Redox Chemistry in Laccase-Catalyzed Oxidation of N-Hydroxy Compounds

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1-Hydroxybenzotriazole, violuric acid, and *N*-hydroxyacetanilide are three N-OH compounds capable of mediating a range of laccase-catalyzed biotransformations, such as paper pulp delignification and degradation of polycyclic hydrocarbons. The mechanism of their enzymatic oxidation was studied with seven fungal laccases. The oxidation had a bell-shaped pH-activity profile with an optimal pH ranging from 4 to 7. The oxidation rate was found to be dependent on the redox potential difference between the N-OH substrate and laccase. A laccase with a higher redox potential or an N-OH compound with a lower redox potential tended to have a higher oxidation rate. Similar to the enzymatic oxidation of phenols, phenoxazines, phenothiazines, and other redox-active compounds, an "outer-sphere" type of single-electron transfer from the substrate to laccase and proton release are speculated to be involved in the rate-limiting step for N-OH oxidation.

Laccases (EC 1.10.3.2) are multi-Cu oxidases that can catalyze the oxidation of a range of reducing substances with the concomitant reduction of O_2 (for recent reviews, see reference 24 and references therein). Because of their capability of catalyzing the oxidation of aromatic compounds, laccases are receiving increasing attention as potential industrial enzymes in various applications, such as pulp delignification, wood fiber modification, dye or stain bleaching, chemical or medicinal synthesis, and contaminated water or soil remediation (15, 37).

Laccases contain one type 1 (T1) Cu center, one type 2 (T2) Cu center, and one type 3 (T3) Cu center. The T2 and T3 sites form a trinuclear Cu cluster onto which O_2 is reduced. The T1 Cu oxidizes the reducing substrate and transfers electrons to the T2 and T3 Cu. Laccase is able to oxidize certain phenols with E_0 values higher than its own (0.5 to 0.8 V versus the normal hydrogen electrode [NHE]) (36). However, many inorganic and organic compounds with comparable E_0 values (such as 1,2,3,5-tetramethoxybenzene [18]) are not laccase substrates due to unfavorable kinetics. Under certain conditions, however, these compounds can be indirectly oxidized by laccase through the mediation of small, redox-active laccase substrates. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was the first compound found capable of efficiently mediating the laccase oxidation of high-E_0, nonsubstrate lignin model compounds (such as veratryl alcohol and nonphenolic lignin model dimers) (8). Based on product structure analysis, it has been proposed that laccase-oxidized ABTS can abstract an H atom from the lignin model compounds, leading to indirect laccase catalysis upon the oxidation of the compounds

(25). To date, other types of mediators, particularly phenoxazines and N-OH compounds, also have been recognized for their mediation function in laccase catalysis (1, 6, 17, 29).

Mediated laccase catalysis has been applied to a wide range of applications, such as pulp delignification (9, 10, 12, 22, 32), textile dye bleaching (31), polycyclic aromatic hydrocarbon degradation (16, 23), pesticide or insecticide degradation (1, 29), and organic synthesis (13, 28). For the paper and pulp industry, novel biological or enzymatic bleaching technologies (including mediated laccase catalysis) have attracted increasing attention (9, 10, 12, 14, 22, 27, 32) because of concerns regarding the environmental impact of the chlorine-based oxidants currently being used in delignification or bleaching.

Detailed, comparative information on the interaction between mediator and laccase remains to be reported (22), although various physical and chemical characterizations have been performed on several well-known laccase mediators (2, 4, 7, 11, 21, 35). For N-OH-type mediators, it has not been clear whether their oxidation by laccase involves H abstraction or electron transfer, similar to that found with the oxidation of phenol (38). To better understand the mechanism that governs the oxidation of these compounds by laccase, we studied the interactions of three N-OH compounds (Fig. 1) with seven fungal laccases. The observed dependence of the reaction rate on ΔE_0 suggests that the laccase-catalyzed oxidation of N-OH compounds is governed by a mechanism similar to that reported for phenols, phenoxazines, and phenothiazines.

