Mechanistic Features of Lignin Peroxidase Catalyzed Oxidation of Substituted Phenols and 1,2-Dimethoxyarenes

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Running Title:
Mechanistic Features of Lignin Peroxidase-Catalyzed Oxidation
The steady state kinetic parameters $K_m$ and $k_{cat}$ for the oxidation of phenolic substrates by lignin peroxidase correlated with the pre-steady state kinetic parameters $K_d$ and $k$ for the reaction of the enzyme intermediate compound II with the substrates, indicating that the latter is the rate limiting step in the catalytic cycle. $\ln K_m$ and $\ln K_d$ values for phenolic substrates correlated with redox properties, unlike $\ln k_{cat}$ and $\ln k$. This finding suggests that in contrast to horseradish peroxidase, electron transfer is not the rate limiting step during oxidation by lignin peroxidase compound II. A mechanism is proposed for lignin peroxidase compound II reactions, consisting of an equilibrium electron transfer step followed by a subsequent rate limiting step. Analysis of the correlation coefficients for linear relationships between $\ln K_d$ and $\ln K_m$ and different calculated redox parameters supports a mechanism in which the acidic forms of phenols are oxidized by lignin peroxidase and that electron transfer is coupled with proton transfer. 1,2-Dimethoxyarenes did not comply with the trend for phenolic substrates, which may be a result of more than one substrate-binding site on lignin peroxidase and/or alternative binding modes. This behavior was supported by analogue studies with the 1,2-dimethoxyarenes, veratric acid and veratryl aldehyde, both of which are not oxidized by lignin peroxidase. Inclusion of either had little effect on the rate of oxidation of phenolic substrates, yet resulted in a decrease in the oxidation rate of 1,2-dimethoxyarene substrates, which was considerable for veratryl alcohol and less pronounced for 3,4-dimethoxyphenethylalcohol and 3,4-dimethoxycinnamic acid, in particular in the presence of veratric acid.
INTRODUCTION

Lignin peroxidases (LIP)\(^1\) play a central role in the biodegradation of the plant cell wall constituent lignin by white-rot fungi, the most extensively studied of which is \textit{Phanerochaete chrysosporium} (1,2). LIP possesses a higher redox potential and lower pH value than any other peroxidase and in accordance, exhibits broader substrate specificity (3). Like other peroxidases, it is capable of oxidizing phenolic compounds (4-6) as well as ring- and N-substituted anilines (7-9). However, it is unique in its ability to oxidize certain non-phenolic aromatic substrates possessing high redox potential values, such as substituted aromatic ethers (e.g. veratryl alcohol) (10-13) and thioethers (14).

The catalytic cycle of LIP is similar to that of other peroxidases (15,16). Reaction of native ferric enzyme (Fe-LIP: Fe\(^{3+}\) P (porphyrin)) with H\(_2\)O\(_2\) yields LIP-compound I (LIPI), a complex of high valent oxo-iron and porphyrin cation radical (Fe\(^{4+}=\text{O}^+\)).

One-electron-oxidation of a reducing substrate by LIPI yields a radical cation and the one-electron-oxidized enzyme intermediate, LIP-compound II (LIPII: Fe\(^{4+}=\text{O}^+\)). A single one-electron oxidation of a second substrate molecule by LIPII, results in the formation of another radical cation, and the release of a molecule of water, with the enzyme being returned to Fe-LIP, completing the catalytic cycle. The oxidation of phenols by LIPI follows second order kinetics and is linearly proportional to substrate concentration (4,5). Kinetic studies indicate a hyperbolic concentration dependence for the conversion of LIPII to Fe-LIP by guaiacol (4,5), β-O-4 phenolic lignin

\(^{1}\)Abbreviations used: LIP, lignin peroxidase; Fe-LIP, native ferric enzyme; LIPI, LIP-compound I; LIPII, LIP-compound II; HRP, horseradish peroxidase; RZ, Reinheitszahl; IP\(_{\text{ad}}\)(UHF), UHF adiabatic ionization potential; IP\(_{\text{ad}}\)(ROHF), ROHF adiabatic ionization potential; IP\(_{\chi}\)(UHF), UHF vertical ionization potential; IP\(_{\chi}\)(ROHF), ROHF vertical ionization potential; OP, oxidation potential; OP\(_{\text{acidic}}\), oxidation potential of undissociated phenol; OP\(_{\text{basic}}\), oxidation potential of phenolate ion; OP\(_{\text{pH3.5}}\), oxidation potential calculated at pH 3.5; BEP, bond energy parameter.
oligomers (5), ferulic acid (6) and nonphenolic veratryl alcohol (17), which suggests at least a two step mechanism for this stage. In this mechanism, rapid equilibrium is ascribed to binding of a substrate molecule to LIPII, and the rate limiting step is commonly considered as an electron transfer from the bound substrate molecule to LIPII (4,5).

The present study focused on investigating the kinetics and thermodynamics for the oxidation of substituted phenols by LIP. The working hypothesis was that if electron transfer is the rate limiting step of the oxidation of substituted phenols by LIPII, then 

\[ -RT\ln k \text{ or } -RT\ln k_{cat} \]

will depend on the thermodynamic driving force of the reaction according to the Marcus equation (18), as was found for the oxidation of different organic compounds by horseradish peroxidase (HRP) compound I (19-22) and compound II (21,22). Furthermore, the research also addressed the kinetics and thermodynamics for the oxidation of 1,2-dimethoxyarenes by LIP. The findings provide new insights into the mechanism of oxidation of phenolic substrates by LIP and furthermore, provide support for the presence of two or more binding sites (23-26) and/or alternative binding modes for phenolic substrates and 1,2-dimethoxyarenes.

**EXPERIMENTAL PROCEDURES**

**Enzyme Purification**

LIP isoenzyme H1 was produced from high-nitrogen cultures of *P. chrysosporium* Burds BKM-F-1767 (27) and purified as described previously (6). The purified enzyme had an RZ (\(A_{409}/A_{280}\)) value > 4.0. LIP concentration was determined at 409 nm using an extinction coefficient of 169 mM\(^{-1}\)cm\(^{-1}\) (15) and activity (U/I) was assayed according to Tien and Kirk (28). The catalytic activity of the stock enzyme
solution was calculated to be 2.14 U per nmol heme protein. The enzyme was extensively dialyzed against double-distilled water before use.

**Steady-State Kinetics Studies**

Steady-state kinetic studies were conducted with 0.2 µM LIP, substrate (the range varied for different substrates but was generally between 0-2000 µM and at least 7 different concentrations were examined) and 300 µM H₂O₂ in 50 mM sodium tartrate buffer, pH 3.5. Reactions were stopped after 10 s by adding an equal volume of acetonitrile to inactivate the enzyme and then analyzed by HPLC as described below. The amount of substrate consumed in 10 s (calculated from blanks without H₂O₂ and authentic compounds) for different initial substrate concentrations was determined. Three replicates of both blanks and reactions were analyzed. $K_m$ and $k_{cat}$ were calculated by non-linear least-squares fits to plots of the initial rate of oxidation as a function of substrate concentration.

