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Dioxygen Reactivity of Laccase

Dependence on Laccase Source, pH, and Anion Inhibition

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Abstract

A study was carried out on the dioxygen reactivity of the laccases from *Trametes villosa*, *Rhizoctonia solani*, *Myceliophthora thermophila*, *Scytalidium thermophilum*, and *Coprinus cinereus*. At pH 5.5, these laccases showed an apparently constant K_m (about 20–50 μ*M*) for O_2 with either 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) or methyl syringate as the reducing substrate, in contrast to the k_{cat}, which varied up to 100-fold. O_2 reactivity of the *Trametes* and *Myceliophthora* laccases was also studied at various pH and NaF concentrations. The apparent K_m of *Trametes* and *Myceliophthora* laccases varied only slightly when pH changed from 3.0 to 8.0 or when the laccases were inhibited by F^- at pH 5.5, although the apparent k_{cat} were more significantly affected by both factors. The dependence of the apparent K_m on the source of laccase, pH, and F^- inhibition suggested that the fungal laccases might have a conserved O_2 pocket and that the F^- or OH^- inhibition might affect the O_2 reduction step (k_{cat}) more than the O_2 binding step (K_m) under steady-state conditions.

Index Entries: Laccase; dioxygen reactivity; pH profile; anion inhibition; fungi.

Introduction

Laccases (EC 1.10.3.2) are a family of multicopper oxidases capable of catalyzing the oxidation of a range of inorganic and aromatic substances with the concomitant reduction of O_2 to water. It is believed that the catalysis involves oxidation of reducing substrate at the type 1 (T1) Cu site, internal electron transfer from the T1 Cu to type 2/type 3 (T2/T3) trinuclear Cu cluster, and O_2 reduction at the T2/T3 site *(1–4)*. Laccases are receiving increasing interest as potential industrial and diagnostic enzymes, because they can catalyze the oxidation (by O_2) of a wide range of organic compounds. Their potential applications include use in delignification *(5–9)*,

dye and stain bleaching *(10)*, detoxification and bioremediation *(3,11,12)*, chemical and medicinal synthesis *(12–14)*, and biosensors *(3,4)*.

The binding and reduction of O_2 in laccase and other multi-Cu oxidases (such as ascorbate oxidase and ceruloplasmin) take place at the T2/T3 Cu sites. Extensive spectroscopic studies have been carried out to characterize the Cu-O bonding and reaction intermediates *(3,4)*. Recently the crystallographic structure of a *Coprinus cinereus* laccase was determined *(15)*. A detailed homologous analysis of the O_2-binding pocket in other laccases still remains difficult owing to the high variation among the corresponding primary structure segments of different laccases *(3)*. In comparison to biophysical studies, the enzymology of O_2 binding and reduction has not been fully explored. For instance, the K_m of O_2 has been reported for only about four laccases *(16–19)*, even though close to 100 laccases have been detected or characterized from various insect, plant, fungal, and bacterial sources. A systematic and comparative measurement of the K_m and k_{cat} of O_2 would help us to understand better the O_2-binding pocket as well as the O_2 reduction kinetics of laccase and other multi-Cu oxidases.

One of the most important characteristics of laccase enzymology is the pH-activity profile, which can be either biphasic or monophasic depending on whether the reducing substrate is phenolic or nonphenolic *(20)*. Various structural and mechanistic factors of laccase, reducing substrate, and O_2 may contribute to the pH-activity profile of laccase. Previous studies have focused mostly on the protic equilibria, OH^- binding, and redox potential at the Cu sites *(17,20–22)*. However, the influence of other factors related to the binding/activation of O_2 has not been fully evaluated. Do laccases (and other multi-Cu oxidases) have pH-dependent K_m and k_{cat} for O_2? How does the binding of an exogenous anion (such as F^- or OH^-) at the T2 Cu affect the O_2 binding in laccase (and other multi-Cu oxidases)? To address these questions, I investigated the O_2 reactivity of several fungal laccases as a function of laccase source, pH, and F^- inhibition. The study showed that these laccases had a similar K_m for O_2, that the K_m was not sensitive to pH change over the range of 3.0–8.0, and that the inhibitory binding of OH^- or F^- onto T2 Cu had only a minor effect on the K_m.