MATERIALS AND METHODS

Materials. The chemicals used were commercial products of at least reagent grade. *Botrytis cinerea* laccase (BcL) (22), *Coprinus cinereus* laccase-1 (CcL) (30), *Myceliophthora thermophila* laccase (MtL) (5), *Myrothecium verrucaria* bilirubin oxidase (MvBO) (39), *Pycnoporus cinnabarinus* laccase (PcL) (22), *Rhizoctonia solani* laccase 4 (RsL) (34), *Scytalidium thermophilum* laccase (StL) (39), and *Trametes villosa* (*Polyporus pinsitus*) laccase 1 (TvL) (40) were purified as previously reported. Violuric acid (VA) and 1-hydroxybenzotriazole (HBT) were purchased from Aldrich. Promazine and chlorpromazine were purchased from Sigma. *N*-Hydroxyacetanilide (NHA) and phenothiazine-10-propionic acid (PP) were synthesized as described previously (26, 33). 10-Methyl phenothiazine, 3,10-dimethyl phenothiazine, 10-ethyl phenothiazine, 10-(2-hydroxyethyl) phenothiazine, phenothiazine 10-methylpropionate, phenothiazine 10-propionamide,

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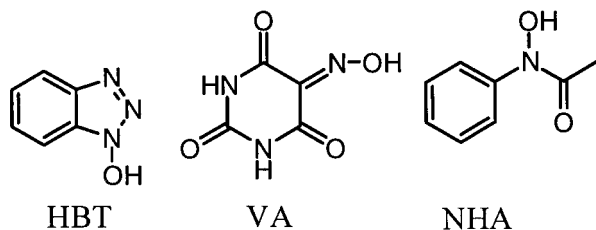
FIG. 1. Structures of HBT, VA, and NHA.

phenothiazine 10-propionitrile, 10-methyl-1-carboxylic acid phenothiazine, 10-methyl-2-carboxylic acid phenothiazine, 10-methyl-3-carboxylic acid phenothiazine, 10-ethyl-4-carboxylic acid phenothiazine, 10-(3-hydroxypropyl) phenothiazine, 10-(2-ethoxy-2′-hydroxyethyl) phenothiazine, 2-acetyl-10-methyl phenothiazine, 10-methyl-3-(2-hydroxyethyl) phenothiazine, 2-chloro-10-methyl phenothiazine, 2-methoxy-10-methyl phenothiazine, 10-methyl phenoxazine, 10-(2-hydroxyethyl) phenoxazine, and phenoxazine 10-propionic acid were synthesized as described elsewhere (20a).

Instruments. UV-visible absorption spectroscopy (including kinetic spectral measurements) was performed either on a spectrophotometer (Shimadzu UV160U or Gilford Instruments 2600) and a quartz cuvette or on a microplate reader (Molecular Devices Thermomax) and 96-well microplates (Costar tissue culture plates). Cyclic and differential pulse voltammetry analyses were performed on a computer-controlled electroanalytical system (Cypress Systems), with a glass carbon working electrode (Cypress Systems model CS-1087), a KCl-saturated calomel reference electrode (Radiometer model K-401), and a platinum wire counterelectrode (0.2-mm diameter, 4-cm length, mounted on the end of the reference electrode). Surface cleansing of the working electrode was carried out by polishing with alumina and washing with water.

Electrochemistry. To determine the E_0 of the N-OH compounds, cyclic voltammetry was performed at 25°C in (aerobic) solutions containing 1 mM N-OH compound, 0.1 M KCl, 33 mM sodium phosphate, 33 mM sodium borate, and 33 mM sodium carbonate (pH 4 to 10). The scanning rate was 0.1 V/s. Measured potentials were compared to the NHE by considering the E_0 of the KCl-saturated calomel reference electrode to be 0.242 V against the NHE.

The E_0 values of the phenoxazines and phenothiazines were measured by differential pulse voltammetry in 50 mM sodium phosphate (pH 7) at room temperature, with a 50-mV pulse height, a 2-mV step height, a 40-ms pulse width, an 0.8- to 0.3-V electrode potential change, and a 12 to 21 μM concentration. E_0 values of 0.66 to 0.95 V were observed (20a).

