N-Substituted p-Phenylenediamines as Peroxidase and Laccase Substrates

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N-Substituted p-phenylenediamines, such as 1-(N,N-dimethylamino)-4-(4-morpholinyl)benzene (AMB), 1-(1-piperidinyl)-4-(4-morpholinyl)benzene (PMB), 1,4-di(1-piperidinyl)benzene (DPB), 1,4-di(4-morpholinyl)benzene (DMB) and N-methyl-N-ethyl-4-(4-morpholinyl) benzylamine (MEMB) are oxidized in a peroxidase-catalyzed process following radical cation formation. At pH 7 an apparent bimolecular constant (k_{ox}) varied from 1.8 to 43.6 $\mu M^{-1} s^{-1}$ for horseradish peroxidase (HRP) and peroxidase from *Coprinus cinereus* (CiP). The limiting step of the substrate oxidation was reduction of compound II, and the steady-state constant of AMB oxidation (13.2 $\mu M^{-1} s^{-1}$) was consistent with a constant (11.9 $\mu M^{-1} s^{-1}$) determined by a stopped-flow method. k_{ox} of compound I reduction with the same substrates was 23.1 $\mu M^{-1} s^{-1}$. CiP showed a broad pH activity at pH 6–10, whereas the activity of the HRP-catalyzed reaction dropped at pH 8.0. The apparent pK_a value for HRP was 7.35 and 9.7 for CiP. The same substrates are also catalytically oxidized by fungal laccase from *Polyporus pinsitus*. k_{ox} constants were considerably lower than in peroxidase catalysis and varied from 0.08 $\mu M^{-1} s^{-1}$ to 1.74 $\mu M^{-1} s^{-1}$. Optimal activity for AMB oxidation was obtained at pH 5–5.5 with apparent pK_a values of 5.5 and 5.3. The correlation between the reaction driving force and electron transfer rate was analyzed for both types of reaction.

Many p-phenylenediamines undergo one-electron oxidation to the corresponding radical cations, the so-called Wurster salts.[1] Radical cations produced from N,N,N'N'-tetraalkyl-substituted p-phenylenediamines are blue and are stable in water solutions. One commercially available compound, N,N,N'N'-tetramethyl-p-phenylenediamine (TMPD), has been used for investigation of the respiratory chain, enzyme kinetics and other biochemical phenomena, but it was found to have limited application owing to fast auto-oxidation.[2] Auto-oxidation of TMPD yields not only radical cation but also superoxide radical and hydrogen peroxide.

In an attempt to find new redox mediators with controllable redox potentials and hydrophobic/hydrophilic properties, new tetrasubstituted p-phenylenediamines were synthesized.[3] The redox potentials of these compounds varied from 0.27 to 0.53 V vs. NHE.[3] Some of them were soluble in lipophilic solvents, such as paraffin oil, silicone oil, tricaprylin.[4]

Both horseradish peroxidase (HRP) and that from *Coprinus cinereus* (CiP) belong to the class of heme peroxidases (donor: H_2O_2 oxidoreductases EC 1.11.17)

and catalyze the oxidation of a wide range of organic compounds such as phenols and aromatic amines,[5–7] azo dyes,[8] and phenothiazines[9] by hydrogen peroxide. The same compounds can be substrates of copper-containing laccases (benzenediol: oxygen oxidoreductase EC 1.10.3.2), but oxidation is performed with oxygen as the electron acceptor. Both types of enzyme are widespread among plants, fungi and bacteria. Their prosthetic groups take part in one-electron oxidation of substrates to yield the corresponding phenoxyl radicals or radical cations. HRP, fungal peroxidases and microbial laccases have gained increased attention as a result of their practical application in lignin biodegradation[10,11] and in clinical analysis.[12] Peroxidase and laccase action can be described by a ping-pong scheme.[13,14] At least two intermediates, compound I and compound II, should be included in a more detailed investigation of peroxidase catalysis. Four copper centers participate in laccase catalysis, and these are divided into three types: a mononuclear 'blue' copper site (type 1), a mononuclear Cu center (type 2) and binuclear Cu site (type 3). Types 2 and 3 constitute the so-called trinuclear copper center.

The scope of this work was to investigate N-substituted p-phenylenediamines as peroxidase and laccase sub-

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strates. To achieve this goal steady-state and pre-steady-state kinetics of peroxidase- and laccase-catalyzed p-phenylenediamine oxidation were studied. As peroxidases HRP and CiP were used. In steady-state laccase-catalyzed oxidation of p-phenylenediamines microbial laccase from *Polyporus pinsitus* (PpL) was used.

