zero-order in Ru^{II} in oxidation by laccase due to the formation of reactive Michaelis complex between Ru^{II} and the enzyme as shown in the **1** mechanism of Scheme 1 is unlikely.

DISCUSSION

As a rule, cyclometalated $Ru^{II/III}$ complexes of **1**−**4** type are drastically more reactive toward oxidoreductases, if compared with acido ruthenium(II/III) complexes such as **5**−**7**, which do not possess a Ru−C σ-bond. This observation holds for glucose oxidase from *A. niger* (*19*), HRP (*19*), peroxidases from sweet potato and royal palm tree (*53*), PQQ-dependent dehydrogenases (*54*), and the blue copper protein, laccase from *C. hirsutus*, as established in this work. So far there is one enzyme, viz. glucose oxidase from *Penicillium funiculosum G-15* in its reduced form, that reacts with both the cyclometalated and the acido ruthenium (III) complexes with rate constants of ca. $\sim 10^5\ M^{-1}\ s^{-1}$ (*20*). Rate constants k_1 (Table 2) for the oxidation of **1**−**3** by laccase from *C. hirsutus* are large, and this highlights the cycloruthenated com-

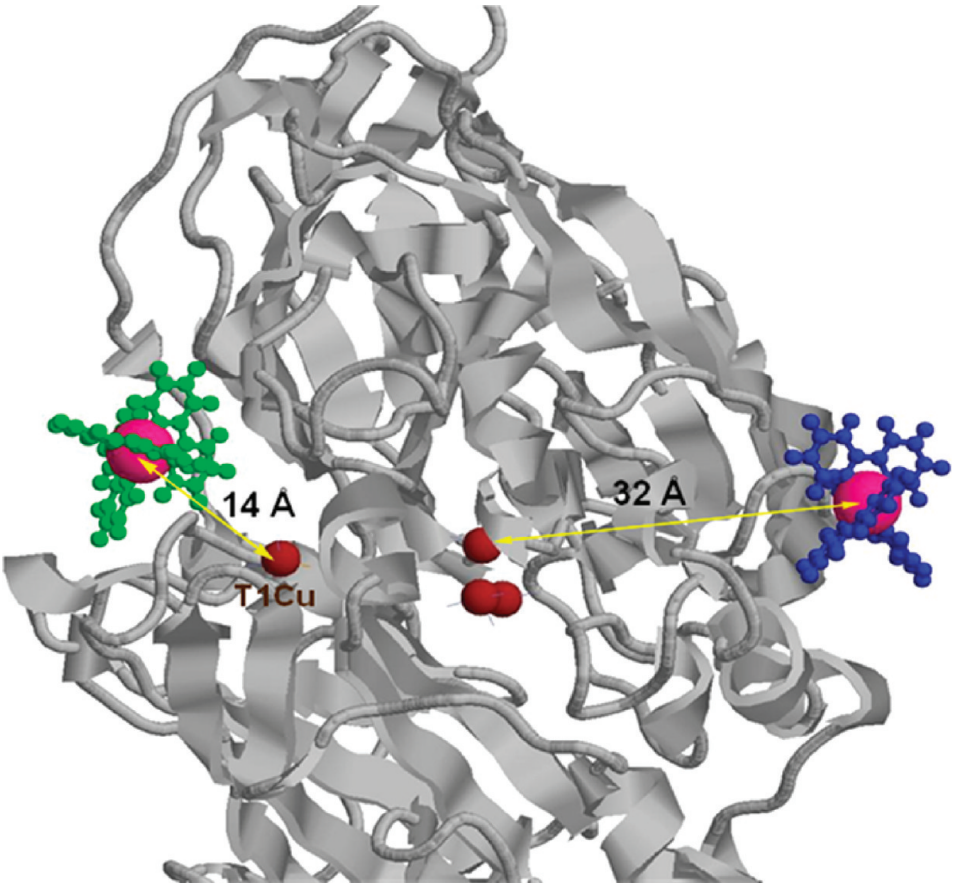


FIGURE 7: "Extreme" positions of cyclometalated complex **4** on the laccase surface evaluated by the Monte Carlo simulations. See text for details.

plexes as plausible mediators for the oxidation of organic compounds resistant to laccase itself. In fact, it has previously been shown that complex **4** serves as a mediator in the peroxidase-catalyzed oxidation of catechol (*53*).