**Pre-Steady-State Kinetics**

Pre-steady-state kinetics were studied using an SFA-20 stopped flow apparatus (HI-TECH Scientific, Sussex, UK) attached to a Shimadzu UV-260 spectrophotometer as previously reported (6). Due to differences in rates between LIPI and LIPII, rate constants for LIPII could be determined from experiments where it was formed from the reaction of LIPI with the substrate (17). LIPI was prepared by adding 1 µM H₂O₂ to 1µM LIP (4) in one of the syringes of the stopped flow apparatus. One minute after preparation, the pneumatic drive was operated and LIPI was mixed in the spectrophotometer flow cell with an equal volume of the contents of another syringe containing substrate and buffer. After mixing, reactions contained 0.5 µM LIPI, 50 mM sodium tartrate buffer, pH 3.5 and varying amounts of substrate (0-1000 µM). The conversion of LIPII to Fe-LIP as a function of substrate concentration was
monitored at 426 nm, the isobestic point between Fe-LIP and LIPI (17). Five replicates of each of at least 8 different substrate concentrations were studied.

**Analogue studies**

Analogue studies were conducted with 0.2 µM LIP, 100 µM substrate and 300 µM H₂O₂, in the presence and absence of either 1500 µM veratryl aldehyde or veratric acid. Reactions were stopped after 10 s by adding an equal volume of acetonitrile to inactivate the enzyme and then analyzed by HPLC as described below. The amount of substrate consumed in 10 s was calculated from blanks without H₂O₂ and authentic compounds. Three replicates of both blanks and reactions were analyzed. The oxidation of each substrate in the presence of veratryl aldehyde or veratric acid is presented as a percent of the oxidation in their absence.

**HPLC analysis**

HPLC analysis was conducted on a Hewlett Packard HPLC (HP1100 series) equipped with a diode array detector. A Lichrospher 100 RP-18 column (25 cm × 5 mm i.d., 5 µm; Merck, Darmstadt, Germany) was employed for analysis. 3,4-Dihydroxybenzoic acid was analyzed using a mobile phase consisting of component A (1% v/v, acetic acid) and component B (1% v/v acetic acid in 90% v/v methanol) (29). All other substrates were analyzed using a mobile phase that consisted of component A (10% v/v, aqueous acetonitrile, 1 mM trifluoroacetic acid) and component B (40% v/v, aqueous methanol, 40%, v/v aqueous acetonitrile, 1 mM trifluoroacetic acid) (30). The relative amounts of each component of the mobile phase varied according to the substrate analyzed. The flow rate in all cases was maintained at 1 ml/min. Quantification was performed by integration of peak-areas at 280 nm for all substrates, with reference to calibrations, which were made using known amounts of authentic compounds.
Quantum chemical calculations

Optimal geometries and heat of formation of the substrates were determined using the restricted Hartree-Fock (RHF) AM1 Hamiltonian (31). For radical cations, unrestricted Hartree-Fock (UHF) (high spin contamination was found) and restricted open shell Hartree-Fock (ROHF) AM1 Hamiltonians were employed (31). The UHF and ROHF adiabatic ionization potentials (IP_{ad}^{UHF} and IP_{ad}^{ROHF}, respectively) were calculated as the difference between the heats of formation of the substrates and their radical cations after the optimal geometries were determined. Vertical ionization potentials (IP_{v}^{UHF} or IP_{v}^{ROHF}) were calculated using the heats of formation of radical cations assuming the geometries of the substrates. All the calculations were performed with the Gaussian 94 (32) and Spartan 5.1 programs.

The difference in the heat of formation of radical cations and parent compounds calculated for aqueous solutions corresponds to the oxidation potential (OP).

Chemicals

H_{2}O_{2} (30% v/v solution) was obtained from Sigma (Rehovot, Israel). The concentration of stock solutions of H_{2}O_{2} was determined at 240 nm using an extinction coefficient of 39.4 M^{-1}cm^{-1} (33).

The substrates chosen for this study are given in Table 1. Phenolic substrates are given in the upper part of the table and are divided into subfamilies on account of their substitution patterns. They are followed by 1,2-dimethoxyarenes in the lower part of the table. All substrates were obtained from Aldrich (Rehovot, Israel), except for ferulic acid, caffeic acid, catechol, guaiacol, vanillic acid and syringic acid which were obtained from Sigma and veratrole, which was obtained from Fluka (Rehovot, Israel).
Stock solutions of substrates were made in 95% (v/v) ethanol and checked using the extinction coefficients given in Table 1. The extinction coefficients were determined by preparing a series of solutions of increasing concentration for each substrate and measuring the absorbance at the pre-determined wavelength of maximum absorbance ($\lambda_{\text{max}}$). All stock solutions were 100 mM, except 3,4-dimethoxycinnamic acid, which was 10 mM.

**(TABLE 1)**

**RESULTS**

**Oxidation of phenolic substrates**

*Kinetic measurements*

In the event that the last stage of the well known ping-pong mechanism (Equation 1) is rate limiting and controls the overall rate of the catalytic cycle, then steady state kinetic parameters for LIP catalyzed oxidation will be representative of the conversion of LIPII to Fe-LIP in the presence of substrate molecules.

\[
\begin{align*}
\text{H}_2\text{O} & \quad \text{LIPI} \\
\text{LIP-H}_2\text{O}_2 & \quad k_2 \\
\text{LIPI-S} & \quad k_3[S] \\
\text{LIP-II} & \quad k_4 \\
\text{P,H}_2\text{O} & \quad k_5[S] \\
\text{LIP-II-S} & \quad k_6 \\
\end{align*}
\]

According to Segel (34), the observed rate (v) for decay of substrate concentration is described by equation 2.
\[
\begin{align*}
2A[H_2O_2][S] \\
AB[H_2O_2] + AC[H_2O_2] + AD + AF[H_2O_2][S] \\
\end{align*}
\]

\[
\begin{align*}
A &= k_1k_2k_3k_4k_5k_6 \\
B &= (k_{-5} + k_5)/k_3k_6 \\
C &= (k_{-3} + k_4)/k_3k_4 \\
D &= (k_{-1} + k_2)/k_1k_2 \\
E &= (k_2k_4 + k_2k_6 + k_4k_6)/k_2k_4k_6
\end{align*}
\]

The overall reaction rate will be determined by the reactivity of LIPII if \(k_2 < k_{-1}, k_4 < k_{-3}, k_6 < k_{-5}\) and \(k_6 < k_2, k_4\). In accordance, equation 2 can be simplified to equation 3.

\[
v = \frac{2k_6[H_2O_2][S]}{k_{-5}[H_2O_2]/k_5 + k_{-3}k_6[H_2O_2]/k_3k_4 + (k_1k_6/k_1k_2 + [H_2O_2])[S]} \tag{3}
\]

If \([H_2O_2] >> k_{-1}/k_1\), the Michaelis-Menten type equation 4 is obtained.

\[
v = \frac{2k_6[S]}{k_{-5}/k_5 + k_{-3}k_6/k_3k_4 + [S]} = \frac{k_{cat}[S]}{Km + [S]} \tag{4}
\]

