Effects of Redox Potential and Hydroxide Inhibition on the pH Activity Profile of Fungal Laccases*

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Feng Xu‡

From Novo Nordisk Biotech, Davis, California 95616

The electronic absorption spectrum, susceptibility to fluoride inhibition, redox potential, and substrate turnover of several fungal laccases have been explored as a function of pH. The laccases showed a single spectrally detectable acid-base transition at pH 6–9 and a fluoride inhibition that diminished by increased pH (indicating a competition with hydroxide inhibition). Relatively small changes in the redox potentials (0.1 V) of laccase were observed over the pH 2.7–11. Under the catalysis of laccase, the apparent oxidation rates (kcat and kcat/Km) of two nonphenolic substrates, potassium ferrocyanide and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid), decreased monotonically as the pH increased. In contrast, the apparent oxidation rates (kcat and kcat/Km) of three 2,6-dimethoxyphenols (whose pH values range from 7.0 to 8.7) exhibited bell-shaped pH profiles whose maxima were distinct for each laccase but independent of the substrate. By correlating these pH dependences, it is proposed that the balance of two opposing effects, one generated by the redox potential difference between a reducing substrate and the type 1 copper of laccase (which correlates to the electron transfer rate and is favored for a phenolic substrate by higher pH) and another generated by the binding of a hydroxide anion to the type 2/type 3 coppers of laccase (which inhibits the activity at higher pH), contributes to the pH activity profile of the fungal laccases.

Laccases (EC 1.10.3.2) are a family of multi-copper oxidases that catalyze the oxidation of a range of inorganic and aromatic substances (particularly phenols) with the concomitant reduction of O2 to water (for recent reviews see Refs. 1–8). Recombinant Polyporus pinsitus (or Trametes villosa) laccase isoform-1 (PpL), Rhizoctonia solani laccase isoform-4 (RsL), and Myceliophthora thermophila laccase (MtL) were purified as reported previously (11, 14, 15). Fe(2,2'-dipyridyl)Cl2 and Fe(2,2'-dipyridyl)Cl3 were made by mixing FeCl3 or FeCl4 with two molar equivalent 2,2'-dipyridyl, respectively. The Britton and Robinson (B&R) buffers were made by mixing 0.1 mM boric acid, 0.1 mM acetic acid, 0.1 mM phosphoric acid with 0.5 M NaOH to the desired pH.

One of the most important characteristics of laccase enzymeology with phenolic substrates is the pH dependence. In general, the phenol oxidase activity of laccase has a bell-shaped (bi-phasic) pH profile whose optimal pH varies considerably among different laccases (1–11). Various structural and mechanistic factors from laccase, phenolic substrate, and O2 may contribute to the pH activity profile. Detailed studies have been carried out with respect to the effect of protic equilibrium (related to the laccase-bound O2 and type 2/type 3 (T2/T3) copper sites) on the rate of laccase-catalyzed O2 reduction (12, 13). However, insight on other potential factors, such as those related to the type 1 (T1) copper and (reducing) substrate, is still limited, and a comprehensive understanding on the mechanism that governs the bi-phasic pH dependence has not been fully established. To address this problem, I investigated several fungal laccases for pH-induced changes in their electronic absorption spectrum, fluoride inhibition, redox potential, and oxidation rate of phenolic and nonphenolic substrates. The study showed that the redox potential difference between a phenolic substrate and the T1 copper of laccase could result in an increased substrate oxidation rate at higher pH, whereas the hydroxide anion binding to the T2/T3 coppers could lead to an inhibition of laccase activity at higher pH. The balance of these two opposing effects might play an important role in determining the pH activity profile of laccase.