Most likely the laccase-catalyzed oxidation of the **5**−**7** acido Ru^{II} complexes adopts the same mechanism as for the **1**−**3** molecules. The oxidation follows a first-order kinetics in Ru^{II} due to slower k_1. In this case, eq 3 becomes the experimentally observed equation $v = k_1[Ru^{II}][E]_t$, since $4k_2[O_2] \gg k_1[Ru^{II}]$. There is a similarity between the reactivity of the cyclometalated versus acido Ru^{II} complexes with respect to HRP (*19*, *39*) and laccase from *C. hirsutus* studied here. There are different rate laws in both instances due to closeness of the speed of oxidation of the cyclometalated Ru^{II} species by both enzymes and the speed of the oxidation of enzymes by natural oxidants, viz. by H_2O_2 for peroxidase and by O_2 for laccase. It is noteworthy that complex **1** is also very reactive toward an oxidized form of a man-made catalase-peroxidase mimic, the so-called Fe-TAML activator of hydrogen peroxide (*55*). The Fe-TAML-catalyzed oxidation of **1** by H_2O_2 is zero-order in **1** under all reaction conditions investigated (*24*).

Direct measurements of the rate of oxidation of reduced laccase by O_2 clarified the mechanism of oxidation of Ru^{II} in favor of the ping-pong sequence 2 in Scheme 1. An average product $k_2[O_2]$ for all complexes **1**−**3** in Table 2 equals 500 s^{-1}. An average solubility of O_2 in water is approximately 0.0005 M (*29*, *56*). Thus, the estimated rate constant k_2 equals $\sim 10 \times 10^5$ M^{-1} s^{-1} to be compared with the matching value of 8×10^5 M^{-1} s^{-1} measured directly using the stopped-flow technique. Complexes studied do not form a kinetically meaningful Michaelis type reactive intermediate. This holds for the acido complexes with hydrolizable chloro (**5** and **7**) and carbonato ligands (**6**) and ruthenacycles **2** and **3**, which possess thermo- and photolabile acetonitrile ligands (*22*, *28*). Evidence for **5**−**7** is in first-order kinetics in Ru^{II}. Evidence for **1**−**3** involves the consistent values of k_2 plus the computational data. The latter suggests that that Ru^{II} may approach the T1 copper of laccase as shown in Figure 7 to ensure ca. 14 Å separation, but this site is thermodynamically unfavorable for the laccase-ruthenium binding. The 14 Å separation between an electron donor and acceptor within a protein framework should correspond to the intramolecular electron transfer rate constant of ca. 10^7 s^{-1} (*51*). If the observed second-order rate constant for the electron transfer is ca. 1×10^7 M^{-1} s^{-1} as in Table 2 and the electron transfer does involve the intermediate Michaelis complex, the corresponding pseudo-Michaelis constant should equal ca. 1 M. Correspondingly, the concentration dependencies for the cyclometalated complexes in Figure 3 are inconsistent with this mechanistic hypothesis.

The results of this study raise a question regarding previous interpretations of the kinetic data on laccase-catalyzed oxidation of *reactive* electron donors. Leveling off the steady-state rate at higher concentrations of an electron donor is traditionally interpreted in terms of the Michaelis−Menten equation assuming the formation of an intermediate enzyme−substrate complex ES (*11*, *31*, *44*, *57*). Mechanism 2 in Scheme 1 and matching eq 3 indicate that the effect may be due to the rate limiting reoxidation of reduced laccase by dioxygen. One may argue that there is a direct X-ray evidence that ABTS, a reactive substrate of laccases, binds to CotA laccase from the endospore coat of *B. subtilis* and the binding site is rather close to the T1 copper (*58*). However, this does not mean that the product of binding is on the

reaction coordinate. ABTS is an artificial electron donor and therefore the binding registered by the X-ray crystallography (*58*) could rather be an artifact than a true kinetic requirement. Therefore, the rate limiting reoxidation of reduced forms of laccases should be taken into account while interpreting the hyperbolic kinetic data.