O$_2$ electrode-based enzymatic assays. Laccase activity was measured in 10 mM morpholineethanesulfonic acid (MES)-NaOH (pH 5.5) at 20°C with a Hansatech O$_2$ cell (38). ABTS was used as a calibrator. The N-OH substrate stock solutions were made in dimethylformamide (1 M for HBT, 0.5 M for VA, and 0.1 M for NHA). At the tested level ($\leq$10%), the dimethylformamide introduced along with the substrate did not alter the kinetic measurements (as tested by ABTS oxidation). The laccase concentrations were 0.8 μM for TvL, 1 to 15 μM for RsL, 2 to 37 μM for MtL, 4 to 55 μM for StL, 3 to 19 μM for CcL, 0.4 to 4 μM for PcL, and 1 to 3 μM for BcL.

The pH-activity profile was measured at 20°C in Britton-Robinson buffer, made by mixing 0.1 M boric acid, 0.1 M acetic acid, 0.1 M phosphoric acid, and 0.5 M NaOH. The substrate concentrations were 40 to 100 mM for HBT, 33 to 58 mM for VA, and 6.7 mM for NHA. The laccase concentrations were 0.8 μM for TvL, 2 to 15 μM for RsL, 2 to 11 μM for MtL, 2 to 16 μM for StL, 3 to 19 μM for CcL, 0.7 μM for PcL, and 3 to 6 μM for BcL.

PP oxidation was performed with 0.01 to 1 mM PP in 10 mM MES (pH 5.5). The concentrations of laccase were 0.1 μM for TvL, 2 μM for RsL, 2 μM for MtL, 5 μM for StL, and 1 μM for MvBO. The stock solution of PP (0.1 M) was made in 0.1 M NaOH.

Spectrophotometric enzymatic assays. Spectrophotometric enzymatic assays were performed with 50 mM sodium acetate (pH 5.5) at 25°C. The oxidation of VA or NHA by laccase was monitored at 310 nm with a molar absorption (ϵ) value of 13.9 or 8.9 mM^{-1} cm^{-1}, respectively. Substrate concentration ranges were 10 to 120 μM for VA and 25 to 200 μM for NHA. Laccase concentration ranges were 0.2 to 1 μM (when oxidizing VA) or 0.2 μM (when oxidizing NHA) for TvL, 1 μM for CcL, and 1 μM (when oxidizing NHA) or 10 μM (when oxidizing VA) for MtL. Third-order polynomial [$c = a + b(t) + c(t^2) + d(t^3)$], where c is the concentration and b is the initial rate constant) nonlinear regression was applied (using the MathCad program) to the kinetic data to extract the apparent rate constant.

For phenoxazines and phenothiazines, their oxidation (into cation radicals) by TvL was monitored at 525 and 514 nm with ϵ values of 16 and 8.9 mM^{-1} cm^{-1}, respectively. The reactions were carried out with 1.5 to 80 μM substrate and 2.5 to 40 nM TvL in 50 mM sodium acetate (pH 5.3) and 1% ethanol at 25°C. K_m values from 6 to 678 μM and k_{cat} (V_{max}/[laccase]) values from 120 to 8,580 min^{-1} were observed (20a).

RESULTS

Electrochemistry and redox potentials of N-OH compounds. Under the conditions used in this study, the cyclic voltammetry of HBT exhibited irreversible oxidation, similar to the observation previously reported (7, 19). Depending on pH, an anodic peak was observed near a peak potential [E_{pa}] of 1.1 to 1.2 V, with a peak current intensity (I_{pa}) corresponding to 2.1 to 2.4 electrons transferred per HBT molecule. Within the scanning rate range, only a small cathodic peak (with a peak potential [E_{pc}] near 0.54 V and a peak current intensity [I_{pc}] $\leq$10% that of I_{pa}) was detected, indicating the residual reduction of oxidized HBT. As shown in Fig. 2, the pH dependence of E_{pa} for HBT was not significant.

Unlike HBT, VA showed a well-shaped cathodic peak, indicating apparent reversibility. The differences between the anodic and cathodic peak potentials ($\Delta E_p = E_{pa} - E_{pc}$) were ~70, 80, and 140 mV for pHs 4 to 8, 9 and 10, respectively. Based on the I_{pa}, ~1.4, 1.5, 1.6, 1.4, 1.3, and 1.1 electrons were transferred per VA molecule during oxidation at pHs 4, 5, 6 to 7, 8, 9, and 10, respectively.