Materials and Methods

Materials

Chemicals used as buffers and substrates were purchased from Aldrich (except for methyl syringate, which came from Acros) and had the highest available grade. O_2 and N_2 were from Airgas (ultrahigh grade, 99.999%). Recombinant *Trametes villosa* (or *Polyporus pinsitus*) laccase-1 (TvL), *Rhizoctonia solani* laccase-4 (RsL), *Myceliophthora thermophila* laccase (MtL), *Scytalidium thermophilum* laccase (StL), and *C. cinereus* laccase (CcL) were purified as reported previously *(15,23)*. The Britton and Robinson (B&R) buffers were made by mixing 0.1 *M* boric acid-0.1 *M* acetic acid-0.1 *M* phosphoric acid with 0.5 *M* NaOH to the desired pH.

Laccase Activity Assay

The laccase-catalyzed O_2 reduction, accompanied by the concomitant oxidation of either 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or methyl syringate (MS), was monitored by a Hansatech (Norfolk, United Kingdom) DW1/AD O_2 cell at 20°C with 0.3 mL of 10 m*M* 4-morpholinoethanesulfonic acid (MES)-NaOH (pH 5.5) or B&R buffer (pH 3.0–9.0), 1 m*M* ABTS or 2.5–10 m*M* MS (2.5 m*M* at pH ≤ 5.0 and 10 m*M* at pH > 5.0), and an adequate amount of laccase (12 µ*M* TvL, 4 µ*M* RsL, 77 µ*M* MtL, 160 µ*M* StL, or 92 µ*M* CcL). The stock solution of MS (0.2 *M*) was made in ethanol. The 1.3% ethanol in the assay solution added along with MS had no detectable effect on the measurement. The O_2 concentration was controlled by bubbling the solution with a selected O_2 and N_2 (both humidified by passing through water) mixture (mixed with two flowmeters [Bel-Art, NJ]) for a few minutes until the O_2 signal from the cell was stabilized. After the voltage reading stabilized, laccase (a few microliters) was added through an airtight syringe into the solution to initiate the reaction. The initial output voltage changes were used to calculate the initial reaction rate (v). The $[O_2] - v$ curves were Michaelis type, and the apparent kinetic parameter K_m was determined by fitting v and $[O_2]$ to $v = V_{max} \times [O_2]/(K_m + [O_2])$ with the Prizm program of GraphPad (San Diego, CA). The apparent k_{cat} was determined from $k_{cat} = V_{max}/[\text{laccase}]$. Standard deviation was used to estimate the range of K_m and k_{cat}. The $[O_2]$ in the air-saturated assay solution was assumed to be 0.28 m*M*, the same as in plain water. The measurements of K_m and k_{cat} for ABTS and MS were reported previously *(23)*. In the same air-saturated assay solution, ABTS had a K_m of 58 ± 8, 52 ± 6, 110 ± 20, 89 ± 14, and 23 ± 3 µ*M* and a k_{cat} of $(2.7 \pm 0.1) \times 10^3$, $(2.5 \pm 0.1) \times 10^3$, $(3.8 \pm 0.3) \times 10^3$, $(4.5 \pm 0.1) \times 10^1$, and $(1.1 \pm 0.1) \times 10^3$ min^{-1} for TvL, RsL, MtL, StL, and CcL, respectively. MS had a K_m of 490 ± 60 and 4100 ± 900 µ*M*, and a k_{cat} of $(2.0 \pm 0.2) \times 10^3$ and $(7.2 \pm 0.1) \times 10^2$ min^{-1} for TvL and MtL, respectively.

The pH profiles of K_m and k_{cat} for TvL and MtL were measured in B&R buffer. At pH ≥ 8.0 or 9.0, respectively, the rate of TvL- or MtL-catalyzed O_2 reduction was too small to allow accurate measurement of K_m and k_{cat}. Thus, only a $k_{cat}(O_2) \leq 10$ min^{-1} was estimated for TvL or MtL at pH 8.0 or 9.0, respectively.

Because the laccase catalysis involved two substrates and the [reducing substrate] was fixed (at saturating level) in this study, the measured K_m for O_2 (oxidizing substrate) derived from the two-substrate Michaelis-Menten kinetics should be considered as apparent. Because of the assumption that 100% of the laccase participated in the catalysis as active enzyme, the measured k_{cat} should be considered as apparent as well.

