Oxidation of Phenols, Anilines, and Benzenethiols by Fungal Laccases: Correlation between Activity and Redox Potentials as Well as Halide Inhibition[†]

Feng Xu

Novo Nordisk Biotech, 1445 Drew Avenue, Davis, California 95616

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ABSTRACT: A comparative study has been performed with several fungal laccases for the oxidation of a series of phenols, anilines, and benzenethiols and for the inhibition by halides. The observed K_m and k_{cat} were correlated to the structure of substrate. The change in log (k_{cat}/K_m) was found to be proportional to the one-electron redox potential difference between laccase's type 1 copper site and substrate. This correlation indicated that the first electron transfer from substrate to laccase was governed by the "outer-sphere" mechanism. Compared to the electronic factor, the steric effect of small *o*-substituents (such as methyl and methoxy groups) was found to be unimportant. The depth of the laccase's type 1 copper site was estimated as ∼10 Å by comparing the steric effect among five 2-methoxyphenols whose 4-substituents ranged from 0.1 to 14 kDa in mass. The observed inhibition potency order of $F^- > Cl^- > Br^-$ was attributed to limited accessibility of laccase's type 2/type 3 trinuclear copper cluster site. Although the enzymes studied have homologous primary sequences and predicted similar backbone structures, the difference exhibited by each enzyme (in interacting with individual substrate or inhibitor) suggested the structural variation in their functional domains.

Phenolic components are widely distributed biogenic compounds. Their oxidation or oxidative derivatization is believed to play an important role in such processes as deleterious cellular oxidation, cellular wall protection, fruit "browning", wine or juice processing, pulp delignification, composite products strengthening, textile dye bleaching, and contaminated water or soil detoxification. The oxidation of phenols can be carried out by either chemical (*e.g.*, O_3, Fe−H_2O_2, or ClO_2) or enzymatic systems (*e.g.*, phenol oxidase−O_2, peroxidase−peroxide or peracid). The chemical oxidation of phenols has been extensively studied and in-depth knowledge has been accumulated with regard to kinetics, mechanism, and structure−reactivity correlation, particularly the correlation between the oxidation potential and the structure of phenols (Steenken & Neta, 1982; Lind et al., 1990; Bordwell & Cheng, 1991; Jovanovic et al., 1991; Hapiot et al., 1992). Interactions of phenol oxidases with certain phenols have also been studied (Mayer, 1962; Fahraeus & Ljunggren, 1964; Duckworth & Coleman, 1970; Lanzarini et al., 1971; Reinhammar, 1972; Froehner & Eriksson, 1974; Dubernet et al., 1977; Wood, 1980; Reinhammar & Malmstrom, 1981; Leatham & Stahmann, 1981; Amin et al., 1985; Mayer, 1987; Kulys & Cenas, 1988; Bekker et al., 1990; Rehman & Thurston, 1992; Robinson et al., 1993; Yaropolov et al., 1994; Salas et al., 1995). However, the lack of systematic and comparative investigation with respect to both substrate and phenol oxidase (*e.g.*, laccase) has limited the elucidation of the mechanism as well as the active centers of the enzyme.

Laccases (EC 1.10.3.1) 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 O_2 to water (Reinhammar & Malmstrom, 1981; Mayer, 1987; Yaropolov et al., 1994; Solomon & Lowery, 1993). The interest of using laccase as a model to study the structure−function relationship of copper-containing proteins and the prospect of applying laccase's ability in catalyzing phenol oxidation to delignification, plant fiber derivatization, textile dye or stain bleaching, and contaminated water or soil detoxification are attracting increasing attention. To better understand the interaction of laccase with phenol, I carried out a comparative kinetic study with several fungal laccases and a series of phenols, benzenethiols, anilines, and other aryl analogs. The study revealed that the one-electron redox potential difference between laccase's type 1 copper site (T1)[1] and substrate plays a key role in activity. I also studied the inhibition of laccase by halide ion and showed that the halide binding to the type 2/type 3 (T2/T3) trinuclear copper sites is different among various laccases.

MATERIALS AND METHODS

Materials. Chemicals used as buffers and substrates (unless mentioned otherwise) were commercial products of the highest available grade. The structures and compound numbers of the substrates studied are summarized in Table 1. Compound **29** was synthesized according to the method

[†] The nucleic acid sequences of the laccases described in this paper have been reported to GenBank (Accession Numbers: PpL, L49376; RsL, Z54277).

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[1] Abbreviations: PpL, recombinant *Polyporus pinsitus* (or *Trametes villosa*) laccase isozyme 1; RsL, recombinant *Rhizoctonia solani* laccase isozyme 4; MtL, recombinant *Myceliophthora thermophila* laccase; StL, recombinant *Scytalidium thermophilum* laccase; MvBO, recombinant *Myrothecium verrucaria* bilirubin oxidase; B&R, Britton and Robinson buffer; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); diimide, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide; E_5, one-electron redox potential at pH 5 (referenced to the normal hydrogen electrode); I_{50}, the halide concentration which causes 50% decrease of the initial laccase activity; T1, type 1 copper site; T2/T3, type 2/type 3 trinuclear copper cluster site. The numeration of the substrates is given in Table 1.