Like VA, NHA had a quasi-reversible cyclic voltammogram. The differences (ΔE_p) were ~130, 110, and 80 mV for pHs 4 to 5, 6 to 7, and 8 to 10, respectively. Based on the I_{pa}, ~1.1, 1.0, 0.8, 0.9, and 1.1 electrons were transferred per NHA molecule during oxidation at pHs 4 to 6, 7, 8, 9, and 10, respectively. As shown in Fig. 2, the formal redox potentials {$E_{1/2} = [(E_{pa} + E_{pc})/2]$} of both VA and NHA were pH dependent. For pH ranges of 6 to 9 and 4 to 7, the $E_{1/2}$-pH plot of VA or NHA had an apparent slope of 50 or 56 mV per pH unit, respectively. For a given pH, the redox potentials of these three N-OH compounds were on the order of HBT > VA > NHA.

Laccase-catalyzed oxidation of N-OH compounds. Serving as a reducing substrate for laccase, the three N-OH compounds exhibited typical Michaelis-Menten kinetics, as monitored by concomitant O$_2$ reduction (Fig. 3). Table 1 shows the K_m and k_{cat} values observed in 10 mM MES (pH 5.5) for the seven laccases and the three N-OH compounds. For MtL, up to 40 mM NHA could not lead to saturation of the initial oxidation rate, thus not allowing an accurate measurement of K_m and k_{cat} values.

The oxidation of VA and NHA by TvL, CcL, and MtL was also monitored spectrophotometrically. Under laccase catalysis, the oxidation of VA led to a decrease of the absorbance
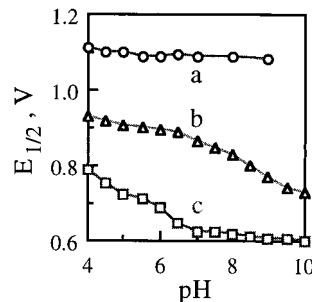

FIG. 2. Formal redox potentials of the N-OH compounds as a function of pH. Traces a, b, and c represent the pH dependence of E_{pa} of HBT, $E_{1/2}$ of VA, and $E_{1/2}$ of NHA, respectively. For HBT, there was no significant acid-base transition for its E_{pa}; thus, no apparent pK_a was extracted from trace a. For VA, two apparent pK_a values of 6.4 and 8.6 were extracted from trace b. For NHA, two apparent pK_a values of 3.7 and 6.3 were extracted from trace c. $E_{1/2}$ values of 0.83 and 0.91 V at pH 4 have been reported for NHA and VA, respectively, by R. Bourbonnais et al. (Oxidative Enzymes for Lignocellulose Processing, Symp. Am. Chem. Soc. 217th Nat. Meet., Anaheim, Calif., 21 to 25 March 1999).
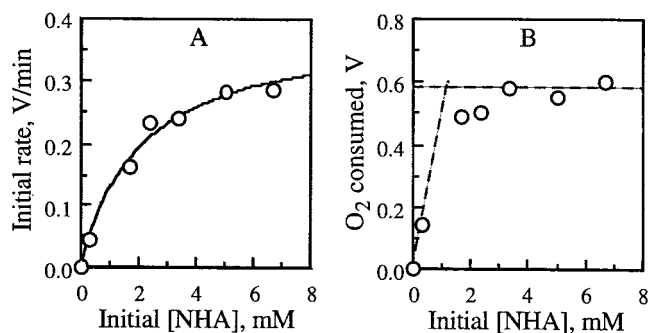
FIG. 3. O_2 consumption in RsL-catalyzed oxidation of NHA at pH 5.5. Plotted against the initial NHA concentration [NHA] are the initial O_2 consumption rate (as the output voltage change rate) (A) and the final O_2 consumed (as the final output voltage change) (B). For graph clarity, two sets of data, obtained with 0.05 and 0.3 mM NHA, are omitted from panel B. Their symbols overlap with those for 0 and 0.33 mM NHA (shown), and their values were included in the graph fitting. In panel A, the solid curve shows the fit to the Michaelis-Menten equation $\{v = -V_{max}[NHA]/(K_m + [NHA])\}$ with a K_m of 2.0 ± 0.5 mM and a V_{max} of 0.39 ± 0.03 V min^{-1} or 0.19 ± 0.01 mM min^{-1} (corresponding to a k_{cat} of 150 ± 10 min^{-1} (mean ± standard deviation). In panel B, the horizontal broken line represents the voltage change (0.58 ± 0.03 V, averaged over the data obtained with 3.3, 5.0, and 6.7 mM NHA) corresponding to maximal O_2 consumption. Its cross point with the other broken line (voltage change, 0.5 × [NHA]; r^2, 0.92), obtained by fitting the data obtained with 0, 0.05, 0.30, and 0.33 mM NHA, yielded a saturating [NHA] of 1.2 mM. By dividing 1.2 mM by 0.28 mM, the dissolved [O_2] in water, we estimated an oxidation stoichiometry number of 4.1.

