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, dyestain 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 on 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, have also 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 (36). 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 $E_0$ 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 (MLL) (5), Myrothecium verrucaria bilirubin oxidase (MVBO) (39), Pycnoporus cinnabarinus laccase (PcL) (22), Rhizoctonia solani laccase 4 (RlL) (34), Serratia marcescens laccase (SML) (39), and Trametes versicolor (Polyporus versicolor) laccase 1 (TvL) (40) were purified as previously reported. Violuric acid (VA) and 1-hydroxybenzotriazole (HBT) 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,
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'-hydroxethyl) 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 UV1600) or a 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 counter electrode (0.2-mm diameter, 4-cm length, mounted on the end of the reference electrode). Surface cleaning of the working electrode was carried out by polishing with alumina and washing with water.

**Electrochemistry.** To determine the Epa of the N-OH compounds, cyclic voltammery 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 E0 of the KCl-saturated calomel reference electrode (Radiometer model K-401).

**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 [Epa] of 1.1 to 1.2 V, with a peak current intensity [Ipa] 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 [Epc] near 0.54 V and a peak current intensity [Ipc] ≤10% that of Ipa) was detected, indicating the residual reduction of oxidized HBT. As shown in Fig. 2, the pH dependence of Epa 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 (ΔEpa = Epa - Epc) were ~70, 80, and 140 mV for pHs 4 to 8, 9 and 10, respectively. Based on the Ipa, ~1.1, 1.0, 0.8, 0.9, 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 (ΔEpa) were ~130, 110, and 80 mV for pHs 4 to 5, 6 to 7, and 8 to 10, respectively. Based on the Ipa, ~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 (E1/2 = [(Epa + Epc)/2]) of both VA and NHA were pH dependent. For pH ranges of 6 to 9 and 4 to 7, the E1/2-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 O2 reduction (Fig. 3). Table 1 shows the Km and kcat values obtained 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 Km and kcat 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...
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 $[\lambda_{\text{max}}]$ at 229, 283, and 308 nm; trough wavelengths $[\lambda_{\text{min}}]$ at 261 and 290 nm), a transient product seemed to be formed, as demonstrated by a spectrum with $[\lambda_{\text{max}}]$ at 245 and 323 nm and $[\lambda_{\text{min}}]$ at 293 nm. A linear dependence of rate on substrate concentration was observed at the selected concentration ranges. For VA, apparent rate constants of $5.9 \times 10^{-2}$ M and 5.9 M were obtained for MtL, respectively. For NHA, apparent rate constants of $1.0 \times 10^{-2}$ and $1.0 \times 10^{-1}$ (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 V) indicates the involvement of H$_1$ (pH 5.5) in the oxidation of NHA increased the oxidation of NHA to form catalytical-

**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 $E_{\text{1/2}}$ 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 $E_{\text{1/2}}$ values, which were 0.2 to 0.3 V lower than that of HBT. The reduction in $E_{\text{1/2}}$ might decrease the oxidative potency or activity of N-O’, thus enhancing stability.

As shown in Fig. 2, the $E_{\text{1/2}}$ 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, $E_0 = E_0 + (RT/F) \ln \left(\frac{[\text{N-O}]}{[\text{N-OH}]}ight)$. According to the Nernst equation, $E_0 = E_0 + (RT/F) \ln \left(\frac{[\text{N-O}]}{[\text{N-OH}]}ight)$. For graph clarity, two sets of data, obtained with HBT, VA, and NHA, the $E_{\text{pa},E_0}$, determined from cyclic voltammetry, respectively, were used to calculate $\Delta E_{\text{p}}$. For HBT, the use of $E_{\text{pa}}$ would slightly overestimate $\Delta E_{\text{p}}$

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

<table>
<thead>
<tr>
<th>Laccase</th>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>Optimal pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVL</td>
<td>HBT</td>
<td>15 ± 3</td>
<td>84 ± 6</td>
<td>5-6</td>
</tr>
<tr>
<td>VA</td>
<td>5 ± 1</td>
<td>260 ± 20</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHA</td>
<td>0.9 ± 0.3</td>
<td>470 ± 60</td>
<td>5</td>
</tr>
<tr>
<td>BC</td>
<td>HBT</td>
<td>12 ± 4</td>
<td>10 ± 1</td>
<td>6</td>
</tr>
<tr>
<td>VA</td>
<td>11 ± 1</td>
<td>40 ± 5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHA</td>
<td>1.5 ± 0.5</td>
<td>160 ± 20</td>
<td>5</td>
</tr>
<tr>
<td>PC</td>
<td>HBT</td>
<td>29 ± 7</td>
<td>22 ± 2</td>
<td>6</td>
</tr>
<tr>
<td>VA</td>
<td>9 ± 1</td>
<td>370 ± 20</td>
<td>4-5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHA</td>
<td>2.2 ± 0.6</td>
<td>1,500 ± 200</td>
<td>4</td>
</tr>
<tr>
<td>RS</td>
<td>HBT</td>
<td>10 ± 2</td>
<td>0.57 ± 0.02</td>
<td>5</td>
</tr>
<tr>
<td>VA</td>
<td>2.7 ± 0.4</td>
<td>46 ± 2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHA</td>
<td>2.0 ± 0.5</td>
<td>150 ± 10</td>
<td>7</td>
</tr>
<tr>
<td>CC</td>
<td>HBT</td>
<td>7 ± 2</td>
<td>0.45 ± 0.05</td>
<td>6</td>
</tr>
<tr>
<td>VA</td>
<td>5 ± 1</td>
<td>10 ± 1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHA</td>
<td>3 ± 2</td>
<td>17 ± 5</td>
<td>6</td>
</tr>
<tr>
<td>SI</td>
<td>HBT</td>
<td>31 ± 16</td>
<td>1.3 ± 0.3</td>
<td>5</td>
</tr>
<tr>
<td>VA</td>
<td>0.35 ± 0.08</td>
<td>3.2 ± 0.3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHA</td>
<td>12 ± 3</td>
<td>6 ± 1</td>
<td>7</td>
</tr>
<tr>
<td>MT</td>
<td>HBT</td>
<td>10 ± 8</td>
<td>0.12 ± 0.05</td>
<td>6</td>
</tr>
<tr>
<td>VA</td>
<td>18 ± 2</td>
<td>27 ± 1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHA</td>
<td>≥20</td>
<td>≥36</td>
<td>7</td>
</tr>
</tbody>
</table>

* The $K_m$ and $k_{\text{cat}}$ data for TVL-, BC-, PC-, and ML-catalyzed HBT and VA oxidations are taken from reference 22.
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, $\Delta E_0$ (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 $\Delta E_0$ (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 $\Delta E_0$ can be found (Fig. 6). Thus, for as 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 $\Delta E_0$ that dominates the oxidation rate. The higher $E_0$ (laccase) or the lower $E_0$ (N-OH), the faster the oxidation rate tends to be. Other factors (such as the composition, structure, or pH of the substrate) seem to be minor, but they could fine-tune the activity for a given $\Delta E_0$ (an effect that might contribute to the scattering shown in Fig. 6).

The apparent negative correlation between log($K_m$) and $\Delta E_0$ suggests that substrate affinity tends to increase when $\Delta E_0$ 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 $\Delta E_0$ 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, $\Delta E_0$ (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 $\Delta E_0$, 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.

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$^-$), 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$^-$ could be less active but more stable than a phenoxy radical. In laccase-catalyzed delignification, a desirable redox mediator should be a good laccase substrate, have a half-life at its oxidized form long enough.
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 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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