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Table 1: Structure of Phenols and Other Analogs Studied[a]

no.	substituents	no.	substituents
	2-OCH_3 Phenols		Phenols
1	4-NO_2	**20**	2-H
2	4-CHO	**21**	2-CH_3
3	4-$COCH_3$	**22**	2-C_2H_5
4	4-CO_2CH_3	**23**	2-OH
5	4-$CH_2NH_3^+$	**24**	4-CO_2^-
6	4-CO_2^-	**25**	2-OH, 4-CO_2^-
7	4-CH_2OH	**26**	2-NO_2, 4-$CH_2CO_2^-$
8	4-H	**27**	4-$COCH_3$
9	4-$CH_2CO_2^-$	**28**	2,6-$(C(CH_3)_3)_2$, 4-CO_2^-
10	4-CH_3	**29**	2,6-$C(CH_3)_3)_2$, 4-$C(CH_2)_2CH_2NH_3^+$
	2,6-$(OCH_3)_2$ Phenols		Phenol Analogs
11	4-CHO	**30**	1,2-$(NH_3^+)_2$
12	4-$COCH_3$	**31**	1,2-$(SH)_2$
13	4-$CHNNHCC_6H_2(OCH_3)_2OH$	**32**	1-NH_3^+, 2-OCH_3
14	4-H	**33**	1-SH, 2-OCH_3
15	4-CO_2^-	**34**	1-CH_3, 2-OCH_3, 4-CO_2^-
		35	1-OCH_3, 2-OCH_3, 4-CO_2^-
	2-OCH_3 Phenols	**36**	1-F, 2-OCH_3, 5-NH_3^+
16	4-$CH_2CONH(CH_2)_6NH_3^+$	**37**	1-OH, 2-OCH_3, 5-NH_3^+
17	4-$CH_2CONHCH(C_6H_5)CO_2CH_3$		
18	4-CH_2CONH-lysozyme		
19	4-$CH_2CONH(CH_2)_6NHCO$-lysozyme		

[a] The charge states are referred to that at pH 5.

of Traylor et al. (1984). Recombinant laccases and bilirubin oxidase were purified by a combination of ion-exchange, gel-filtration, and hydrophobic interaction chromatography (Xu et al., 1996; Wahleithner et al., 1996; Yaver et al., 1996).[2] *Rhus vernicifera* luccase was kindly provided by Professor E. I. Solomon at the Department of Chemistry, Stanford University. The Britton and Robinson (B&R) buffer with pH 5 was made by mixing 0.1 M boric acid−0.1 M acetic acid−0.1 M phosphoric acid with 0.5 M NaOH. Although the laccases studied have optimal activity at pH 5−8 in oxidizing syringaldazine (**13**) and are stable at pH 3−9 (Xu et al., 1996; Wahleithner et al., 1996; Yaver et al., 1996), the pH of 5 was chosen in this study so that the phenoxy group of all the substrates would be protonated. The stock solutions (0.1 M) of substrates **5**, **32**, **36**, and **37** were made with the addition of equivalent HCl, the stock solutions of substrates **4**, **13**, **27**, **29**, **31**, and **33** were made in ethanol, the stock solutions of substrates **8**, **16**−**20**, **23**, **30**, and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were made in plain water, and the stock solutions of the rest substrates were made with the addition of equivalent NaOH. The presence of ≤10% ethanol in the final assay solution showed minimal effect on enzyme activity.

Syntheses of Homovanillic Acid Derivatives. Different groups, ranging from 0.1 to 14 kDa in mass, were linked to homovanillate (**9**) at the 4-CH_2CO_2H site *via* amidation catalyzed by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (diimide). In general, equal amounts (20−500 μmol) of homovanillate (**9**), a coupling compound, and the diimide were mixed in 5−8 mL of water at pH 4.75 and reacted for 0.5−2 h. The pH of the reacting solutions was kept at 4.75 by HCl or NaOH and the completion of reaction was judged by the stabilization of pH. The products were separated by either ether extraction (followed by air-drying and redissolving in methanol) or, when lysozyme was attached, dialysis on a Spectrum membrane with molecular weight cutoff of 10 000. The identification was made by thin-layer chromatography (TLC) (on Aldrich's silica gel plates) with $CHCl_3/CH_3OH$ (10/1.7) and by difference spectra at pH 11 and 5. The yield estimations for the syntheses were made with difference spectra at pH 11 and 5 (at 304 nm) as well as oxidation stoichiometry measurement. For the homovanillic derivatives coupled to lysozyme (**18** and **19**), the pH-induced spectral change in lysozyme itself was subtracted from the observed total spectral change.

Under these conditions, homovanillate (**9**) was amidated with 1,6-hexanediamine, or phenylalanine methyl ester, or lysozyme (through a free amine group from its lysines or the N-terminus) to yield, respectively, the putative 2-OCH_3-4-$CH_2CONH(CH_2)_6NH_2$-phenol (**16**, 65% yield), or 2-OCH_3-4-$CH_2CONHCH(C_6H_5)CO_2CH_3$-phenol (**17**, 31% yield), or 2-OCH_3-4-CH_2CONH-lysozyme phenol (**18**, 24% yield). The phenols **16** and **17** showed R_f values of 0.53 and 0.61, respectively, in the TLC assay, in comparison with the R_f of 0.76 for homovanillate (**9**). A lysozyme molecule was also coupled (through a free carboxy group from its glutamates or aspartates or C-terminus) to compound **16** at the free amine site on its 4-$CH_2CONH(CH_2)_6NH_2$ substituent, by reacting with equivalent amount of the diimide, to generate the putative 2-OCH_3-4-$CH_2CONH(CH_2)_6NHCO$-lysozyme phenol (**19**, 21% yield). For control study purpose, lysozyme was acetylated/amidated in B&R buffer, pH 4.75, which served as the buffer as well as the acetate source, with equivalent diimide, which served as the catalyst as well as the primary amine source.