centered at 310 nm. The oxidation of NHA increased the absorbances at 220 to 230 and 266 to 370 nm (with maxima at 229, 283, and 308 nm) and decreased the absorbance centered at 245 nm (with two apparent isobestic points at 230 and 266 nm). Before the full formation of the apparently stable product (peak wavelengths [λ_{max}] at 229, 283, and 308 nm; trough wavelengths [λ_{min}] at 261 and 290 nm), a transient product seemed to be formed, as demonstrated by a spectrum with λ_{max} at 245 and 323 nm and λ_{min} at 293 nm. A linear dependence of rate on substrate concentration was observed at the selected concentration ranges. For VA, apparent rate constants of ~350, 170, and 5.9 M^{-1} s^{-1} were observed for TvL, CcL, and MtL, respectively. For NHA, apparent rate constants of ~2,100, 330, and 30 M^{-1} s^{-1} were observed for TvL, CcL, and MtL, respectively.

Dependence on E_0 and pH. Figure 4 shows the dependence of k_{cat}, K_m, and k_{cat}/K_m on ΔE_0 $\{E_0$ [laccase (T1 Cu)] − E_0 [substrate]$\}$ at pH 5.5. For HBT, VA, and NHA, the E_{pa}, $E_{1/2}$, and $E_{1/2}$ determined from cyclic voltammetry, respectively, were used to calculate ΔE_0. For HBT, the use of E_{pa} would slightly overestimate ΔE_0, since the irreversible decay of the immediately oxidized HBT would yield E_{pa} greater than or equal to $E_{1/2}$ (~1.11 V at pH 4 [7]), according to the kinetic characteristics of a homogeneous redox catalysis electrochemical reaction (3). An apparently linear, positive correlation was observed between $\log(k_{cat})$ and ΔE_0 as well as between $\log(k_{cat}/K_m)$ and ΔE_0, while an apparently linear, negative correlation was observed between $\log(K_m)$ and ΔE_0. All three N-OH substrates showed bell-shaped pH-rate profiles with an optimal pH ranging from 4 to 7 (Table 1).

Laccase-catalyzed oxidation of phenoxazines and phenothiazines. Serving as a reducing substrate for laccase, the oxidation of PP exhibited typical Michaelis-Menten kinetics, as monitored by concomitant O_2 reduction. The K_m and k_{cat} values (mean ± standard deviation) observed in 10 mM MES (pH 5.5) were 120 ± 50 μM and 2,500 ± 400 min^{-1} for TvL, 32 ± 5 μM and 8 ± 1 min^{-1} for RsL, 120 ± 40 μM and 11 ± 4 min^{-1} for MtL, 47 ± 5 μM and 4.7 ± 0.1 min^{-1} for StL, and

30 ± 5 μM and 21 ± 1 min^{-1} for MvBO, respectively. An apparently linear correlation was observed between $\log(k_{cat}/K_m)$ and ΔE_0 (Fig. 5), similar to the data obtained for the TvL-catalyzed oxidation of more than 20 phenothiazines and phenoxazines (20a).