Fluoride Inhibition

The inhibition of the oxidase activity of TvL or MtL by NaF was assayed in 10 m*M* MES-NaOH, pH 5.5, with ABTS as the reducing substrate.

Table 1
O_2 Reactivity of Various Laccases Measured
with 1 m*M* ABTS or 10 m*M* MS in 10 m*M* MES-NaOH, pH 5.5, at 20°C

	ABTS as reducing substrate		MS as reducing substrate	
Laccase	K_m (μ*M*)	k_{cat} (min^{-1})	K_m (μ*M*)	k_{cat} (min^{-1})
TvL	46 ± 8	$(1.2 \pm 0.1) \times 10^3$	39 ± 11	$(1.7 \pm 0.1) \times 10^3$
RsL	29 ± 6	$(8.4 \pm 0.1) \times 10^2$	24 ± 6	$(4.3 \pm 0.3) \times 10^2$
MtL	24 ± 3	$(1.4 \pm 0.1) \times 10^3$	24 ± 6	$(1.0 \pm 0.1) \times 10^3$
StL	31 ± 11	$(2.1 \pm 0.2) \times 10^1$	22 ± 2	$(1.5 \pm 0.1) \times 10^1$
CcL	21 ± 2	$(2.0 \pm 0.1) \times 10^2$	34 ± 6	$(1.9 \pm 0.1) \times 10^2$

For TvL, O_2 reactivity was measured in the presence of 0, 0.03, or 1 m*M* NaF, whereas for MtL, O_2 reactivity was measured in the presence of 0, 5, or 15 m*M* NaF. In these [NaF] ranges, about 20–80% of the original activity of these laccases would be inhibited.

Results and Discussion

Dependence of K_m *and* k_{cat} *on Laccase Source*

Table 1 shows the Michaelis-Menten constants for O_2 reactivity of the five fungal laccases at pH 5.5. For a given laccase, the use of ABTS or MS as the reducing substrate did not significantly affect the K_m of the oxidizing substrate O_2. The $K_m(O_2)$ of all five laccases was found at about 20–50 μ*M* at pH 5.5, close to the values reported for the laccases from the fungi *Lactarius piperatus* (11 μ*M*, pH 5.6) and *Polyporus versicolor* (16 μ*M*, pH 5.0), and the plants *Pinus taeda* (37 μ*M*, pH 5.9) and *Rhus vernicifera* (21 μ*M*, pH 7.5) *(16–19)*. The small variation of $K_m(O_2)$ suggested that the laccases might have a similar binding interaction with O_2 at steady state, different from their variable interactions with reducing substrate (as demonstrated by the variable K_m for reducing substrate) *(23)*.

In contrast to the small variation (≤ 2-fold) in $K_m(O_2)$, the difference in $k_{cat}(O_2)$ could be up to 100-fold for different laccases (Table 1). However, individual laccases had a $k_{cat}(O_2)$ similar to the k_{cat} measured on the reducing substrate, k_{cat}(ABTS) or k_{cat}(MS), as anticipated for a steady state with efficient coupling of the two redox halves of the catalysis. The $k_{cat}(O_2)$ dependence on laccase observed in this study should reflect the previously reported k_{cat}(reducing substrate) dependence on laccase *(23)*. Because the redox potential (E°) of the T1 Cu in laccase controls the electron transfer from the reducing substrate to laccase and consequently the k_{cat}, the up to 100-fold variation in $k_{cat}(O_2)$ among the five laccases could be attributed to the up to 0.3 V difference in their E° *(23)*.

Dependence of K_m *and* k_{cat} *on pH*

Figure 1 shows the pH dependence of the apparent $K_m(O_2)$ and $k_{cat}(O_2)$ observed for TvL and MtL, two representatives of the "high redox poten-