Oxidation Assays. The laccase-catalyzed phenol oxidation was monitored by O_2 consumption with a Hansatech DW1/AD O_2 electrode (Norfolk, England), with 0.04−15 μM

[2] The details of the purification and characterization, along with the cloning and expression, for the recombinant *Myceliophthora thermophila* and *Scytalidium thermophilum* laccases have been previously presented at the Western Regional ACS Meeting and Pacific Conference, held on October 19−22, 1994, in Sacramento, CA (authors: Berka, B. M., Thompson, S. A., Brown, S. H., Golightly, E. J., Brown, K. M., and Xu, F.) and in the patent applications WO9533836A1 and WO9533837A1. The nucleic acid sequences of the laccases have been reported to GenBank.

laccases in 0.3−0.5 mL of B&R buffer, pH 5, at 20 °C. After the voltage reading stabilized, laccase was added into the solution to initiate the reaction. Duplicate data were obtained for each concentration setting. Without laccase or substrate, no significant O_2 consumption took place during the experimental time frame. The O_2 concentration in air-saturated buffer solution was assumed as the same in plain water (0.28 mM). The performance of the Hansatech apparatus as well as the measurement of phenol oxidation were calibrated by laccase-catalyzed ABTS oxidation whose one-electron oxidation led to the formation of its stable, spectrally characteristic cation radical.

Direct spectrophotometric measurements were also carried out for guaiacol (**8**) at 418 nm with $\Delta\epsilon$ of 1.6 mM^{-1} cm^{-1}; 2,6-$(OCH_3)_2$-phenol (**14**) at 468 nm with $\Delta\epsilon$ of 22 mM^{-1} cm^{-1}; syringaldehyde (**11**) at 370 nm with $\Delta\epsilon$ of 1.2 mM^{-1} cm^{-1}; syringaldazine (**13**) at 530 nm with $\Delta\epsilon$ of 65 mM^{-1} cm^{-1} (Bauer & Rupe, 1971); and ABTS at 418 nm with $\Delta\epsilon$ of 36 mM^{-1} cm^{-1} (Childs & Bardsley, 1975) in B&R buffer, pH 5, on a Shimadzu UV160U spectrophotometer with 1-cm quartz cuvettes.

Halide Inhibition. The inhibitory effect of F^-, Cl^-, and Br^- on laccase activity was assayed in laccase- (0.03−1 μM) catalyzed ABTS (at saturating 2 mM level) oxidation in 0.1 M sodium acetate, pH 5. The oxidation was monitored at 405 nm with various sodium halide concentrations in 96-well plates (Costar, tissue culture grade) with a Molecular Devices Thermomax microplate reader.

Kinetic Analysis. K_m was determined by fitting experimental data (≥6 duplicates for each substrate) $\Delta V_i/\Delta t$ and [substrate] to $\Delta V_i/\Delta t = (\Delta V_{max}/\Delta t)$[substrate]/($K_m$ + [substrate]), where $\Delta V_i/\Delta t$ was the initial output voltage change of the O_2 cell. The parameter k_{cat} was determined from $k_{cat} = (\Delta V_{max}/\Delta t)/(\Delta V_f)n[O_2]/$[laccase], where ΔV_f was the final output voltage change corresponding to total O_2 consumption, n was the oxidation stoichiometry number determined by saturating [substrate], $[O_2]$ was the dissolved O_2 concentration (0.28 mM), and [laccase] was the laccase concentration based on subunit. In the case of spectrophotometric measurements, ΔV was replaced by change in absorbance ΔA and $n[O_2]$ was replaced with $1/\Delta\epsilon$ to determine k_{cat}. Nonlinear regression fitting was performed using GraphPad's Prizm program. The errors for both K_m and k_{cat} were given as SD.

The pK_a values of the phenolic substrates were obtained from Jovanovic et al. (1991), Perrin et al. (1981), Stewart (1985), and Xu et al. (1996). The one-electron redox potentials at pH 5 (E_5) of syringaldazine (**13**), ABTS, and the laccases (T1 site) were from Xu et al. (1996). The E_5 values of compounds **1**, **3**, **6**, **8**, **10**, **12**, **14**, **15**, **20**, **23**−**25**, **27**, and **30** were converted from the potential values at pH 7 (E_7) or other pH (Steenken & Neta, 1982; Lind et al., 1990; Jovanovic et al., 1991) as described by Jovanovic et al. (1991). The E_5 values of compounds **2**, **4**, **5**, **7**, **9**, **12**, **11**, **21**, and **22** were converted from E_7 values which were estimated by the correlation of $E_7 = 0.95 + 0.31\sigma^+$ (Jovanovic et al., 1991) and the published σ^+ values (McGary et al., 1955) for the substituents involved. An E_5(**32**) of 0.9 V was estimated from E_5(**8**) + {E_5(4-NH_2-C_6H_5OH) − E_5-(1,4-$C_6H_4(OH)_2$)}, in which E_5(1,4-$C_6H_4(OH)_2$) of 0.58 V and E_5(4-NH_2-C_6H_5OH) of 0.53 V were converted from the values of Steenken and Heta (1982). An E_5(**31**) of −0.7 V was estimated from E_5 (**23**) + {E_5(C_6H_5SH) − E_5(**20**)}, in which an E_5(C_6H_5SH) of −0.3 V was converted from the value of Ryklan and Schmidt (1994); whereas an E_5(**33**) of −0.5 V was estimated from E_5(**8**) + {E_5(C_6H_5SH) − E_5-(**20**)}.