DISCUSSION

Redox chemistry of N-OH compounds. It is known that the oxidation of HBT generates a highly unstable intermediate, putatively an N-O· radical, that quickly decays into catalytically inactive secondary product(s), including benzotriazole (21). An apparent $E_{1/2}$ of ~1.1 V has been reported for a two-electron electrochemical oxidation of HBT at pH 4 (7). In our study, instability of the putative HBT radical was observed over the pH range of 4 to 10. The better stability observed for the immediate oxidation products (likely N-O· in nature) of VA and NHA could be related to their $E_{1/2}$ values, which were 0.2 to 0.3 V lower than that of HBT. The reduction in $E_{1/2}$ might decrease the oxidative potency or activity of N-O, thus enhancing stability.

As shown in Fig. 2, the $E_{1/2}$ of VA and NHA decreased when pH increased. Since phenyl-N-OH is a heteroatomic homolog of phenol, the oxidation of an aromatic N-OH compound could lead to H$^+$ release (N-OH → N-O· + e$^-$ + H$^+$), as for phenol (C-OH → C-O· + e$^-$ + H$^+$). According to the Nernst equation, $E_0 = E_0 + \{RT/F\} \ln \{[N\text{-}O^·][H^+]/[N\text{-}OH]\} = E_0 + \{RT/F\} \ln \{[N\text{-}O^·]/[N\text{-}OH]\} - RT/\{F \log(e)\}pH$, such H$^+$ release would lead to a lower potential at a higher pH (~0.06-V reduction per pH unit at room temperature), reflecting the fact that the quenching of H$^+$ by OH$^-$ at a higher pH facilitates thermodynamically the N-OH oxidation. Thus, the decrease in $E_{1/2}$ of VA and NHA with an increase in pH indicated the involvement of H$^+$ release and the concomitant production of N-O· during the oxidation.

TABLE 1. Kinetic properties of the laccases on HBT, VA, and NHA at pH 5.5[a]

Laccase	Substrate	Mean ± SD		Optimal pH
		K_m (mM)	k_{cat} (min^{-1})	
TvL	HBT	15 ± 3	84 ± 6	5–6
	VA	5 ± 1	260 ± 20	6
	NHA	0.9 ± 0.3	470 ± 60	5
BcL	HBT	12 ± 4	10 ± 1	6
	VA	11 ± 1	40 ± 2	5
	NHA	1.5 ± 0.5	160 ± 20	5
PcL	HBT	29 ± 7	22 ± 2	6
	VA	9 ± 1	370 ± 20	4–5
	NHA	2.2 ± 0.6	1,500 ± 200	4
RsL	HBT	10 ± 2	0.57 ± 0.02	5
	VA	2.7 ± 0.4	46 ± 2	4
	NHA	2.0 ± 0.5	150 ± 10	7
CcL	HBT	7 ± 2	0.45 ± 0.05	6
	VA	5 ± 1	10 ± 1	5
	NHA	3 ± 2	17 ± 5	6
StL	HBT	31 ± 16	1.3 ± 0.3	5
	VA	0.35 ± 0.08	3.2 ± 0.3	6
	NHA	12 ± 3	6 ± 1	7
MtL	HBT	10 ± 8	0.12 ± 0.05	6
	VA	18 ± 2	27 ± 1	4
	NHA	≥20	≥36	7