EXPERIMENTAL PROCEDURES

Materials—Chemicals used as buffers and substrates were purchased from Aldrich (except for methyl syringate, which was from Acros) with the highest available grade. Recombinant Polyporus pinsitus (or Trametes villosa) laccase isoform-1 (PpL), Rhizoctonia solani laccase isoform-4 (RsL), and Myceliophthora thermophila laccase (MtL) were purified as reported previously (11, 14, 15). Fe(2,2'-dipyridyl)Cl2 and Fe(2,2'-dipyridyl)Cl3 were made by mixing FeCl3 or FeCl4 with two molar equivalent 2,2'-dipyridyl, respectively. The Britton and Robinson (B&R) buffers were made by mixing 0.1 mM boric acid, 0.1 mM acetic acid, 0.1 mM phosphoric acid with 0.5 M NaOH to the desired pH. Spectrophotometrical Redox Titration of Laccase—The spectrum of laccase was recorded in B&R buffer, pH 2.7–11, on a Shimadzu UV1600 spectrophotometer with an 1-cm quartz cuvette. The redox potential (E') of the T1 copper in laccase was measured as reported previously (11), except that B&R buffer was used. Briefly, the E'(PpL) was measured with 21 mM PpL, 0.2 mM Fe(bipyridyl)Cl2, and 0.1–0.4 mM Fe(bipyridyl)Cl3, E'(Fe(bipyridyl)Cl2/Fe(bipyridyl)Cl3) = 0.780 V; the E'(ReL) was measured with 17 mM ReL, 0.2 mM Fe(bipyridyl)Cl2, and 0.05–0.2 mM Fe(bipyridyl)Cl3 and the E'(MtL) was measured with 0.14 mM MtL, 23 mM K4Fe(CN)6, and 0–200 mM K3Fe(CN)6, E'(K3Fe(CN)6/K4Fe(CN)6) = 0.433 V. Under various potentials of the solution poised by various concentration ratio of the redox titrant couples, the absorbance changes of laccase in the range of 550–800 nm were monitored, and the concentrations of the copper(II) and copper(I) states were calculated after the spectral change reached an equilibrium. An aerobicity was achieved by repetitive evacuating and argon flushing of the reaction chamber at 4 °C.

†To whom correspondence should be addressed: Novo Nordisk Biotech, 1445 Drew Ave., Davis, CA 95616. Tel.: 916-757-8100; Fax: 916-758-0317; E-mail: fengxu@nnbt.com.
The pKₐ value of the phenolic substrate studied was determined spectrally in B&R buffer. At alkaline pH, the phenolic substrates showed a deprotonation-induced spectral change. For syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde), the absorption maximum at 302 nm was replaced by two maxima at 250 and 365 nm (isosbestic points: 238, 266, and 327 nm); for acetylsyringone (4’-hydroxy-3,5’-dimethoxycacetophenone), the absorption maximum at 294 nm was replaced by two maxima at 250 and 359 nm (isosbestic points: 237, 263, and 321 nm); and for methyl syringate (methyl 4-hydroxy-3,5-dimethoxybenzoate), the absorption maximum at 273 nm was replaced by one shoulder around 236 nm and one maximum at 322 nm (isosbestic points: 231, 251, and 293 nm). The pH profiles of the absorption at 302 and 365 nm yielded a pKₐ of 7.0 ± 0.2 for syringaldehyde, a pKₐ of 7.8 ± 0.2 for acetylsyringone, and a pKₐ of 8.7 ± 0.2 for methyl syringate. Under the same conditions, a pKₐ of 8.2 and 2.2 were previously found for syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) and 2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), respectively (11).

**Laccase Activity Assays**—The laccase-catalyzed oxidation of syringaldehyde, acetylsyringone, or methyl syringate was monitored by O₂ consumption as described in Ref. 16, and the oxidation of syringaldazine or ABTS was determined photometrically as described in Ref. 11. Briefly, the phenol oxidation was monitored by a Hansatech DWI/AD O₂ electrode with 0.04–15 μM laccases in 0.3–0.5 ml of B&R buffer at 20 °C. After the voltage reading stabilized, laccase was added into the solution to initiate the reaction, and the initial output voltage changes were used to calculate the initial reaction rate (v). The oxidation of the nonphenolic substrates were photometrically monitored on either a Shimadzu UV160U spectrophotometer with a 1-cm quartz cuvette (for ABTS; Δε at 418 nm = 36 mM⁻¹ cm⁻¹) or on a Molecular Devices Thermomax microplate reader with a 96-well plate (Costar, tissue culture grade) (for K₅Fe(CN)₆; Δε at 405 nm = 0.90 mM⁻¹ cm⁻¹). The initial absorbance changes were used to calculate the initial reaction rate (v) and the apparent kinetic parameter Kₐ was determined by fitting the initial reaction rate (v) and substrate concentration to v = Vmax × [substrate]/(Kₐ + [substrate]) with the Prizm program of GraphPad (San Diego, CA), and the apparent kₐ was determined from kₐ = Vmax/[laccase]. All the experiments were carried out in air-saturated solutions. Thus the initial [O₂] was kept constant (and was assumed to be the same as in plain water (0.25 mM)), and the observed Kₐ and kₐ were “apparent” values ([O₂] needs to be systematically varied in order to measure the true Kₐ and kₐ.)