Pre-steady kinetics of the conversion of LIPII to Fe-LIP, the last stage in equation 1, involves monitoring the change in concentration of LIPII upon reaction with substrate molecules and is depicted in equation 5. If rapid equilibrium is assumed for the first step, the observed rate of oxidation \(v'\) can be given by equation 6, as previously described by Koduri and Tien (4).

\[
\begin{align*}
LIPII + S &\overset{k_5}{\underset{k_{-5}}{\rightleftharpoons}} LIPII-S \overset{k_6}{\rightarrow} LIP + P + H_2O \tag{5}
\end{align*}
\]

\[
v' = \frac{k_6[S]}{k_{-5}/k_5 + k_6/k_5 + S} = \frac{k[S]}{Kd + [S]} \tag{6}
\]

If steady-state kinetic parameters are representative of the conversion of LIPII to Fe-LIP (i.e., the conversion of LIPII to Fe-LIP is the rate limiting step in the catalytic...
cycle) then according to eq. 4 and 6, it can be expected that $k_{s5}/k_{5} >> k_{s6}/k_{6}k_{4}, k_{6}/k_{5}$ and thus $k_{\text{cat}} = 2k$ and $K_{m} = K_{d}$.

Steady state kinetic parameters were determined for all of the substituted phenols. Pre-steady state kinetic parameters were determined for six of them, chosen to cover the entire range of redox potentials of the substrates studied under steady state kinetic conditions. Under steady state conditions, the plots of the initial reaction rate ($v$) vs. substrate concentration were hyperbolic for all of the substituted phenols studied. Non-linear least squares fits were then used to determine $K_{m}$ and $k_{\text{cat}}$, which are presented in Table 2. Similarly, the plots of the initial reaction rate ($v'$) vs. substrate concentration for the pre-steady kinetics of the conversion of LIPII to Fe-LIP were also hyperbolic, in full accordance with equation 5 and as noticed previously for the conversion of LIPII to Fe-LIP by other substrates (4-6). The kinetic parameters $k$ and $K_{d}$, calculated from equation 6 using a non-linear least-squares fit to the data are presented in Table 2. The average value of $k$ for the six substrates, 17 s$^{-1}$, was similar to the average value of $k_{\text{cat}}/2$ for the same six substrates, 16 s$^{-1}$. The average value of $k_{\text{cat}}/2$ for all of the studied phenols was calculated as 15 s$^{-1}$. Furthermore, a linear relationship was observed between $K_{d}$ and $K_{m}$ for the six substrates studied by both approaches (Fig. 1). Since in accordance with equations 4 and 6, $k_{\text{cat}} \approx 2k$ and $K_{m} \propto K_{d}$, kinetic parameters obtained under steady-state conditions can be considered to reflect the reactivity of LIPII, which is thus the rate limiting step in the catalytic cycle.

*(TABLE 2, FIG. 1)*
Estimation of the thermodynamic driving force of electron transfer

To characterize the thermodynamic driving force for the oxidation of substituted phenols by LIPII, the redox potential of both the enzyme intermediate and the substrates must be known. The redox potential of LIPII is estimated to be about 1.15-1.25 V vs. NHE (2).

The redox characteristics of phenols in aqueous solution are dependent on pH. Oxidation of phenolate leads to formation of the corresponding phenoxy radical (equation 7).

\[
\text{PhO}^- \rightarrow \text{PhO}^* + e \quad (7)
\]

Redox potentials \(E^\circ(\text{PhO}^-/\text{PhO}^*)\) of a large number of substituted phenolates have been determined experimentally (35-42). The one electron oxidation of undissociated phenols is usually described as a dissociative electron transfer (middle reaction in equation 8) and is characterized by higher redox potentials than for the corresponding phenolates. The redox potential of an undissociated phenol may be calculated using equation 9 based on the thermodynamic cycle represented in the upper part of equation 8. When both the undissociated phenol and the phenolate ion are present in aqueous solution, then equation 10 may be applied to estimate the effective redox potential at a given pH (43).

\[
E^\circ(\text{PhO}^* + H^+/\text{PhOH}) = E^\circ(\text{PhO}^-/\text{PhO}^*) + 0.059 \text{ pKa} \quad (9)
\]

\[
E^\circ(\text{PhO}^- + H^+/\text{PhOH}) = E^\circ(\text{PhO}^-/\text{PhO}^*) + 0.059 (\text{pKa} - \text{pH}) \quad (10)
\]
Another way to estimate the redox potential of an undissociated phenol is based on the thermodynamic cycle shown in the bottom part of equation 8. In this case the redox potential is equal to the sum of Bond Dissociation Energy (BDE) of the phenol in eV and the redox potential of the $\text{H}^+ \rightarrow \text{H}^+$ transformation.

TABLE 3

The experimentally measured redox potentials are known for only some of the phenols studied (Table 3). Therefore a calculated parameter that adequately represents the redox properties of the substrates was sought. Previous studies have relied on three approaches to calculate parameters that describe the reactivity of phenols with HRP (44-46). These involve predicting the rate of oxidation either on account of the energy of the highest occupied molecular orbital (HOMO) of the substrates (44-46), on account of the relative difference in the heat of formation of the substrate and its radical cation obtained by one-electron abstraction (45), or on account of the energy required for hydrogen atom abstraction, since initial hydrogen atom abstraction is thought to represent an alternative to the initial electron abstraction for the reaction of phenolic substrates with HRP (45). Nevertheless, these quantum chemical calculations were performed for systems in vacuum and do not take into account the possible differences in solvation energies of parent compounds, radical cations, free radicals or other intermediates. This is especially important for phenolic substrates and their radical cations, since they are involved in acid-base transformations in aqueous solutions. As a result, in the present study several additional parameters were tested as potential representatives of the redox properties of the substrates studied. The first is based on the suggestion that LIPII is reduced by phenolate anion and involved calculation of the difference between the heat of formation of phenolate and the corresponding phenoxy radical in aqueous solution. In this way, the OP of the basic
form \((\text{OP}_{\text{basic}})\) in aqueous solution was calculated. The second parameter is based on the suggestion that LIPII oxidizes a phenolic substrate in its acidic form and a radical cation intermediate is formed as the primary product. The difference between the heat of formation of phenolic substrates and their radical cations in aqueous solution was calculated and is referred to as \(\text{OP}_{\text{acidic}}\). The third parameter involves correcting for the influence of pH, and for this purpose equation 11 was employed, which is similar to equation 10, that describes the dependence of the \(E^0\) on pH.

\[
\text{OP (pH)} = \text{OP (of the basic form)} + 0.059 (pK_a - pH) \tag{11}
\]

\(pK_a\) values for each of the phenols were retrieved from the literature (47) or calculated using the \textit{SPARC} on-line calculator (48). The fourth redox parameter is based on the possibility that the acidic form of the phenolic compound is oxidized by LIPII according to the dissociative electron transfer mechanism (middle reaction in equation 8). In this case the calculated redox parameter is associated with the difference between a phenolic compound and its corresponding phenoxy radical in aqueous solution. It is referred to here as the bond energy parameter (BEP). This computer-calculated redox parameter represents the free energies of reactions on a relative scale and is not an exact representation. Therefore it only reflects the relative differences between the energies required for conversion of a series of compounds.