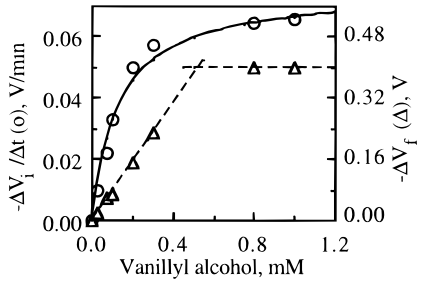

FIGURE 1: O_2 uptake in PpL-catalyzed oxidation of vanillyl alcohol (**7**) monitored by O_2 electrode. The initial output voltage change rate $-\Delta V_i/\Delta t$ (○; in volts per minute) and the final output voltage change $-\Delta V_f$ (△; in volts) of each run were plotted *vs* substrate concentration. The solid curve represents the fitting to the Michaelis−Menten equation $-\Delta V_i/\Delta t = -V_{max}$[substrate]/($K_m$ + [substrate]), with a K_m of 130 ± 20 μM and a V_{max} of 0.075 ± 0.003 V min^{-1}. A k_{cat} of 2600 ± 100 min^{-1} was obtained with [PpL] = 40 nM (subunit) and $n = 2$. The cross point of the two dashed lines yielded a saturating [substrate] of 0.5 mM, giving an oxidation stoichiometry n of 1.8.

RESULTS

Laccase-Catalyzed ABTS Oxidation Monitored by Spectroscopic and O_2 Electrode Methods. In the presence of a laccase, ABTS can surrender one electron and be readily oxidized to its stable cation radical, whose concentration can be easily determined by UV−visible spectroscopy (Childs & Bardsley, 1975). We monitored *Polyporus pinsitus* laccase- (PpL-) catalyzed ABTS oxidation spectrophotometrically and obtained a K_m of 58 ± 8 μM and a k_{cat} of 2700 ± 100 min^{-1}. In the O_2 cell, the oxidation can be monitored by O_2 consumption. In our experiment, the plot of final output voltage reading *versus* ABTS concentration yielded a saturating [ABTS] near 1.1 mM. Since the dissolved $[O_2]$ is around 0.28 mM, this indicates a stoichiometry number of 3.9, in agreement with our expectation that the reduction of one O_2 molecule (to water) requires four electrons. The initial rate of O_2 uptake was found to be proportional to PpL concentration over the tested range of 2−200 nM. Based on the observed Michaelis-type dependence on [ABTS], a K_m of 100 ± 20 μM and a k_{cat} of 3300 ± 200 min^{-1} were obtained, similar to the spectroscopic results.

Laccase-Catalyzed Oxidation of 4-Substituted 2-Methoxy- and 2,6-Dimethoxyphenols. An example of the PpL-catalyzed oxidation of 4-substituted 2-OCH_3 phenols is shown in Figure 1. As monitored by O_2 uptake, the reaction showed typical exponential voltage time course whose initial slope ($-\Delta V_i/\Delta t$) exhibited a Michaelis−Menten-type dependence on initial phenol concentration. The relationship between final voltage change ($-\Delta V_f$) and phenol concentration indicated an oxidation stoichiometry number of 2. Table 2 summarizes the K_m and k_{cat} values observed with PpL for a series of 4-substituted 2-methoxy- and 2,6-dimethoxyphenols. For the oxidation of guaiacol (**8**), syringaldehyde (**11**), and 2,6-dimethoxyphenol (**14**), the photometrically monitored product formation and the O_2 electrode-monitored O_2 consumption gave similar K_m and k_{cat} values (Table 2), indicating that the substrate oxidation (or the T1 copper reduction) was rate-limiting. A linear correlation was found

Table 2: Effect of Substituents in 4-Substituted 2-Methoxy- and 2,6-Dimethoxyphenols

no.	pK_a	K_m (mM)	k_{cat} (min^{-1})
	4-Substituted 2-OCH_3 Phenols		
1	7.2	9.90 ± 2.10	320 ± 40
2	7.6	2.30 ± 0.30	1300 ± 100
3	8.0	1.20 ± 0.20	1100 ± 100
4	8.9	1.10 ± 0.20	1200 ± 100
5	9.3	0.90 ± 0.10	2300 ± 100
6	9.5	0.17 ± 0.03	2500 ± 100
7	9.9	0.13 ± 0.02	2600 ± 100
8[a]	9.9	0.13 ± 0.01	2400 ± 200
9	10.0	0.10 ± 0.02	2200 ± 100
10	10.0	0.10 ± 0.02	3600 ± 400
	4-Substituted 2,6-$(OCH_3)_2$ Phenols		
11[a]	7.6	0.11 ± 0.01	2200 ± 100
12	8.0	0.12 ± 0.02	1800 ± 100
13	8.2	0.005 ± 0.0005	2900 ± 100
14[a]	9.6	0.13 ± 0.01	3100 ± 100
15	9.8	0.08 ± 0.01	3100 ± 100

[a] Spectrophotometric measurement of the product formation yielded a K_m of 0.22 ± 0.04 mM and k_{cat} of 3300 ± 500 min^{-1} for **8**; a K_m of 0.11 ± 0.02 mM and k_{cat} of 3200 ± 600 min^{-1} for **11**; and a K_m of 0.11 ± 0.02 mM and k_{cat} of 3300 ± 500 min^{-1} for **14**.