**Fluoride Inhibition**—The inhibition of laccase by NaF was assayed with laccase-catalyzed ABTS oxidation in B&R buffer. The assay solutions contained 2 mM ABTS and 0.6 μM ML or 40 nM PpL. Being a complex “linear mixed type,” the inhibition showed convex type correlations when the slope and the y intercept of the Lineweaver-Burk plots (1/rate versus 1/[substrate]) were plotted against [NaF], similar to that observed with the *Rhus* laccase (12). The inhibition was quantitated by the parameter I₅₀, the NaF concentration at which only 50% of the initial laccase activity remained, because the complexity of the plots complicated the extraction of the inhibition constant Kₐ.

**RESULTS**

The pH-induced changes in the E’ and the wavelength corresponding to the maximal absorption of the blue band of laccase are shown in Fig. 1 (A and B). At the pH range of 2.7–11, one spectrally detected acid-base transition occurred around pH 6.5 for ML, pH 8 for RsL, and pH 9 for PpL (Fig. 1B). For ML, the E’ decreased ~90 mV from pH 2.7 to 5 but increased ~20 mV from pH 5 to 11; for RsL, the E’ decreased ~80 mV from pH 2.7 to 7 but increased ~30 mV from pH 7 to 11; and for PpL, the E’ did not change from pH 2.7 to 7 but increased ~30 mV from pH 7 to 11. Under the experimental conditions, no obvious correlation between the two types of pH profiles in Fig. 1 (A and B) was observed.

The pH profiles of the apparent Kₐ and kₐ for the nonphenolic substrates are shown in Fig. 3 (A–D). With both PpL and ML, the profiles of the apparent kₐ for ABTS and K₅Fe(CN)₆ had a monotonic declining nature when the pH changed from acidic range to alkaline range (Fig. 3, A and B). With PpL, K₅Fe(CN)₆ showed an apparent Kₐ that did not change much when the pH changed from 2.7 to 7, whereas ABTS showed an apparent Kₐ that did not change much from pH 2.7 to pH 7 but increased approximately 4-fold from pH 7 to pH 8 (Fig. 3A). With ML, K₅Fe(CN)₆ showed an apparent Kₐ that increased about 3-fold when the pH changed from 2.7 to 4 but did not change much from pH 4 to pH 7, whereas ABTS showed an apparent Kₐ that increased 4-fold from pH 2.7 to pH 6 but 40-fold from pH 6 to pH 8 (Fig. 3C). The catalyzed oxidation of K₅Fe(CN)₆ or ABTS was so slow above pH 7 or pH 8 that no accurate Kₐ and kₐ could be obtained.

The pH profiles of the apparent Kₐ and kₐ for the phenolic substrates are presented in Fig. 4 (A–D). Being oxidized by PpL, all these substrates showed the highest apparent kₐ at pH 5 (Fig. 4B). With PpL, the apparent Kₐ for syringaldehyde and acetylsyringone did not change much from pH 4 to 7 but increased about 10-fold from pH 4 to 2.7, whereas the apparent Kₐ for methyl syringate did not change much from pH 2.7 to 6 but decreased about 10-fold from pH 6 to 7 (Fig. 4A). With ML,
the apparent $K_m$ for methyl syringate and acetosyringone showed minimal change at pH 2.7 to 9, but the apparent $K_m$ for syringaldehyde increased about 3-fold from pH 6 to 2.7 (Fig. 4C). Being oxidized by MtL, all the phenols showed the highest apparent $k_{cat}$ at pH 7, although an increase in the apparent $k_{cat}$ was also observed when the pH changed from 4 to 2.7 (Fig. 4D). The catalyzed oxidation of all three phenols was so slow above pH 7 with PpL or pH 9 with MtL that no accurate $K_m$ and $k_{cat}$ could be obtained. Similar result was previously observed with syringaldazine (11).