[a] The K_m and k_{cat} data for TvL-, BcL-, PcL-, and MtL-catalyzed HBT and VA oxidations are taken from reference 22.
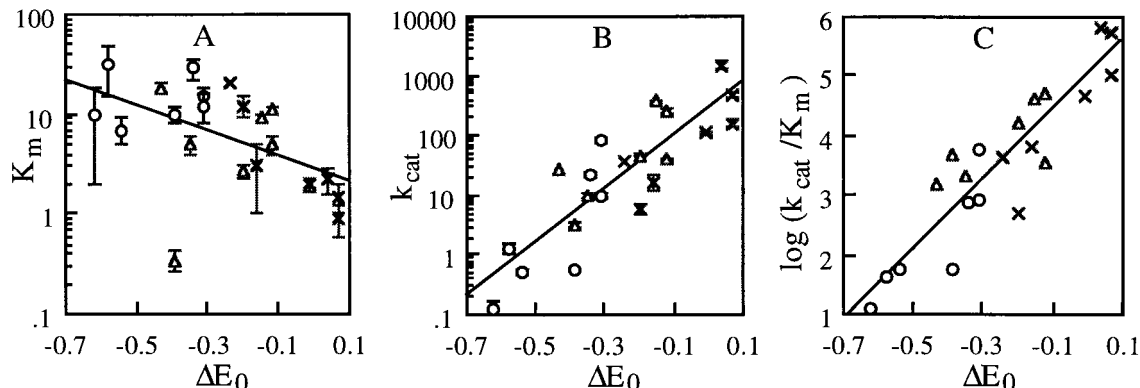
FIG. 4. Dependence of K_m, k_{cat}, and k_{cat}/K_m on ΔE_0. Symbols: ○, HBT; △, VA; ×, NHA. Correlation lines: A, $\log(K_m) = -1.3(\Delta E_0) + 0.46$ (r^2, 0.26); B, $\log(k_{cat}) = 4.5(\Delta E_0) + 2.5$ (r^2, 0.74); C, $\log(k_{cat}/K_m) = 5.8(\Delta E_0) + 5.0$ (r^2, 0.82). Units: A, K_m, mM; B, k_{cat}, min^{-1}; C, k_{cat}/K_m, M^{-1} min^{-1}. ΔE_0 is reported in V. E_0 values for laccase are taken from reference 36. Error bars indicate standard deviations.

Electron transfer from N-OH compounds to laccases. At steady state, the rate-limiting step for phenol oxidation by laccase involves the Marcus "outer-sphere" mechanism. In this mechanism, ΔE_0 (together with reorganization energy and transmission coefficient) determines the electron transfer rate, distinguishing it from other oxidation mechanisms (i.e., H abstraction), where energetic factors related to covalent bond are most important (i.e., homolytic O—H bond dissociation energy). As shown in Fig. 4, a linear correlation existed between $\log(k_{cat})$ or $\log(k_{cat}/K_m)$ (in which k_{cat}/K_m could be approximated as the second-order rate constant of the oxidation) and ΔE_0 (the driving force for electron transfer from the N-OH compound to laccase) for laccase-catalyzed oxidation of the N-OH compounds. When the data for a wide variety of phenols, phenothiazines, phenoxazines, N-OH compounds, and other inorganic and organic redox-active molecules are analyzed together, a common linear correlation between $\log(k_{cat}/K_m)$ and ΔE_0 can be found (Fig. 6). Thus, as for other laccase substrates, the rate-limiting step of laccase-catalyzed N-OH oxidation involves electron transfer from the substrate to the T1 Cu site in laccase. It is ΔE_0 that dominates the oxidation rate. The higher E_0 (laccase) or the lower E_0 (N-OH) is, the faster the oxidation rate tends to be. Other factors (such as the composition, structure, or pK_a of the substrate) seem to be minor, but they could fine-tune the activity for a given ΔE_0 (an effect that might contribute to the scattering shown in Fig. 6).

The apparent negative correlation between $\log(K_m)$ and ΔE_0 suggests that substrate affinity tends to increase when ΔE_0 increases (realized by either E_0 [laccase] increase or E_0 [substrate] decrease) (Fig. 4A), a phenomenon also observed for phenolic substrates (36, 39). Prior to electron transfer, the filled (valence) molecular orbitals of N-O in the N-OH compounds (or the phenoxy-O in phenols) overlap with the half-occupied molecular orbitals (HOMO) of T1 Cu when the substrate is bound to laccase. A larger ΔE_0 could create a transitional energy state more favorable for the molecular orbital interaction, resulting in better substrate binding and consequently faster electron transfer.

Dependence of activity on pH. When oxidizing a phenolic substrate, laccase generally possesses a bell-shaped pH-activity profile. Two opposing factors, ΔE_0 (involving substrate and laccase T1 Cu) and OH$^-$ inhibition (involving T2 Cu in laccase), are suggested to play important roles in determining the pH-activity profile (38). Like phenols, HBT, VA, and NHA have redox potentials that decrease when pH increases (Fig. 2). Since the E_0 of laccase is often quite insensitive to pH change (38), the decrease in the E_0 of N-OH as pH increases would

increase ΔE_0, which in turn would enhance the oxidation rate through the correlation shown in Fig. 4. However, the OH$^-$ inhibition of laccase would become overwhelming at an alkaline pH. The combination of these two effects might contribute to the bell-shaped pH-activity profiles of N-OH compounds.