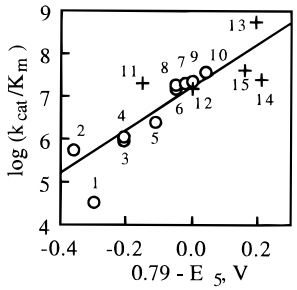

FIGURE 2: Correlation of log (k_{cat}/K_m) with E_5 for 4-substituted phenols. Symbols: (○) 4-substituted 2-methoxyphenols; (+) 4-substituted 2,6-dimethoxyphenols. The linear regression correlation is log (k_{cat}/K_m) = 5.1 ΔE + 7.2, R^2 = 0.83, in which ΔE = E_5 (PpL-T1 site) − E_5 = 0.79 − E_5. The unit of k_{cat}/K_m is M^{-1} min^{-1}.

Table 3: Steric Effect in 4-Substituted 2-Methoxyphenols

no.	4-substituent's MW	K_m (mM)	k_{cat} (min^{-1})
9	58	0.10 ± 0.02	2200 ± 100
16	158	0.20 ± 0.02	1700 ± 100
17	191	0.27 ± 0.03	2100 ± 100
18	14000	0.40 ± 0.06	1400 ± 100
19	14200	0.33 ± 0.06	1400 ± 100

between log (k_{cat}/K_m) and E_5 for the 2-OCH_3 and 2,6-$(OCH_3)_2$ phenols studied (Figure 2).

Table 3 summarizes the K_m and k_{cat} values observed for a series of 2-methoxyphenols and 2,6-dimethoxyphenols which have bulky substituents at the 4-position. Although the size of these substituents varies from 0.1 to 14 kDa, only a relatively small effect was seen. For the lysozyme-coupled phenols **18** and **19**, the presence of the hexyl linker did not affect the reactivity much. Neither native nor diimide-treated (acetylated/amidated) lysozyme showed any reactivity toward PpL under the same conditions.

Oxidation of Phenolic Analogs That Have Different o-Substitutions. Table 4 summarizes the K_m and k_{cat} values observed for a series of phenols which have different substitutions at the *o*-position. A correlation between log (k_{cat}/K_m) and E_5 of these substrates was found (Figure 3), which was similar to that shown in Figure 2. The replacement of the 2-OCH_3 in **9** by a 2-NO_2, which resulted in **26**, led to a K_m of 3.0 ± 0.5 mM and k_{cat} of 340 ± 20 min^{-1}. For phenols **27**−**29**, $K_m \geq 10$ mM and $k_{cat} \leq 40$ min^{-1} were estimated due to detection limits.

Table 4: Effect of Different Substituents in *o*-Substituted Phenols

no.	K_m (mM)	k_{cat} (min^{-1})
	Substituted Phenols	
20	44 ± 12	1600 ± 300
21	1.90 ± 0.20	1300 ± 100
22	2.20 ± 0.30	3500 ± 200
23	0.19 ± 0.02	3300 ± 100
8	0.13 ± 0.01	2400 ± 200
14	0.13 ± 0.01	3100 ± 100
	Substituted 4-CO_2^- Phenols	
24	3.1 ± 1.4	120 ± 20
25	0.57 ± 0.06	3000 ± 100
6	0.17 ± 0.03	2500 ± 100
15	0.08 ± 0.01	3100 ± 100

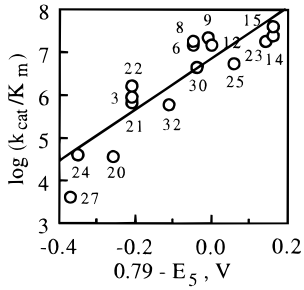

FIGURE 3: Correlation of log (k_{cat}/K_m) with E_5 for phenol analogs with different *o*-substitutions as well as for phenol and aniline analogs. The linear regression gives a line of log (k_{cat}/K_m) = 6.1ΔE + 6.8, R^2 = 0.78, in which ΔE = E_5(PpL-T1 site) − E_5 = 0.79 − E_5. The data for benzenethiols **31** and **33** are located at −0.5 to −0.7 V and are not included in the figure. The unit of k_{cat}/K_m is M^{-1} min^{-1}.

Table 5: Difference between Phenoxyl and Other Functional Groups

no.	K_m (mM)	k_{cat} (min^{-1})
	Substituted Benzenes	
30	0.47 ± 0.03	2100 ± 100
31	0.80 ± 0.20	45000 ± 4000
23	0.19 ± 0.02	3300 ± 100
	Substituted Anisoles ($C_6H_5OCH_3$)	
32	1.6 ± 0.4	970 ± 80
33	0.12 ± 0.02	2900 ± 100
8	0.13 ± 0.01	2400 ± 200

Oxidation of Phenol, Benzenethiol, Aniline, and Other Aryl Analogs. With PpL, 1,2-catechol (**23**) and 1,2-phenylenediamine (**30**) showed oxidation stoichiometry numbers of 2.0 and 2.2, respectively. 1,2-Benzenedithiol (**31**) showed an oxidation stoichiometry number of 3.3, indicating possible formation of disulfide (one-electron oxidation product which would result in an oxidation stoichiometry number of 4) and sulfenic acid (two-electron oxidation product which would result in an oxidation stoichiometry number of 2). When 40 nM PpL was replaced by 160 nM $CuSO_4$, no significant O_2 uptake occurred with 1,2-benzenedithiol. Guaiacol (**8**) and *o*-anisidine (**32**) gave oxidation stoichiometry numbers close to 2. For 2-methoxybenzenethiol (**33**), a stoichiometry number of 2 was observed when [**33**] ≤ 1 mM. Table 5 summarizes the K_m and k_{cat} values observed for a series of aryl compounds with replacement of the aryl OH group by aryl SH and NH_2. The replacement of the aryl OH group in