The addition of NaF resulted in an immediate laccase inhibition with $I_{50} \approx 0.1 \text{ mM}$ at acidic pH. Because HP has a $pK_a$ of 3.5, the laccase inhibition shown in Fig. 5 was most likely caused by $F^−$. As the pH increased, the $I_{50}$ became larger, indicating a weaker $F^−$ inhibition at higher pH (Fig. 5). This increase of $I_{50}$ at higher pH did not correspond to an increase in laccase activity, however, because in the absence of NaF, the activity of laccase diminished as pH increased (Fig. 2, C and D).

**DISCUSSIONS**

Based on a wide range of physical and chemical characterizations, it is generally accepted that the catalysis of fungal laccase involves (a) the binding of a reducing substrate to the T1 pocket and subsequent reduction of the T1-Cu(II) to Cu(I), (b) the internal electron transfer from the T1 to the T2/T3 center, and (c) the binding and subsequent reduction of an $O_2$ to $H_2O$ at the T2/T3 center (1–8). Potentially, any pH-induced structural or mechanistic changes in either the reducing substrate, $O_2$, or laccase (particularly on its T1 and T2/T3 centers) could contribute to the observed pH activity profiles.

The oxidation of phenol by laccase depends on the redox potential difference between the phenol and the T1 copper (16). Due to the oxidative proton release, the $E^0$ of a phenol decreases when pH increases. At a rate of $\Delta E/\Delta pH = 0.059 \text{ V}$ at $25^\circ C$, a pH change from 2.7 to 11 would result in an $E^0$(phenol) decrease of 0.49 V. However, over the same pH range, the $E^0$ changes for the laccases studied were much smaller ($\approx 0.1 \text{ V}$), similar to the case of the *Rhus* laccase (17). Such different pH dependences of the $E^0$ for phenolic substrate and laccase would then result in a larger difference in redox potential $[\Delta E^0 = E^0$(laccase, T1) $- E^0$(substrate, single electron)] or driving force (for the electron transfer from phenol to T1 copper) at higher pH (Fig. 6, A and C). Given the correlation of log(rate) = $7.1 \times \Delta E^0 + 7.0$ observed (at pH 5) for a wide variety of substrates and laccases (16), this $\Delta E^0$ effect should lead to a pH dependence in which the activity increases as the pH increases, thus contributing to the ascending part of the bell-shaped pH activity profile for phenols shown in Figs. 2, 4, and 6. The increase in $k_{cat}$ from pH 4 to 2.7 for MtL-catalyzed phenol oxidation (Fig. 4D) could also be related to the $\Delta E^0$ effect, because, as shown in Fig. 1A, the $E^0$(MtL) decreased 80 mV when pH changed from 2.7 to 4, thus reducing the oxidation potency of MtL.

The loss of $F^−$ inhibition at high pH did not result in recovery or increase of laccase activity. Likely the observed pH dependence of the $F^−$ inhibition was mainly due to an $OH^−$ competition (with $F^−$) for inhibiting MtL and PpL, similar to the cases of other laccases in which $OH^−$ and $F^−$ are shown to competitively bind to the T2/T3 center and inhibit activity (1, 3, 19–22). Such $OH^−$ inhibition interrupts the internal electron transfer from the T1 to the T2/T3 centers in laccase and, together with other rate-diminishing deprotonations (to be discussed later), could contribute to the descending part of the pH activity profile of phenols shown in Figs. 2, 4, and 6.