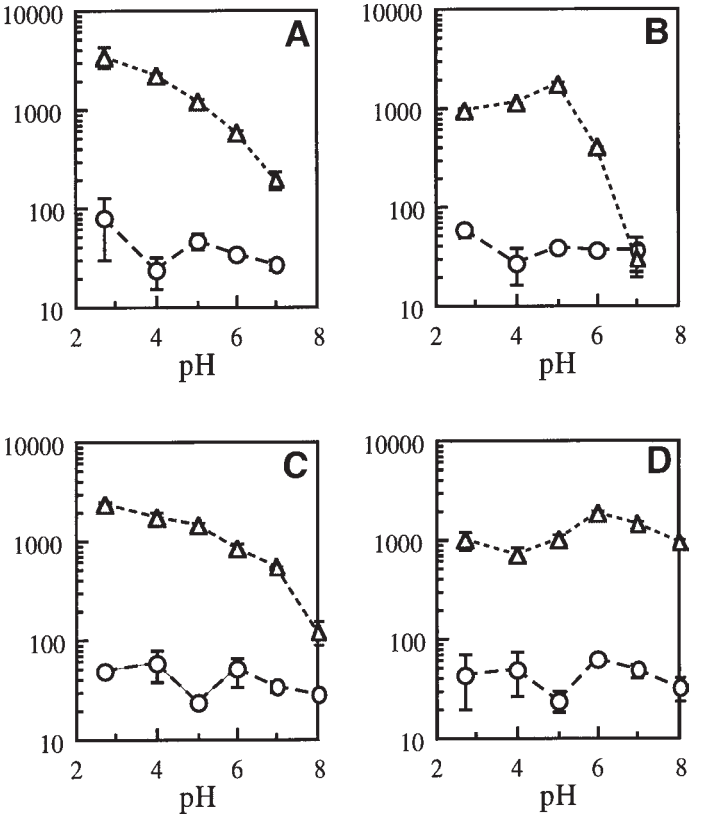

Fig. 1. pH profiles for $K_m(O_2)$ and $k_{cat}(O_2)$. Laccases studied: **(A,B)** TvL; **(C,D)**, MtL. Reducing substrates used: (A,C), ABTS; (B,D), MS. (○), K_m (μ*M*); (△), k_{cat} (min^{-1}).

tial" and "low redox potential" laccase families *(20)*. With ABTS as the reducing substrate, both TvL and MtL had a $k_{cat}(O_2)$ profile that declined monotonically as pH increased from 3.0 to 8.0, whereas with MS as the reducing substrate, TvL and MtL had an optimal pH for the $k_{cat}(O_2)$ at pH 5.0 and 6.0, respectively. The former profiles were similar to the previously reported k_{cat}(ABTS) profiles of TvL and MtL, and the latter were similar to the previously reported k_{cat}(MS) profiles of TvL and MtL *(20)*. This was anticipated for a steady-state with efficient coupling of the two redox halves of the catalysis. Thus, the observed dependence of the pH-$k_{cat}(O_2)$ profile on reducing substrate should correlate with the pH-k_{cat}(reducing substrate) profile and be explained by the previously proposed mechanism involving E° and OH^- inhibition *(20)*. Under this mechanism, the OH^- inhibition would result in a loss of laccase activity at a higher pH, such as that observed when ABTS served as the reducing substrate. For a phenol-type reducing substrate (such as MS), however, the decrease of its E° at a higher pH would increase the E° difference with laccase and consequently enhance its oxidation rate. The combination of the two opposite effects would then lead to an optimal pH.

Protic equilibria on T2 Cu-bound O_2 or its intermediates, which take place at a site (T2/T3 Cu cluster) away from the reducing substrate pocket (T1 Cu) in laccase, have been shown to be capable of affecting laccase activity *(21,22)*. However, their effect on the pH-k_{cat} profile seemed minor under the conditions of this study, because the profile varied as the type of the reducing substrate changed. With ABTS as the reducing substrate, an apparent "pK_a" of ~4 or 5 was estimated for TvL or MtL, respectively, from the pH profile of $\log(k_{cat}/K_m)$. With MS as the reducing substrate, an apparent "pK_a" of ~5 was estimated for TvL and MtL from the pH profile of $\log(k_{cat}/K_m)$.

The pH dependence of $K_m(O_2)$ for both TvL and MtL was less significant than that of $k_{cat}(O_2)$. This indicates that at steady state neither the binding of OH^- onto T2 Cu nor the deprotonation of amino acid residues in the O_2 pocket of laccase significantly impacted O_2 binding (onto the T2/T3 trinuclear cluster). This finding is in sharp contrast to the major inhibitory effect of T2 Cu-bound OH^- on the O_2 reduction rate *(22)*.