Table 6: Comparison of Kinetic Parameters for Selected Substrates with Non-PpL Laccases[a]

substrate		RsL	MtL	StL	MvBO
8	K_m	0.42 ± 0.06	0.48 ± 0.13	0.13 ± 0.01	0.51 ± 0.06
	k_{cat}	15 ± 1	45 ± 6	7.2 ± 0.3	4.4 ± 0.1
2	K_m	12 ± 3	15 ± 4	12 ± 2	0.9 ± 0.1
	k_{cat}	6.5 ± 0.9	3.6 ± 0.6	7 ± 1	4.7 ± 0.2
22	K_m	2.8 ± 0.4	4.3 ± 0.8	1.3 ± 0.2	1.0 ± 0.1
	k_{cat}	3.5 ± 0.2	1.7 ± 0.1	1.8 ± 0.1	5.8 ± 0.2
33	K_m	0.18 ± 0.02	0.77 ± 0.08	0.08 ± 0.02	0.34 ± 0.05
	k_{cat}	180 ± 10	190 ± 10	16 ± 1	20 ± 1
32	K_m	0.38 ± 0.07	0.41 ± 0.04	0.31 ± 0.04	0.32 ± 0.06
	k_{cat}	5.1 ± 0.3	0.99 ± 0.03	0.66 ± 0.01	2.5 ± 0.1
9	K_m	0.44 ± 0.06	0.92 ± 0.08	0.13 ± 0.02	0.75 ± 0.13
	k_{cat}	77 ± 3	23 ± 1	4.9 ± 0.2	2.3 ± 0.1
18	K_m	0.05 ± 0.01	0.52 ± 0.08	0.07 ± 0.01	1.4 ± 0.5
	k_{cat}	3.4 ± 0.3	9.9 ± 0.6	1.7 ± 0.1	3.0 ± 0.7

[a] The units for K_m are millimolar, and for k_{cat}, reciprocal minutes.

6 by a -CH_3 or -OCH_3, which resulted in **34** or **35**, respectively, led to a $K_m \geq 10$ mM and $k_{cat} \leq 40$ min^{-1} (both estimated due to detection limits). The replacement of the aryl OH group in **37** by -F, which resulted in **36**, led to a K_m of 0.72 ± 0.06 mM and k_{cat} of 240 ± 60 min^{-1} (which actually corresponded to the oxidation of the aryl NH_2, rather than the aryl F). Except for the benzenethiol analogs (**31** and **33**), the phenol and aniline analogs (**8**, **23**, **30**, and **32**) seemed to have a correlation between log (k_{cat}/K_m) and E_5 similar to that observed for the 2-methoxy- and 2,6-dimethoxyphenols (Figure 2) as well as that observed for the *o*-substituted phenols (Figure 3).

Phenol Oxidation Catalyzed by Other Laccases. From the 37 substrates whose oxidations by PpL were studied, guaiacol (**8**) and six derivatives were selected for testing with other laccases and bilirubin oxidase. In two chosen derivatives, the effect of *p*-substitution [4-CHO in vanillin (**2**), mainly electronic] and change in the *o*-substitution [2-OCH_3 to 2-C_2H_5 switch in 2-ethylphenol (**22**), both electronic and steric] was studied by comparing to guaiacol (**8**). In other two chosen derivatives, the effect of replacing aryl OH by aryl SH [in 2-OCH_3-benzenethiol (**33**)] or aryl NH_2 [in *o*-anisidine (**32**)] was studied by comparing to guaiacol (**8**). In the remaining two chosen derivatives, the steric effect of the 4-substituents different in size was studied by comparing compounds **9** and **18**. Under our conditions, *Rhizoctonia solani* laccase (RsL), *Myceliophthora thermophila* laccase (MtL), *Scytalidium thermophilum* laccase (StL), and *Myrothecium verrucaria* bilirubin oxidase (MvBO) all exhibited Michaelis-type kinetics in oxidizing the selected substrates and showed oxidation stoichiometry close to 2. Table 6 shows the K_m and k_{cat} values of these substrates for RsL, MtL, StL, and MvBO. The effects discussed above for PpL (electronic effect, steric effect, replacement of aryl OH by aryl SH and aryl NH_2) were also observed with RsL, MtL, StL, and MvBO.

When individual phenol substrates were compared, PpL showed the highest activity as judged by the value of k_{cat}/K_m (Tables 2−6). The difference in k_{cat}/K_m could be attributed mostly to the difference in k_{cat}, and it appeared that the substitution on the substrates affected k_{cat} more than K_m for the enzymes tested. As shown in Figure 4, the difference between the E_5 of the T1 site in laccases and the E_5 of the substrate correlates with the substrate activity for all the phenols tested.

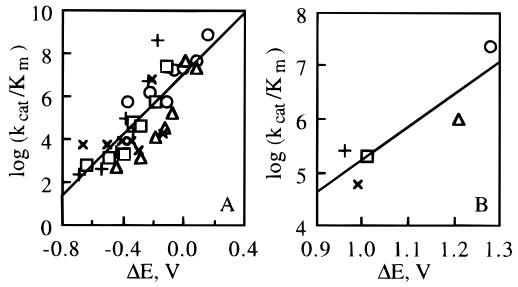

FIGURE 4: Correlation of log (k_{cat}/K_m) with ΔE [= E_5 (laccase-T1 site) − E_5(substrate)]. The values of log (k_{cat}/K_m) and E_5(substrate) for compounds **2**, **8**, **9**, **22**, **33**, and **32** are from Tables 2−6. The E_5 for the T1 site in PpL (0.78 V; ○), RsL (0.71 V; △), MtL (0.46 V; +), StL (0.51 V; □), and MvBO (0.49 V; ×), as well as the E_5 and k_{cat}/K_m for ABTS and syringaldazine are from Xu et al. (1996). The linear regression line of log (k_{cat}/K_m) = 7.1ΔE + 7.0, R^2 = 0.65, in (A) is made for all substrates except **33**, whose data are shown in (B) with a correlation log (k_{cat}/K_m) = 6.2ΔE − 1.0, R^2 = 0.80.