For phenol substrates, a bell-shaped pH activity profile with an optimal pH dependent on laccase (not substrate) is consistent with the mechanism in which the opposing effects of the $OH^−$ inhibition and $\Delta E^0$ contribute, respectively, at alkaline and acidic pH mainly. The oxidation of ABTS (to the stable, preferred cation radical) or $K_2Fe(CN)_6^−$ (to $K_3Fe(CN)_6$) does not involve protons, and thus possesses an $E^0$ independent of pH (17). This would make any pH effect of $\Delta E^0$ minimal for these two substrates. The contribution of the $OH^−$ inhibition would then result in a monotonic pH activity profile consistent with the data presented in Figs. 2, C and D, and 3, C and D. As shown in Fig. 6, B and D, the descending part of the pH profile for the phenols (at neutral alkaline pH range) is similar to the profile for the nonphenolic substrates (ABTS and K$_2$Fe(CN)$_6$), suggesting the contribution of a common mechanism involving
The contribution of the obtained from Fig. 4 (A–D) apparent acid-base transitions (on their T1 center) with a in independent of the reducing substrate. The effect also seems to be small in this study, because a dominant intermediate product (and the T2/T3 pocket could diminish the docking (mediate product) and the T1 pocket could affect substrate libria related to the reducing substrate (or its oxidized intermediate product) and the T1 Cu(II) related to protic equilibria of transforming phenol to phenoxide or vice versa. However, the lines for syringaldehyde and acetosyringone, which are superposable to line α, and the line for KFe(CN)$_6$ which is superposable to line β, are omitted in B and D.)

the OH$^-$ inhibition of laccase.

The laccases studied showed different, spectrally detected acid-base transitions (on their T1 center) with a $pK_a$ ranging from 6.5 to 9. The pH-induced change in the blue absorption band around 600 nm was probably caused by an energy perturbation (on the T1 Cu(II)) related to the OH$^-$ or proton Eo dependence on pH. The values for the Eo(phenol, single electron) are from Ref. 18. B and D, dependence of log(rate) on pH. For syringaldehyde (C), acetosyringone (ΔΔ), and methyl syringate (Δ), the log(rate) = log([H]/[R]) values are obtained from Fig. 4 (A–D) and presented relative to the value at pH 5. The contribution of the ΔE$^0$ to the pH activity profile is represented by the dashed line α, which is derived from the correlation log(rate) = 7.1 × ΔE$^0$ + 7.0 (16) with the ΔE$^0$ values from A and C and is presented relative to the value at pH 5. The contribution of the OH$^-$ inhibition to the pH activity profile is represented by the dotted line β, which is derived from the apparent $k_{cat}$ and $K_m$ of ABTS (Fig. 3, A–D) and presented relative to the value at pH 8 (B) or 9 (D). For clarity purpose, the lines for syringaldehyde and acetosyringone, which are superposable to line α, and the line for KFe(CN)$_6$, which is superposable to line β, are omitted in B and D.)

In summary, this study demonstrated that both the OH$^-$ inhibition at the T2/T3 center and the redox potential difference between a reducing substrate and the T1 center could affect the pH activity profile of a laccase. For a reducing substrate (such as KFe(CN)$_6$) whose oxidation does not involve protons and has a minimal Eo dependence on pH, the activity of a laccase could decline monotonically when the pH increases, as the result of the possible involvement of an OH$^-$ inhibition on the T2/T3 center. For a reducing substrate (such as syringaldehyde), whose oxidation involves protons and has a significant Eo dependence on pH, the pH activity profile of a laccase could be bi-phasic, reflecting possible combinatory contribution from the opposing effects of the pH-induced redox potential change (on both the T1 center and substrate) and the OH$^-$ inhibition. It should be pointed out that laccase is a two-substrate enzyme and to obtain the true kinetic parameters, both substrates should be subjected to concentration variation. The $K_m$ and $k_{cat}$ reported above were observed with various reducing substrate concentrations in air-saturated solutions only and hence are apparent values. Thus the hypothesis proposed above should be further tested by a full kinetic analysis based on experiments in which [O$_2$] is also systematically varied.