The absolute reactivity of laccase from *C. hirsutus* with respect to **1**−**3** is remarkable in comparison with that for the oxidation of ABTS by the most reactive, high potential recombinant laccases from *Polyporus pinsitus* (0.79 V vs NHE) and *Rhizoctonia solani* (0.73 V vs NHE) (*44*). Xu et al. analyzed the kinetic data using the Michaelis−Menten equation (*44*). Hence, the kinetic parameters reported by Xu et al. should be compared with the present data quantified in terms of the 1 mechanism in Scheme 1 (Table 2). The "Michaelis constants" (the $(k_{-1} + k_2)/k_1$ ratio in Table 2) are comparable for ABTS and **1**−**3**, though the "catalytic" rate constants (k_2 in Table 2) are by the order of magnitude higher for **1**−**3**. Syringaldazine is less reactive than ABTS and therefore the reactivity gap is even higher. Kulys et al. introduced *N*-substituted phenothiazines and phenoxazines as reactive electron donors of laccase from *Polyporus pinsitus* (*57*). *N*-Substituted (methyl and 2-hydroxyethyl) phenoxazines are the most reactive. The corresponding Michaelis constants K_M equal 5.8×10^{-6} and 5.9×10^{-6} M, and catalytic rate constants k_{cat} equal 1.0×10^2 and 1.2×10^2 s^{-1}, respectively. The pseudo-second-order rate constants (k_{cat}/K_M) of 1.7×10^7 and 2.0×10^7 M^{-1} s^{-1} are just slightly lower than k_2 reported in Table 2. Again, there is a certain reactivity advantage of ruthenium(II) species **1**−**3**. The reduction potential for laccase from *C. hirsutus* used in this work has been previously approximated as ca. 0.8 V vs NHE (*35*). The rate comparisons confirm this estimate. The enzyme belongs to a family of high potential of laccases, the reduction potential of which is higher than 0.7 V vs NHE. Higher potential cyclometalated compound *P,P-trans*-[Ru(*o*-C_6H_4-2-py)(PPh_3)$_2$($MeCN$)$_2$]PF_6 (*59*) (1.05 V vs NHE in MeCN 0.1 M $^nBu_4NPF_6$) is unreactive and this sets up the upper limit for the reduction potential for laccase from *C. hirsutus*.

In conclusion, the *C,N*-cyclometalated chelate increases the reactivity of Ru^{II} complexes **1**−**3** in the enzymatic oxidation by laccase from *C. hirsutus* by 3 orders of magnitude in comparison with the related acido complexes **5**−**7**. This is not due to a decrease in reduction potentials of **1**−**3** compared to **5**−**7**. The effect likely arises from more advantageous self-exchange rate constants for **1**−**3** (*14*). This study adds laccases to the list of enzymes that react very rapidly with the cyclometalated ruthenium and osmium complexes. There are mechanistic similarities between the kinetics of oxidation of **1**−**3** by peroxidases and laccase. There is no evidence for a kinetically meaningful Michaelis complex formation between the enzymes and the ruthenacycles and there is a rate law change on going from acido (**5**−**7**) to cyclometalated (**1**−**3**) complexes. Theoretical modeling supports the kinetic conclusion of the unimportance of the Michaelis complex formation and favors the bimolecular electron transfer from the T1 copper center of laccase at Ru^{II} of either an acido or cyclometalated complex.

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SUPPORTING INFORMATION AVAILABLE

EPR spectra of complex **2a**, Eyring plot for reoxidation of reduced laccase by dioxygen, UV−vis spectrum of *C. hirsutus* laccase at pH 6.2. This material is available free of charge via the Internet at http://pubs.acs.org.

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