The speculation of an H$^+$ release step during laccase-catalyzed N-OH oxidation, together with the observation that the reduction of one O$_2$ was accompanied by the oxidation of about four N-OH groups, indicates that the reaction N-OH → N-O$^\bullet$ + e$^-$ + H$^+$ might be involved in the rate-limiting step, similar to the reaction C-OH → C-O$^\bullet$ + e$^-$ + H$^+$, which is involved in laccase-catalyzed phenol oxidation (36).

Overall remarks. The results of this study suggest that the initial oxidation of a phenol (aryl C-OH) compound by laccase is quite similar to the oxidation of an aryl N-OH (phenol homolog) compound in terms of the dependence of the initial rate on E_0 and pH. In general, phenol is first oxidized to a highly unstable phenoxy radical (aryl C-O$^\bullet$), which then surrenders an additional e$^-$ (at a rate faster than that of the first e$^-$ transfer) to yield a stable, but much less active, quinone. Oxidation of N-OH compounds also involves a single e$^-$ transfer at the initial oxidation step. N-O$^\bullet$ could be less active but more stable than a phenoxy radical. In laccase-catalyzed pulp delignification, a desirable redox mediator should be a good laccase substrate, have a half-life at its oxidized form long
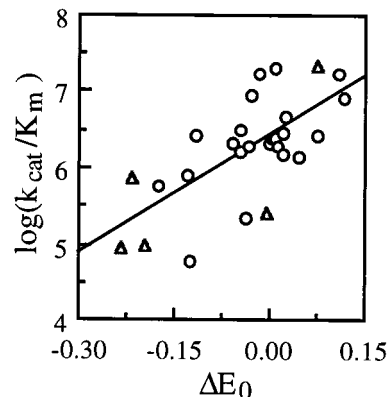

FIG. 5. Correlations between $\log(k_{cat}/K_m)$ and ΔE_0 for phenothiazines and phenoxazines. Symbols: ○, oxidation by TvL of 3 phenoxazines and 20 phenothiazines (see Materials and Methods for their formulas) (20a); △, oxidation of PP (E_0, 0.71 V) by TvL, RsL, MtL, StL, and MvBO at pH 5.3 to 5.5. Correlation: $\log(k_{cat}/K_m) = 5.1(\Delta E_0) + 6.4$ (r^2, 0.47). Units: k_{cat}/K_m, M^{-1} min^{-1}; ΔE_0, V.
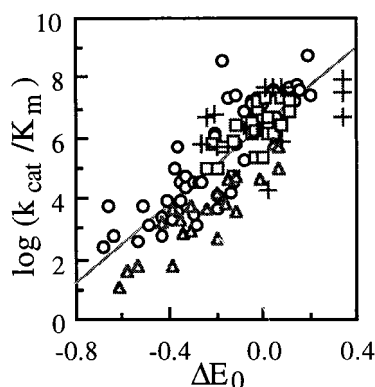
FIG. 6. Correlations between $\log(k_{cat}/K_m)$ and ΔE_0 for laccase catalysis. Symbols: ○, oxidation of 24 phenols by 10 fungal on plant laccases (36); □, oxidation of 3 phenoxazines and 22 phenothiazines by TvL, as well as oxidation of PP by 5 fungal laccases (data from Fig. 5); △, oxidation of three N-OH compounds by 7 fungal laccases (data from Table 1 and Fig. 4C); +, oxidation of ABTS, $K_4Fe(CN)_6$ and morpholinoaniline by up to 10 fungal or plant laccases (20, 36). Other conditions: pH, 5.3 to 5.5; temperature, 20 to 25°C. Correlation: $\log(k_{cat}/K_m) = 6.4(\Delta E_0) + 6.4$ (r^2, 0.65). Units: k_{cat}/K_m, M^{-1} min^{-1}; ΔE_0, V.

enough to permit diffusion to heterogeneous lignin, and possess high oxidation potency to effectively oxidize lignin. In comparison with those of a phenoxy radical, the activity and stability of N-O$^{\bullet}$ seem to be better balanced, which could contribute to the better performance of the latter as a mediator for laccase-based delignification.

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