Table 7: I_{50} of Sodium Halides for Various Laccases[a]

halide	PpL	RsL	MtL	StL	MvBO	*Rhus*
NaF	0.02	0.02	0.05	0.5	1.0	0.02
NaCl	40	50	600	0.4	10	0.05
NaBr	200	200	1600	5	10	0.05

[a] In 0.1 M sodium acetate, pH 5, with ABTS (2 mM) as the substrate. I_{50} values are given in millimolar.

Halide Inhibition. Under our conditions, halides inhibited the ABTS oxidase activity of laccase. The effect of the ionic strength change caused by adding sodium halides was minimal. Adding sodium halide salts after ABTS oxidation did not interfere with the spectrum of the cation radical. A similar halide inhibition was also observed with syringaldazine as substrate. The inhibition seemed to be reversible, since a dilution which reduced the halide concentration 100-fold below its I_{50} (the concentration of an inhibitor causing 50% activity reduction) led to full recovery of the laccase activity.

The observed halide inhibition of the laccases showed characteristics of a complex "linear mixed-type" inhibition. For F^-, convex-type correlations were observed when the slope and the *y*-intercept of the Lineweaver−Burk plots (1/rate *versus* 1/[substrate]) are plotted against halide concentrations, similar to that observed with *Rhus* laccase (Koudelka & Ettinger, 1988). For Cl^- and Br^-, however, linear correlations were observed. The parameter I_{50} was recorded for quantitation purposes, since the complexity of the plots complicated the extraction of the inhibition constant K_i. Based on I_{50}, the same order for the inhibitory potency of the halides ($F^- > Cl^- > Br^-$) was observed for all the laccases studied (Table 7). However, the enzymes showed variable tolerance toward individual halides. For instance, MtL has an I_{50} 10^3-fold higher than that of StL for Cl^-.

DISCUSSION

Effect of the o- and p-Substituents on Activity. The comparison of the 4-substituted 2-methoxy- and 2,6-methoxyphenol series listed in Table 2 indicates that the presence of electron-withdrawing substituents diminished the activity of phenol. These substituents (*e.g.*, 4-NO_2 and 4-$COCH_3$) reduce the electron density at the phenoxy group, thus making it more difficult to be oxidized (as shown by higher

E_5), less reactive in surrendering electron to the T1 copper of laccase (as shown by smaller k_{cat}), and less basic (as shown by smaller pK_a). The electron-withdrawing substituents in 2-methoxyphenols led to increased K_m and decreased k_{cat}. For polyphenol oxidase, which uses one pair of coupled type 3 copper atoms to oxidize phenol, a similar or different correlation for k_{cat} or K_m was found, respectively, with respect to the substituent effect (Mayer, 1962; Duckworth & Coleman, 1970; Lanzarini et al., 1971).

The comparison of the correlation shown in Figure 3 to that shown in Figure 2 indicates that, for phenols with small *o*-substitutions (*e.g.*, 2-H, OH, OCH_3, CH_3, or C_2H_5), electronic contribution plays a more important role in determining the activity than steric effect. For instance, the reason that guaiacol (**8**) is more active than 2-ethylphenol (**22**) is most likely because of the electron-donating property of the 2-OCH_3 and the resulting reduction in E_5. However, this observation may not hold when the size of the *o*-substituent becomes significantly larger. In that scenario, steric hindrance of bulky *o*-substituents could severely interfere with the proper docking of a substrate molecule in the substrate pocket (or the T1 copper site) in laccase. This may explain why 2,6-di-*t*-butylphenols (**28** and **29**) are much less active.

The comparison among the phenols possessing 4-substituents that vary significantly in size (Table 3) may shed light on the spatial form of the T1 site in laccase. The relatively small difference observed among homovanillate (**9**) and its derivatives (**16**−**19**) in terms of reactivity suggests that the depth of the substrate pocket in laccase is probably in the order of ∼10 Å [corresponding to the length of a homovanillate (**9**) molecule]. For other T1 copper-containing proteins, X-ray structure analyses have revealed the depth of the T1 copper from the Connolly surface as ∼7 Å in ascorbate oxidase (Messerschmidt et al., 1992), ∼4 Å in nitrate reductase (Godden et al., 1991), and ∼7 Å in azurin (Nar et al., 1991).

Comparison among Phenol, Benzenethiol, Aniline, and Other Aryl Analogs. The lower activities of fluorobenzene, toluene, and anisole in comparison with their phenol analogs as well as the difference between phenols and anilines (Table 5) can be attributed mainly to electronic effect or redox potential. The oxidation potentials of aryl F and aryl CH_3 could be ≥1 V, like that of aryl OCH_3 (Kersten et al., 1990), thus making them highly inactive. The dependence of the log (k_{cat}/K_m) on E_5 for the anilines is closely similar to that observed for the phenols (Figures 2 and 3), suggesting the involvement of a similar oxidation mechanism. Unlike their aniline analogs, the benzenethiols show data that do not fall on the same log (k_{cat}/K_m)−E_5 correlation as that for the phenols (Figure 4). Probably a special interaction between the thiol and the T1 copper (many sulfur-containing groups have high affinity toward copper ion) is involved in addition to the simple substrate docking to the T1 pocket that phenols and anilines are related to; or probably the adaptation of the E_5 values for benzenethiols **31** and **33**, which are derived from measurements made in aqueous ethanol, is not accurate for the redox potential under our conditions (B&R buffer, pH 5). However, possible interference from a nonproteinaceous benzenethiol−copper complex, generated from an extractive chelation of the copper atoms from laccase by benzenethiol, can be ruled out, since the presence of $CuSO_4$ causes the oxidation of 1,2-$C_6H_4(SH)_2$ (**31**) at a rate that is negligible in comparison to that observed with the equivalent laccase.