As shown in Fig. 1, the $K_m(O_2)$s of TvL and MtL were well below the dissolved O_2 concentration of 0.28 m*M*, making both laccases nearly saturated in O_2 in aerated aqueous solutions over the pH range of 3.0 to 9.0. Thus, the previously reported pH-activity profiles, which are measured on the reducing substrate, should reflect the true pH dependence of the substrate oxidation by laccase *(23)*.

Dependence of K_m *and* k_{cat} *on* F^- *(as well as* OH^-*) Inhibition*

Table 2 shows the $K_m(O_2)$ and $k_{cat}(O_2)$ of TvL and MtL measured in the presence of the laccase inhibitor NaF. On the increase of [NaF] and the subsequent decrease of $k_{cat}(O_2)$, the $K_m(O_2)$ in TvL or MtL decreased slightly or remained nearly constant, respectively. Like OH^-, the binding of F^- onto the T2 Cu is well known, and its negative effect on k_{cat} is attributed to a perturbed T2/T3 Cu cluster unfavorable toward the internal electron transfer from T1 Cu *(1,3,18,22)*. Under the conditions of this study, the effect of NaF on $K_m(O_2)$ seemed to indicate weak, if any, competition between O_2 and F^- in binding to the T2/T3 cluster, similar to the competition between O_2 and OH^- discussed previously, at steady state.

The integration of T2 and T3 Cu atoms into a trinuclear cluster as well as the effects of bound exogenous small ionic/molecular ligands on the coordination/redox/kinetic properties of the cluster are well established for laccase and other multi-Cu oxidases *(1–4)*. Extensive spectroscopic and X-ray diffraction studies have shown that an exogenous ligand such as O_2, F^-, or OH^- binds to the T2/T3 cluster. However, the results from the two methods disagree on whether the ligand binds onto one T3 Cu (in a terminal mode) or between one T2 Cu and a T3 Cu (in a bridge mode) *(3,21)*. Because F^- and OH^- are known to bind the T2 Cu, a significant impact by the anion on the binding of O_2 onto the T2 Cu should be anticipated if O_2 binds to T2 Cu (the bridge mode). Thus, the observation that the binding of F^- or OH^- did not significantly affect the K_m of O_2 might support the terminal T3 Cu-O_2 mode *(3)*.

Table 2
Effect of NaF on the $K_m(O_2)$ and $k_{cat}(O_2)$ of TvL and MtL Measured with 1 m*M* ABTS in 10 m*M* MES-NaOH, pH 5.5, at 20°C

TvL				MtL			
[NaF] (m*M*)	K_m (μ*M*)	k_{cat} (10^3 min^{-1})	k_{cat}/K_m (10^7 *M* min^{-1})	[NaF] (m*M*)	K_m (μ*M*)	k_{cat} (10^3 min^{-1})	k_{cat}/K_m (10^7 *M* min^{-1})
0	46 ± 8	1.20 ± 0.10	2.6	0	24 ± 3	1.40 ± 0.10	5.8
0.03	32 ± 6	0.92 ± 0.06	2.9	5	15 ± 3	0.70 ± 0.04	4.7
1	17 ± 5	0.35 ± 0.02	2.1	15	18 ± 4	0.30 ± 0.02	1.7

Conclusion

For the first time, a comparative and systematic study on the O_2 reactivity of multi-Cu oxidases was made with five fungal laccases that are quite different in terms of sequence homology (≤56% similarity), redox chemistry, and specificity for reducing substrate *(23)*. This study demonstrated that these quite different laccases have a similar $K_m(O_2)$ at the tested pH of 5.5. For the *Trametes* and *Myceliophthora* laccases, representing, respectively, the high redox potential and low redox potential laccase families, the $K_m(O_2)$s are quite insensitive to pH as well as the inhibitory binding of F^- or OH^- at the T2 Cu. Based on these data, it is speculated that there is a conserved O_2 binding domain among different laccases, and that O_2 binds on to a T3 Cu by a terminal mode at steady state. However, because of the kinetic (rather than thermodynamic) nature of the parameter K_m, these speculations must be verified by other experiments, especially real O_2 affinity measurement as well as three-dimensional structure analysis of native/oxygenated laccases.

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