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REFERENCES

1. Reinhammar, B. and Malmstrom, R. G. (1981) in Metal Ions in Biology-Copper Proteins (Sprio, T. G. ed.), Vol. 3, pp109–149, J. Wiley, New York, NY
2. Farver, O. and Pecht, I. (1984) in Copper Proteins and Copper Enzymes (Loncaric, R. Ed.), pp. 184–149, CRC Press, Boca Raton, FL
3. Mayer, A. M. (1987) Phytochemistry 26, 11–20
4. Sardianislani, F. S. (1989) Crit. Rev. Biotechnol. 9, 171–257
5. Solomon, E. J., Baldwin, M. J. and Lowery, M. D. (1992) Chem. Rev. 92, 541–542
6. Messerschmidt, A. (1994) Adv. Inorg. Chem. 43, 121–185
7. Thorsten, C. F. (1994) Microbiol. 140, 19–26
8. Yaropolov, A., Skorobogat’ko, O. V., Varfolomeyev, S. D. (1994) Appl. Biochem. Biotechnol. 49, 257–280
9. Mayer, A. M. and Harel, E. (1979) Phytochemistry 18, 193–215
10. Bollag, J-M. and Leonowicz, A. (1984) Appl. Environ. Microbiol. 48, 849–854
11. Xu, F., Shin, W., Brown, S. H., Wahleithner, J. A., Sundaram, U. M., and Solomon, R. I. (1996) *Biochim. Biophys. Acta* **1292**, 303–311
12. Koide, K. B., Hansen, F. B., and Ettinger, M. J. (1985) *J. Biol. Chem.* **260**, 15561–15565
13. Naki, A. and Varfolomeyev, S. D. (1982) *Biochemistry (Engl. Transl. Biokhimiya)* **46**, 1344–1350
14. Wahleithner, J. A., Xu, F., Brown, K. M., Brown, S. H., Golightly, E. J., Halkier, T., Kauppinen, S., Pederson, A., and Schneider, P. (1996) *Curr. Genet.* **29**, 395–403
15. Yaver, D. S., Xu, F., Golightly, E. J., Brown, K. M., Brown, S. H., Rey, M. W., Schneider, P., Halkier, T., Monderk, K., and Dalbøge, H. (1996) *Appl. Environ. Microbiol.* **62**, 834–841
16. Xu, F. (1996) *Biochemistry* **35**, 7608–7614
17. Nakamura, T. (1958) *Biochim. Biophys. Acta* **30**, 44–52
18. Jovanovic, S. V., Tosic, M., and Simic, M. G. (1991) *J. Phys. Chem.* **95**, 10824–10927
19. Andressen, L. E. and Reinhammar, B. (1979) *Biochim. Biophys. Acta* **568**, 145–156
20. Hansen, F. B., Noble, R. W., and Ettinger, M. J. (1984) *Biochemistry* **23**, 2049–2056
21. Tolin, G., Meyer, T. E., Cusanovich, M. A., Curir, P., and Marchesini, A. (1993) *Biochim. Biophys. Acta* **1183**, 309–314
22. Peyratout, C. S., Severns, J. C., Holm, S. R., and McMillin, D. R. (1994) *Arch. Biochem. Biophys.* **314**, 405–411
23. Reinhammar, B. R. (1972) *Biochim. Biophys. Acta* **275**, 245–249
24. Farver, O., Goldberg, M., Wherland, S., and Pecht, I. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5245–5249
25. Hoffmann, P. and Esser, K. (1977) *Arch. Microbiol.* **112**, 111–114
26. Rosenberg, R. C., Wherland, S., Holwerda, R. A., and Gray, H. B. (1976) *J. Am. Chem. Soc.* **98**, 6364–6369
27. Bourbonnais, R. and Paice, M. G. (1992) *Appl. Microbiol. Biotechnol.* **36**, 823–827
28. Fukushima, Y. and Kirk, T. K. (1995) *Appl. Environ. Microbiol.* **61**, 872–876
29. Omura, T. (1961) *J. Biochem. (Tokyo)* **59**, 264–272