Mechanism of Electron Transfer from Substrate to Laccase. The discussion made above with PpL is also applicable to RsL, MtL, StL, and MvBO. As shown in Figure 4, the different activity of various laccases toward each substrate can be attributed mainly to the difference in the redox potential. The correlation in Figure 4 is closely similar to that in Figures 2 and 3. Higher E_5 (laccase-T1 site) or lower E_5 (substrate) makes the driving force $\Delta E = E_5$(laccase-T1 site) − E_5(substrate) larger, which in turn leads to a faster reaction rate [or larger log (k_{cat}/K_m)]. All these indicate that the enzymes tested have similar T1 sites and involve similar mechanisms that govern the electron transfer from the substrate to the T1 copper.

When expressed as log (k_{cat}/K_m) = $\alpha\{E_5$ (laccase-T1 site) − E_5(substrate)$\} + \beta$, the correlation in Figures 2, 3, and 4 shows respectively $\alpha = 5.1$ and $\beta = 7.2$, $\alpha = 6.1$ and $\beta = 6.8$, and $\alpha = 7.1$ and $\beta = 7.0$. The correlation indicates that the first electron transfer between substrate and laccase can be described by the "outer-sphere" mechanism in which the rate (or activation energy) is predominantly regulated by the redox potential difference (Marcus & Sutin, 1985). However, it should be noted that some E_5 (substrate) values used in the correlation are estimated and it would be better to directly measure them by methods such as radiolysis or laser flash photolysis (Jovanovic et al., 1991).

Interaction of Laccases with Halides. As shown with *Rhus* laccase-F^- system, the inhibition of laccase by halide is likely at the T2/T3 site (Koudelka & Ettinger, 1988). The inhibition order of $F^- > Cl^- > Br^-$ is attributed to limited accessibility of the T2/T3 copper atoms (Naki & Varfolomeev, 1981). As shown in Table 7, different laccases can have quite different tolerances toward inhibition by various halide species. If the inhibition is mainly limited by the size of the putative channel that leads to the T2/T3 site, then the observed variation in I_{50} indicates a significant difference among various laccases. While the plant *Rhus* laccase might have a "wide-open" channel leading to the T2/T3 site (as indicated by the similar I_{50} values for F^-, Cl^-, and Br^-), fungal laccases seem to have T2/T3 channels with a defined "cutoff diameter" that corresponds to the diameter of hydrated Cl^- or Br^-. The halide inhibition study does not include I^-, because I^- reacts with the T1 site and serves as a substrate for laccase (Xu, 1996).

Overall Remarks. Previously, the activities of phenols toward various copper-containing enzymes have been related to their pK_a and other typical substitution parameters such as Hammett σ constant (Fahraeus & Ljunggren, 1964; Reinhammar, 1972; Froehner & Eriksson, 1974; Dubernet et al., 1977; Wood, 1980; Reinhammar & Malmstrom, 1981; Leatham & Stahmann, 1981; Amin et al., 1985; Mayer, 1987; Bekker et al., 1990; Rehman & Thurston, 1992; Robinson et al., 1993; Yaropolov et al., 1994; Salas et al., 1995). Although pK_a is primarily a parameter for the acidity of phenols, it has been correlated to oxidation potential or aryl O−H bond dissociation energy in certain chemical systems (Steenken & Neta, 1982; Lind et al., 1990; Bordwell & Cheng, 1991). In this study, a wide range of phenols and other aryl analogs have been comparatively investigated and their activities are correlated to E_5, the oxidation potential of the first step in phenol oxidation. The correlation between log (k_{cat}/K_m) and E_5 indicates that the first electron transfer

from phenol to laccase is rate-limiting in laccase-catalyzed multielectron phenol oxidation and is governed by the redox potential difference between the substrate and laccase's T1 copper through the "outer-sphere" mechanism. However, our assumption about the rate-limiting step in fungal laccase catalysis should be further tested by independent kinetic methods capable of measuring the intramolecular electron-transfer rate between the T1 and T2/T3 sites (Tollin et al., 1993). Although the effect of one-electron redox potentials on activity is emphasized in this study, we cannot ignore other structural and mechanistic factors whose effects may result in the data scattering (from the correlation line) shown in Figures 2−4. Nevertheless, the important contribution from the potentials is evident when sufficient ranges for ΔE (from −0.7 to 0.2 V) and log (k_{cat}/K_m) (from 2 to 9) are considered. The observed difference in halide inhibition may reflect the structural difference of the T2/T3 channel in laccase. All these are consistent with the belief that although laccase and other multicopper oxidases are quite homologous in terms of primary sequence and backbone structure (Messerschmidt & Huber, 1990), they can have significant variation in molecular and enzymatic properties, such as redox potential and substrate/inhibitor specificity. These results, combined with general knowledge on copper proteins, could assist us not only in probing the active sites and catalytic mechanism of laccase but also in identifying and optimizing their potential industrial/medicinal applications.

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