Values of \(pK_a\), \(\text{IP}_v\) (UHF and ROHF), \(\text{IP}_{\text{ad}}\) (UHF and ROHF), \(\text{OP}_{\text{basic}}\) (UHF and ROHF), \(\text{OP}_{\text{pH3.5}}\) (UHF and ROHF), \(\text{OP}_{\text{acidic}}\) (UHF and ROHF), and BEP (UHF and ROHF) are presented in Table 3. For all of the calculated redox parameters, a higher value indicates that the oxidation is less thermodynamically favorable.

Correlation between kinetic parameters and redox parameters.

Plots of \(\ln K_m\), \(\ln k_{\text{cat}}\), \(\ln K_d\) and \(\ln k\) vs. \(\text{IP}_v\), \(\text{IP}_{\text{ad}}\), \(\text{OP}_{\text{basic}}\), \(\text{OP}_{\text{pH3.5}}\), \(\text{OP}_{\text{acidic}}\) and BEP yielded the correlation coefficients given in Table 4. \(\ln K_m\) and \(\ln K_d\) values exhibited
a linear relationship with all calculated redox parameters (Table 4), the best of which were obtained with BEP (Figs. 2a and 2b). It is clear from these graphs that the values of lnK$_m$ and lnK$_d$ are larger for thermodynamically less favorable reactions. Analysis of the data for phenolic substrates presented in Table 4 leads to the following conclusions. The correlation coefficients are generally higher for UHF than for ROHF calculated redox parameters. Comparison of the correlation coefficients within the same group of redox parameters (UHF or ROHF) indicates that correlations were slightly better when values were calculated in aqueous solution.

*(TABLE 4, FIG. 2)*

In contrast to lnK$_m$ and lnK$_d$ values, lnk and lnk$_{cat}$ values did not show any dependence on the redox parameters of the phenols (Table 4). This finding is in contrast to previous reports for HRP (45), which indicated that such correlations exist in the case of HRP-catalyzed oxidation of phenols.

**Oxidation of 1,2-dimethoxyarenes**

The kinetic and thermodynamic parameters for the oxidation of several 1,2-dimethoxyarenes are presented in Table 5. It is clear that these substrates did not comply with the trend witnessed for phenolic substrates in which lnK$_d$ and lnK$_m$ increased for thermodynamically, less favorable reactions. The differential behavior between the 1,2-dimethoxyarene and phenolic substrates is illustrated in Figs. 3a and 3b, which show lnK$_m$ and lnK$_d$ as a function of OP at pH 3.5 for the two sets of substrates. 1,2-Dimethoxyarenes clearly do not comply with the trend witnessed for phenolic substrates.

*(TABLE 5, FIG. 3)*
Analogue studies

The differential behavior between the 1,2-dimethoxyarenes and phenolic substrates prompted analogue studies in which the 1,2-dimethoxyarenes, veratric acid and veratryl aldehyde, both of which are not oxidized by LIP, were included in reactions containing phenolic or 1,2-dimethoxyarene substrates. Inclusion of either veratric acid or veratryl aldehyde had little if any effect on the rate of oxidation of phenolic substrates (Fig. 4). On the contrary, the rate of oxidation of 1,2-dimethoxyarene substrates was reduced in the presence of either veratric acid or veratryl aldehyde. The most pronounced decrease was noticed for veratryl alcohol, whereas the decrease in rate of oxidation of 3,4-dimethoxyphenethylalcohol and 3,4-dimethoxycinnamic acid in the presence of veratric acid was the least significant (Fig. 4).

(DISCUSSION)

Mechanistic features of oxidation of phenolic substrates

The dependence of \( \ln K_m \) and \( \ln K_d \) and independence of \( \ln k \) and \( \ln k_{cat} \) on redox parameters of the phenolic substrates may be explained as follows. After formation of the binding complex, a fast equilibrium electron transfer from substrate molecule \( (K_{et}) \) takes place with a subsequent rate limiting step \( (k_6) \) in which the products of substrate oxidation do not participate. Accordingly, equation 5 must be modified as follows:

\[
LIP_{II} + S \xrightleftharpoons[k_{-5}]{k_5} LIP_{II}-S \xrightarrow{K_{et}} LIP_{II}-P \xrightarrow{k_6} LIP + P + H_2O \quad (12)
\]

Kinetic analysis of equation 12 leads to equation 13, a Michaelis-Menten like equation:
\[ v' = \frac{(k_6K_{et}/(K_{et} + 1))[S]}{k_5/k_5(K_{et} + 1) + k_6K_{et}/k_5((K_{et} + 1) + S)} = \frac{k[S]}{K_d + [S]} \]  

(13)

When the oxidation is carried out under steady state conditions, a similar equation may be derived for the observed rate (the transformation LIPII → Fe-LIP is rate limiting).

Two principal situations evolve from equation 13.

I) In the event that the electron transfer step is exergonic, then \( K_{et} \gg 1 \), and equation 13 is modified to equation 14:

\[ v' = \frac{k_6[S]}{k_5/k_5K_{et} + k_6/k_5 + S} = \frac{k[S]}{K_d + [S]} \]  

(14)

In this case \( k = k_6 \) and \( K_d = k_5/k_5K_{et} \), if \( k_5/k_5K_{et} \gg k_6/k_5 \). A linear relationship between \( \ln K_d \) and the thermodynamic driving force of the reaction \( (\Delta G_{et}) \) may be expected if \( \ln(k_5/k_5) \) does not depend significantly on the structure of the phenolic substrate: \( \ln K_d = \ln(k_5/k_5) - \ln K_{et} = \ln(k_5/k_5) + \Delta G_{et}/RT \). According to this expression, \( \ln K_d \) increases with an increase in \( \Delta G_{et} \) (the thermodynamic driving force of the reaction). Taking into account the above mentioned restrictions, we can conclude that equation 12 fits well with our observations. The nature of the rate limiting step is unclear, but in view of the above mentioned suggestion that substrate molecules are not involved in this step, a least two possibilities may exist: a) since the mechanism of transformation of LIPII to Fe-LIP involves transfer of two protons to the oxygen atom of \( \text{Fe}^{4+} = \text{O} \) and subsequent elimination of a water molecule, this could be the rate limiting step; b) alternatively reorganization of the reduced heme moiety, that precedes elimination of water molecule, may be the rate limiting step.
II) If the electron transfer step is endergonic ($K_{et}<<1$), then a completely different situation arises (equation 15).

$$v' = \frac{k_6K_{et}[S]}{k_{-5}/k_5 + k_6K_{et}/k_5 + S} = \frac{k[S]}{Kd + [S]}$$

(15)

In this case, $\ln k = \ln k_6K_{et} = \ln k_6 + \ln K_{et}$ depends on the redox parameters of the substrate molecules, even though the one electron oxidation of the substrate is not rate limiting. Therefore, the dependence of $\ln k$ or $\ln k_{cat}$ on the redox parameters of the substrate in endergonic enzymatic oxidation does not provide unambiguous evidence of the redox nature of the rate limiting step.

The specific mechanistic features of LIPII-substituted phenol interactions – an equilibrium electron transfer step with subsequent rate limiting step - differs from the commonly accepted suggestion that the rate of compound II reactions is limited by the electron transfer step. The latter has been well established for the oxidation of substituted phenols by HRP (21,22,45) and automatically expanded to other less studied peroxidases. In light of our results, it is very likely that despite the fact that the general scheme of peroxidase action (equation 1) is similar for all types of peroxidases, the mechanisms of Compound I- or Compound II-substrate interactions may differ.

Analysis of the correlation coefficients for linear relationships between $\ln K_d$ and $\ln K_m$ and different calculated redox characteristics (Table 4) sheds some light on the nature of the electron transfer step. It is obvious that at pH=3.5 all of the phenols studied are in their acidic form. As a result, any one of three mechanisms may depict the electron transfer step. The first involves oxidation of phenolate ion obtained by preliminary abstraction of a proton from the parent phenol. This case is represented by the redox parameter, $OP_{basic}$. The second mechanism involves one electron oxidation of phenol...
with subsequent abstraction of a proton from the obtained radical cation and is represented by IP_{ad}, IP_{v} or OP_{acidic}. The third mechanism suggests coupled electron and proton transfer and is represented by BEP or OP_{pH3.5}. Comparison of the correlation coefficients for the linear relationships reveals that the best fits were obtained for BEP and OP_{pH3.5}. This supports the third mechanism in which the acidic forms of phenols are oxidized by LIP and that electron transfer is coupled with proton transfer.

**Oxidation of 1,2-dimethoxyarenes**

The results obtained for 1,2-dimethoxyarenes did not comply with trends observed for phenolic substrates. This finding may be a consequence of different substrate binding sites and/or alternative binding modes. Indeed, previous research has suggested that LIP contains at least two substrate binding sites (23-26). Site directed mutagenesis indicated that veratryl alcohol and probably other hydrophobic substrates are oxidized at the surface of the enzyme with Trp 171 being implicated in catalysis by long range electron transfer routes (23). Two mutants lacking Trp 171 lost all activity towards veratryl alcohol, yet retained substantial activity towards two dye substrates. After a charge neutralization mutation in the “classical heme edge” of the enzyme, in which Glu 146 was substituted by Gly, LIP showed substantial activity with respect to veratryl alcohol and a marked (2.4 pH units) increase in pK_{a} for the oxidation of a negatively charged difluoroazodye. This indicates that hydrophilic substrates are most probably oxidized at the “classical heme edge” of the enzyme. To verify whether or not our findings were a consequence of different binding sites and/or alternative binding modes, analogue studies were conducted, in which the 1,2-dimethoxyarenes, veratryl aldehyde and veratric acid, both of which are not oxidized by LIP, were incorporated into reaction mixtures containing either substituted phenols (4-bromo-
and 4-chlorophenol, catechol, ferulic acid, and syringaldehyde) or 1,2-dimethoxyarenes (3,4-dimethoxyphenethylalcohol, 3,4-dimethoxytoluene, 3,4-dimethoxycinnamic acid, and veratryl alcohol). Both veratryl aldehyde and veratric acid suppressed the oxidation of 1,2-dimethoxyarene substrates as reflected by a decrease in the rate of their oxidation. The decrease was considerable for veratryl alcohol and less pronounced for 3,4-dimethoxyphenethylalcohol and 3,4-dimethoxycinnamic acid, in particular in the presence of veratric acid. Overall, these findings suggest that veratric acid and veratryl aldehyde interfered with the interaction between the 1,2-dimethoxyarene substrates and the enzyme, competitively inhibiting their oxidation. Since the oxidation of phenolic substrates was unaffected by the presence of veratric acid and veratryl aldehyde, it is possible that they are either oxidized at another site on the enzyme, in accordance with the reports that LIP possesses more than one binding site (23-26) and/or the modes of binding of phenolic substrates and 1,2-dimethoxyarenes differ.

The higher OP of veratryl alcohol at pH 3.5 (1.36 V vs. NHE) (49) in comparison to phenolic substrates (0.79 – 1.17 V vs. NHE, Table 3) is probably the driving force for veratryl alcohol mediated oxidation of phenolic substrates, which has been extensively documented (4, 6, 17, 50-52). The radical cation formed upon oxidation of veratryl alcohol is preferentially reduced by phenolic substrate. Consequently, the oxidation of veratryl alcohol to veratryl aldehyde begins only after all of the phenolic substrate has been oxidized. A recent study showed that in the presence of a saturating concentration of veratryl alcohol, the rate of oxidation of low concentrations of ferulic acid by LIP approached the $k_{cat}$ value of veratryl alcohol (6). As the concentration of ferulic acid was increased, the rate of its oxidation increased to a value between the $k_{cat}$ for veratryl alcohol and the $k_{cat}$ for ferulic acid, suggesting that direct oxidation and mediated oxidation of ferulic acid were occurring concomitantly. This
phenomenon has two possible explanations. According to the first, both compounds compete for the same interaction site on the enzyme. The second suggests two distinct interaction sites for phenol and veratryl alcohol. In light of the findings from the analogue studies in the present paper as well as previous studies which indicate two or more different substrate binding sites (23-26), the second explanation is more attractive.

Recently it has been shown that 3,4-dimethoxycinnamic acid and veratrole, which also have significantly higher OP values than phenolic substrates (Tables 3 and 5), are also capable of mediating their oxidation (50). On account of the aforementioned, it is predicted that the mediation is dependent on the difference between the OP value of the mediator and the target substrate. Therefore, other 1,2-dimethoxyarenes are also expected to serve as mediators.

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REFERENCES


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**FIGURE LEGENDS**

**Fig. 1.** Pre-steady state dissociation constant, $K_d$, vs. steady-state dissociation constant, $K_m$, for phenolic substrates. $R^2=0.90$. The slope is $0.71 \pm 0.18$, whereas the Y-intercept is $-70 \pm 36$.

**Fig. 2.** Ln $K_m$ values (A) and Ln $K_d$ values (B) vs. bond energies for phenolic substrates. Bromophenols (Δ), chlorophenols (∇), dihydroxy derivatives (◊), hydroxy methoxy derivatives (○), hydroxy dimethoxy derivatives (□).

**Fig. 3.** (A): Ln $K_m$ values vs. calculated oxidation potential values at pH 3.5 for phenolic and 1,2-dimethoxyarene substrates. Bromophenols (Δ), chlorophenols (∇), dihydroxy derivatives (◊), hydroxy methoxy derivatives (○), hydroxy dimethoxy derivatives (□), 1,2-dimethoxyarene substrates (●). (B): Ln $K_d$ values vs. calculated oxidation potential values at pH 3.5 for phenolic (○) and 1,2-dimethoxyarene substrates (●).

**Fig. 4.** The influence of the analogues, veratryl aldehyde (□) and veratric acid (■) on the rate of oxidation of phenols and 1,2-dimethoxyarenes. The initial rate of oxidation of 100 µM substrate was measured in the presence and absence of 1500 µM veratryl aldehyde or veratric acid. Results are presented as the percentage rate of oxidation relative to that of the pure substrate (mean ± standard deviation of 3 replicates).

Abbreviations: 4BP, 4-bromophenol; 4CP, 4-chlorophenol; CAT, catechol; FA, ferulic acid; SYR, syringaldehyde; DMP, 3,4-dimethoxyphenethylalcohol; DMT, 3,4-dimethoxyltoluene; DMC, 3,4-dimethoxycinnamic acid; VA, veratryl alcohol
Table 1. List of substrates studied.

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Systematic name</th>
<th>Common name</th>
<th>Calculated $\lambda_{\text{max}}$, $\epsilon$ (M$^{-1}$ cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_1=\text{Br}$, $R_2=\text{H}$, $R_3=\text{Br}$</td>
<td>2,4-dibromophenol</td>
<td></td>
<td>286 nm, $1.7\times10^3$</td>
</tr>
<tr>
<td>$R_1=\text{H}$, $R_2=\text{H}$, $R_3=\text{Br}$</td>
<td>4-bromophenol</td>
<td></td>
<td>280 nm, $1.3\times10^3$</td>
</tr>
<tr>
<td>$R_1=\text{H}$, $R_2=\text{Br}$, $R_3=\text{H}$</td>
<td>3-bromophenol</td>
<td></td>
<td>275 nm, $1.7\times10^3$</td>
</tr>
<tr>
<td>$R_1=\text{Cl}$, $R_2=\text{H}$, $R_3=\text{Cl}$</td>
<td>2,4-dichlorophenol</td>
<td></td>
<td>284 nm, $1.6\times10^3$</td>
</tr>
<tr>
<td>$R_1=\text{H}$, $R_2=\text{H}$, $R_3=\text{Cl}$</td>
<td>4-chlorophenol</td>
<td></td>
<td>280 nm, $1.4\times10^3$</td>
</tr>
<tr>
<td>$R_1=\text{H}$, $R_2=\text{Cl}$, $R_3=\text{H}$</td>
<td>3-chlorophenol</td>
<td></td>
<td>274 nm, $1.6\times10^3$</td>
</tr>
<tr>
<td>$R=\text{H}$</td>
<td>1,2-Dihydroxybenzene</td>
<td>Catechol</td>
<td>276 nm, $2.1\times10^3$</td>
</tr>
<tr>
<td>$R=\text{CH}=\text{CH-COOH}$</td>
<td>3-(3,4-Dihydroxyphenyl)-2-propenoic acid</td>
<td>Caffeic acid</td>
<td>316 nm, $1.4\times10^4$</td>
</tr>
<tr>
<td>$R=\text{COOH}$</td>
<td>3,4-dihydroxybenzoic acid</td>
<td>Protocatechuic acid</td>
<td>291 nm, $4.1\times10^3$</td>
</tr>
<tr>
<td>$R=\text{CH}=\text{CH-COOH}$</td>
<td>3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid</td>
<td>Ferulic acid</td>
<td>320 nm, $1.5\times10^4$</td>
</tr>
<tr>
<td>$R=\text{H}$</td>
<td>2-methoxyphenol</td>
<td>Guaiacol</td>
<td>280 nm, $1.7\times10^3$</td>
</tr>
<tr>
<td>$R=\text{CH}_2\text{OH}$</td>
<td>4-Hydroxy-3-methoxybenzyl alcohol</td>
<td>Vanillyl alcohol</td>
<td>278 nm, $2.3\times10^3$</td>
</tr>
<tr>
<td>$R=\text{COOH}$</td>
<td>4-Hydroxy-3-methoxybenzoic acid</td>
<td>Vanillic acid</td>
<td>287 nm, $4.4\times10^3$</td>
</tr>
<tr>
<td>$R=\text{CHO}$</td>
<td>4-Hydroxy-3,5-dimethoxybenzaldehyde</td>
<td>Syringaldehyde</td>
<td>270 nm, $2.9\times10^3$</td>
</tr>
<tr>
<td>$R=\text{H}$</td>
<td>2,6-dimethoxyphenol</td>
<td></td>
<td>268 nm, $8.4\times10^2$</td>
</tr>
<tr>
<td>$R=\text{COOH}$</td>
<td>4-Hydroxy-3,5-dimethoxybenzoic acid</td>
<td>Syringic acid</td>
<td>265 nm, $8.6\times10^3$</td>
</tr>
<tr>
<td>$R=\text{CH}_2\text{CH}_2\text{OH}$</td>
<td>3,4-dimethoxyphenethylalcohol</td>
<td></td>
<td>278 nm, $2.4\times10^3$</td>
</tr>
<tr>
<td>$R=\text{CH}_2\text{COOH}$</td>
<td>(3,4-Dimethoxyphenyl)-acetic acid</td>
<td>Homoveratric acid</td>
<td>278 nm, $2.4\times10^3$</td>
</tr>
<tr>
<td>$R=\text{CH}_3$</td>
<td>3,4-dimethoxytoluene</td>
<td>4-methylveratrole</td>
<td>279 nm, $2.5\times10^3$</td>
</tr>
<tr>
<td>$R=\text{H}$</td>
<td>1,2-Dimethoxybenzene</td>
<td>Veratrole</td>
<td>273 nm, $2.1\times10^3$</td>
</tr>
<tr>
<td>$R=\text{CH}=\text{CH-COOH}$</td>
<td>3-(3,4-Dimethoxyphenyl)-2-propenoic acid</td>
<td>3,4-dimethoxyxinnamic acid</td>
<td>314 nm, $1.6\times10^4$</td>
</tr>
<tr>
<td>$R=\text{CH}_2\text{OH}$</td>
<td>3,4-Dimethoxybenzyl alcohol</td>
<td>Veratryl alcohol</td>
<td>280 nm, $2.5\times10^3$</td>
</tr>
<tr>
<td>$R=\text{COOH}$</td>
<td>3,4-Dimethoxybenzoic acid</td>
<td>Veratric acid</td>
<td>287 nm, $4.5\times10^3$</td>
</tr>
<tr>
<td>$R=\text{CHO}$</td>
<td>3,4-Dimethoxybenzaldehyde</td>
<td>Veratraldehyde</td>
<td>310 nm, $9.3\times10^3$ *</td>
</tr>
</tbody>
</table>

*According to Tien and Kirk; (28) $\lambda_{\text{max}}$: wavelength for maximal absorption; $\epsilon$: absorptivity coefficient.
Table 2. Kinetic parameters of substituted phenols.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$, s(^{-1})</th>
<th>$K_m$, µM</th>
<th>$k$, s(^{-1})</th>
<th>$K_d$, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-dibromophenol</td>
<td>(4.3±0.2)×10(^{1})</td>
<td>(3.8±0.6)×10(^{2})</td>
<td>(2.3±0.2)×10(^{1})</td>
<td>(3.1±0.8)×10(^{2})</td>
</tr>
<tr>
<td>4-bromophenol</td>
<td>(4.0±0.5)×10(^{1})</td>
<td>(5.2±1.5)×10(^{2})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-bromophenol</td>
<td>(3.0±0.5)×10(^{1})</td>
<td>(1.3±0.6)×10(^{3})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4-dichlorophenol</td>
<td>(2.3±0.2)×10(^{1})</td>
<td>(3.3±1.1)×10(^{2})</td>
<td>(1.6±0.2)×10(^{1})</td>
<td>(1.4±0.4)×10(^{2})</td>
</tr>
<tr>
<td>4-chlorophenol</td>
<td>(2.2±0.2)×10(^{1})</td>
<td>(3.2±0.8)×10(^{2})</td>
<td>(1.5±0.2)×10(^{1})</td>
<td>(1.9±0.7)×10(^{2})</td>
</tr>
<tr>
<td>3-chlorophenol</td>
<td>(2.7±0.5)×10(^{1})</td>
<td>(7.9±3.6)×10(^{2})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catechol</td>
<td>(3.6±0.2)×10(^{1})</td>
<td>(1.9±0.4)×10(^{2})</td>
<td>(1.6±0.1)×10(^{1})</td>
<td>(2.0±0.6)×10(^{1})</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>(3.8±0.3)×10(^{1})</td>
<td>(2.2±0.4)×10(^{2})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>(2.5±0.3)×10(^{1})</td>
<td>(4.4±1.3)×10(^{2})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>(3.8±0.1)×10(^{1})</td>
<td>(1.0±0.1)×10(^{2})</td>
<td>(1.6±0.1)×10(^{1})</td>
<td>(2.8±0.4)×10(^{1})</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>(3.8±0.1)×10(^{1})</td>
<td>(1.7±0.2)×10(^{2})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanillyl alcohol</td>
<td>(3.9±0.2)×10(^{1})</td>
<td>(1.2±0.2)×10(^{2})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>(1.8±0.1)×10(^{1})</td>
<td>(1.6±0.4)×10(^{2})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td>(1.3±0.1)×10(^{1})</td>
<td>(1.7±0.3)×10(^{2})</td>
<td>(1.5±0.1)×10(^{1})</td>
<td>(1.3±0.7)×10(^{1})</td>
</tr>
<tr>
<td>2,6-dimethoxyphenol</td>
<td>(2.7±0.3)×10(^{1})</td>
<td>(9.7±4.5)×10(^{1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syringic acid</td>
<td>(1.0±0.1)×10(^{1})</td>
<td>(1.1±0.4)×10(^{2})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$K_m$ and $k_{cat}$ are steady state parameters calculated from non-linear least-squares fits to plots of the initial rate of oxidation as a function of substrate concentration (3 replicates of each of at least 7 different substrate concentrations in the range 0-2000 µM were studied).

$K_d$ and $k$ are pre-steady state kinetic parameters calculated from equation 6 using a non-linear least-squares fit to plots of initial reaction rate vs. substrate concentration (five replicates of each of at least 8 different substrate concentrations were studied). Data are presented as the mean ± standard error.
Table 3. Experimentally determined and calculated redox parameters, and pKa values for studied substituted phenols.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pKa</th>
<th>Eo, V</th>
<th>IP_{ad}(UHF)/IP_{ad}(ROHF), Ev</th>
<th>IP_{v}(UHF)/IP_{v}(ROHF), Ev</th>
<th>BEP, Ev</th>
<th>OP_{acidic}, Ev</th>
<th>OP_{basic}, Ev</th>
<th>OP_{pH3.5}, Ev</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-dibromophenol</td>
<td>7.79</td>
<td>8.70/8.89</td>
<td>8.91/9.08</td>
<td>1.17/1.60</td>
<td>6.51/6.76</td>
<td>5.25/5.68</td>
<td>5.50/5.93</td>
<td></td>
</tr>
<tr>
<td>4-bromophenol</td>
<td>9.17</td>
<td>8.56/8.73</td>
<td>8.78/8.94</td>
<td>1.17/1.59</td>
<td>6.35/6.60</td>
<td>5.12/5.54</td>
<td>5.45/5.87</td>
<td></td>
</tr>
<tr>
<td>3-bromophenol</td>
<td>9.03</td>
<td>8.66/8.86</td>
<td>8.88/9.08</td>
<td>1.17/1.60</td>
<td>6.36/6.57</td>
<td>5.07/5.48</td>
<td>5.39/5.80</td>
<td></td>
</tr>
<tr>
<td>2,4-dichlorophenol</td>
<td>7.89</td>
<td>8.60/8.82</td>
<td>8.82/8.99</td>
<td>1.08/1.51</td>
<td>6.40/6.61</td>
<td>5.09/5.52</td>
<td>5.35/5.78</td>
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</tr>
<tr>
<td>4-chlorophenol</td>
<td>9.35</td>
<td>8.48/8.63</td>
<td>8.72/8.86</td>
<td>1.12/1.52</td>
<td>6.28/6.48</td>
<td>5.04/5.44</td>
<td>5.38/5.78</td>
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</tr>
<tr>
<td>3-chlorophenol</td>
<td>9.13</td>
<td>8.61/8.82</td>
<td>8.83/9.02</td>
<td>1.16/1.58</td>
<td>6.35/6.56</td>
<td>5.07/5.48</td>
<td>5.40/5.81</td>
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</tr>
<tr>
<td>Catechol</td>
<td>9.45</td>
<td>0.84</td>
<td>8.21/8.37</td>
<td>8.44/8.61</td>
<td>0.98/1.32</td>
<td>6.06/6.20</td>
<td>4.84/5.17</td>
<td>5.19/5.52</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>9.07</td>
<td>0.85</td>
<td>8.20/8.48</td>
<td>8.42/8.68</td>
<td>0.93/1.43</td>
<td>6.06/6.35</td>
<td>4.82/5.32</td>
<td>5.14/5.64</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>8.64</td>
<td>0.81</td>
<td>8.66/8.76</td>
<td>8.82/9.00</td>
<td>1.01/1.37</td>
<td>6.15/6.28</td>
<td>4.95/5.31</td>
<td>5.25/5.61</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>9.39</td>
<td>0.90</td>
<td>8.05/8.33</td>
<td>8.27/8.53</td>
<td>0.87/1.35</td>
<td>6.02/6.31</td>
<td>4.78/5.26</td>
<td>5.12/5.60</td>
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<tr>
<td>Guaiacol</td>
<td>9.90</td>
<td>0.89</td>
<td>8.05/8.21</td>
<td>8.29/8.46</td>
<td>0.97/1.32</td>
<td>6.07/6.23</td>
<td>4.79/5.15</td>
<td>5.16/5.52</td>
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<tr>
<td>Vanillyl alcohol</td>
<td>9.99</td>
<td>7.90/8.07</td>
<td>8.12/8.29</td>
<td>0.94/1.30</td>
<td>6.06/6.22</td>
<td>4.77/5.14</td>
<td>5.15/5.52</td>
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<tr>
<td>Vanillic acid</td>
<td>9.39</td>
<td>0.98</td>
<td>8.41/8.58</td>
<td>8.66/8.83</td>
<td>1.01/1.38</td>
<td>6.17/6.32</td>
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<td>Syringaldehyde</td>
<td>7.54</td>
<td>8.03/8.26</td>
<td>8.29/8.51</td>
<td>0.88/1.32</td>
<td>6.21/6.46</td>
<td>4.92/5.36</td>
<td>5.16/5.60</td>
<td></td>
</tr>
<tr>
<td>2,6-dimethoxyphenol</td>
<td>9.69</td>
<td>0.79</td>
<td>7.77/7.96</td>
<td>8.02/8.20</td>
<td>0.83/1.23</td>
<td>6.04/6.24</td>
<td>4.66/5.07</td>
<td>5.02/5.43</td>
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<tr>
<td>Syringic acid</td>
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<td>0.86</td>
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<td>8.35/8.58</td>
<td>0.88/1.31</td>
<td>6.12/6.37</td>
<td>4.76/5.19</td>
<td>5.11/5.54</td>
</tr>
</tbody>
</table>

Abbreviations: Eo, experimentally determined redox potential; IP_{ad}(UHF), UHF adiabatic ionization potential; IP_{ad}(ROHF), ROHF adiabatic ionization potential; IP_{v}(UHF), UHF vertical ionization potential; IP_{v}(ROHF), ROHF vertical ionization potential; BEP, bond energy parameter; OP_{acidic}, oxidation potential of undissociated phenol; OP_{basic}, oxidation potential of phenolate ion; OP_{pH3.5}, oxidation potential calculated for pH 3.5
Table 4. Correlation coefficients ($R^2$) between the natural logarithm of the empirical kinetic parameters and experimentally determined and calculated redox parameters of substituted phenolic substrates.

<table>
<thead>
<tr>
<th></th>
<th>Ln $k_{cat}$</th>
<th>Ln $K_m$</th>
<th>Ln $k$</th>
<th>Ln $K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eo, V (pH=3.5)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$IP_{ad}$</td>
<td>UHF</td>
<td>7.7x$10^{-3}$</td>
<td>7.4x$10^{-1}$</td>
<td>3.0x$10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>ROHF</td>
<td>7.9x$10^{-3}$</td>
<td>7.3x$10^{-1}$</td>
<td>2.7x$10^{-1}$</td>
</tr>
<tr>
<td><strong>IP_v</strong></td>
<td>UHF</td>
<td>4.5x$10^{-3}$</td>
<td>7.4x$10^{-1}$</td>
<td>2.9x$10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>ROHF</td>
<td>2.7x$10^{-3}$</td>
<td>7.4x$10^{-1}$</td>
<td>2.9x$10^{-1}$</td>
</tr>
<tr>
<td><strong>BEP</strong></td>
<td>UHF</td>
<td>8.7x$10^{-2}$</td>
<td>7.8x$10^{-1}$</td>
<td>4.3x$10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>ROHF</td>
<td>7.2x$10^{-2}$</td>
<td>7.2x$10^{-1}$</td>
<td>4.6x$10^{-1}$</td>
</tr>
<tr>
<td><strong>OP_{acidic}</strong></td>
<td>UHF</td>
<td>7.4x$10^{-5}$</td>
<td>5.5x$10^{-1}$</td>
<td>2.3x$10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>ROHF</td>
<td>1.5x$10^{-4}$</td>
<td>4.5x$10^{-1}$</td>
<td>3.9x$10^{-1}$</td>
</tr>
<tr>
<td><strong>OP_{basic}</strong></td>
<td>UHF</td>
<td>2.0x$10^{-2}$</td>
<td>6.4x$10^{-1}$</td>
<td>3.8x$10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>ROHF</td>
<td>1.6x$10^{-2}$</td>
<td>5.6x$10^{-1}$</td>
<td>3.9x$10^{-1}$</td>
</tr>
<tr>
<td><strong>OP_{pH3.5}</strong></td>
<td>UHF</td>
<td>5.4x$10^{-2}$</td>
<td>6.8x$10^{-1}$</td>
<td>4.4x$10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>ROHF</td>
<td>4.6x$10^{-2}$</td>
<td>6.1x$10^{-1}$</td>
<td>4.9x$10^{-1}$</td>
</tr>
</tbody>
</table>

Abbreviations: $IP_{ad}$(UHF), UHF adiabatic ionization potential; $IP_{ad}$(ROHF), ROHF adiabatic ionization potential; $IP_v$(UHF), UHF vertical ionization potential; $IP_v$(ROHF), ROHF vertical ionization potential; BEP, bond energy parameter; OP_{acidic}, oxidation potential of undissociated phenol; OP_{basic}, oxidation potential of phenolate ion; OP_{pH3.5}, oxidation potential calculated at pH 3.5
Table 5. Calculated oxidation potential and experimentally determined kinetic parameters for 1,2-dimethoxyarenes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>OP&lt;sub&gt;pH3.5&lt;/sub&gt;, eV</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;, s&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;, µM</th>
<th>k, s&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>K&lt;sub&gt;d&lt;/sub&gt;, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-dimethoxyphenethylalcohol</td>
<td>6.05</td>
<td>(1.3±0.1)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(6.1±1.6)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(1.5±0.1)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(8.7±2.9)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Homoveratric acid</td>
<td>6.11</td>
<td>(1.9±0.1)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(1.4±0.2)&lt;sup&gt;2&lt;/sup&gt; × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>(1.5±0.1)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(5.1±1.9)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-methylveratrole</td>
<td>6.01</td>
<td>(1.8±0.1)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(5.0±1.1)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(9.7±0.7)&lt;sup&gt;0&lt;/sup&gt; × 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>(5.0±1.6)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Veratrole</td>
<td>6.04</td>
<td>(6.9±1.3)&lt;sup&gt;0&lt;/sup&gt; × 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>(3.1±1.4)&lt;sup&gt;2&lt;/sup&gt; × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,4-dimethoxycinnamic acid</td>
<td>6.08</td>
<td>(1.5±0.2)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(2.4±0.9)&lt;sup&gt;2&lt;/sup&gt; × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>6.09</td>
<td>1.3×10&lt;sup&gt;1&lt;/sup&gt;*</td>
<td>(7.2±1.0)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(1.9±0.1)&lt;sup&gt;1&lt;/sup&gt; × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(1.9±0.2)&lt;sup&gt;2&lt;/sup&gt; × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

OP<sub>pH3.5</sub> is the oxidation potential calculated at pH 3.5. K<sub>m</sub> and k<sub>cat</sub> are steady state parameters calculated from non-linear least-squares fits to plots of initial rate of oxidation as a function of substrate concentration (3 replicates of each of at least 7 different substrate concentrations in the range 0-2000 µM were studied). K<sub>d</sub> and k are pre-steady state kinetic parameters calculated from equation 6 using a non-linear least-squares fit to plots of initial reaction rate vs. substrate concentration (five replicates of each of at least 8 different substrate concentrations were studied). Data are presented as the mean ± standard error.

*Standard error for veratryl alcohol was less than 0.05 s<sup>-1</sup>.
Fig. 1
Fig. 2

A

B

$\ln K_m$

$\ln K_d$

BEP, eV

A

B
Fig. 4

Relative oxidation rate (%)

Phenols

1,2-dimethoxyarenes