Experimental

Horseradish peroxidase (HRP, type I) was obtained from Sigma, peroxidase from *Coprinus cinereus* (CiP) and laccase from *Polyporus pinsitus* (PpL) were products of Novo Nordisk A/S, Denmark. The RZ values (absorbance ratio at 403 and 280 nm) of the peroxidases were 0.6 (HRP) and 2.8 (CiP). All enzymes were used without further purification. The molecular weight of the fungal laccase is 65 000 and pI is ca. 3.5. 1-(N,N-dimethylamino)-4-(4-morpholinyl)benzene (AMB), 1-(1-piperidinyl)-4-(4-morpholinyl)benzene (PMB), 1,4-di(1-piperidinyl)benzene (DPB), 1,4-di(4-morpholinyl)benzene (DMB), and N-methyl-N-ethyl-4-(4-morpholinyl)-benzylamine (MEMB) were synthesized at the University of Copenhagen and were used as crystalline products.[15] $N,N,N'N'$-Tetramethyl-1,4-phenylenediamine (TMPD) was purchased from Aldrich (USA). Sodium acetate, acetic acid, potassium phosphate, KOH, HCl were obtained from Reachim (Russia). Buffers and other solutions were prepared using doubly distilled water.

The concentrations of HRP and CiP were determined spectrophotometrically at 403[16] and 405[17] nm using the molar absorbance of 95 mM^{-1} cm^{-1} and 108 mM^{-1} cm^{-1}, respectively. The concentration of laccase (PpL) was measured at 280 nm by using an absorbance of 1.2 ml mg^{-1} cm^{-1}. Solutions of H_2O_2 were prepared from perhydrol (30%) and concentrations were controlled using the molar absorbance[18] (39.4 M^{-1} cm^{-1}) at 240 nm. Stock solutions of substrates were prepared in ethanol. The molar absorbance of the oxidized forms of substrates, with the exception of MEMB, was taken from literature.[3] The extinction coefficient of MEMB radical cation was determined in this work according to the method given in Ref. 3. The concentration of oxygen dissolved in buffer solution was assumed to be 0.254 mM at 25 °C.[19] The absorption spectrum of AMB radical cation was recorded in 0.05 M phosphate buffer, pH 7.0 containing 1 nM CiP, 50 μM H_2O_2 and 100 μM AMB.

The kinetics of p-phenylenediamine substrate oxidation were measured by a spectrophotometric method. Kinetic curves and absorption spectra were registered by means of a computer-controlled spectrophotometer Beckman DU-8B. The generation of oxidized forms of the p-phenylenediamines was measured at wavelengths as indicated in Table 1. Reaction mixtures contained 50 μM H_2O_2, 1–10 nM of peroxidases and 5–320 μM of substrates. The amount of ethanol from the substrate stock solutions did not exceed 1% (v/v) of the mixture in the reaction cell. The reaction was initiated by addition of

H_2O_2. Owing to the weak stability of TMPD in buffer solution the kinetics of the enzymatic generation of TMPD$^{+\cdot}$ was corrected according to the kinetics of TMPD auto-oxidation. Auto-oxidation of other p-phenylenediamines was insignificant, therefore no additional corrections were made. The measurements were carried out at 25 ± 0.1 °C in 0.05 M K-phosphate buffer, pH 7.0 and in 0.05 M sodium carbonate buffer, pH 10.0. In the laccase-catalyzed reaction 6–190 nM of PpL and 5–640 μM of substrates were used. PpL-catalyzed oxidation of p-phenylenediamines was performed in 0.05 M sodium acetate buffer, pH 5.3. pH-dependence measurements were made in 33 mM disodium phosphate, 33 mM boric acid and 33 mM sodium carbonate buffer in which the pH (6–11) was changed by adding 3 M hydrochloric acid or 3 M sodium hydroxide solution.

Pre-stationary CiP-catalyzed AMB oxidation was performed by a stopped-flow method using an Otsuka RA-401 stopped-flow spectrophotometer interfaced with a computer system. Measurements were conducted by rapid scanning of spectra. The gate time was 4 ms, slit width 1.4 nm and wavelength range 325–515 nm. The temperature was maintained at 25 °C using a circulating water bath. One syringe of the spectrophotometer contained 2 μM of CiP and 2 μM of H_2O_2; the other contained 2–20 μM of AMB in 20 mM phosphate buffer, pH 7.0. The redox potentials of the substrates investigated were determined according to the method described in Ref. 3.

An apparent Michaelis constant (K_m) and catalytic constant (k_{cat}) in steady-state peroxidase and laccase catalyzed oxidation of p-phenylenediamines were determined using the initial rate (v), and enzyme (E) and substrate (S) concentrations according to the Michaelis–Menten equation:

$$v = k_{cat} E S/(K_m + S) \qquad (1)$$

The bimolecular rate constant (k_{ox}) is defined as the ratio k_{cat}/K_m. All data are presented as mean ± standard deviation. Kinetic parameters were determined by means of the data analysis program for non-linear regression, ENZFITTER- version 1.05.

Quasi-first-order rate constants of compound I (k_2) and compound II (k_3) reductions were calculated by fitting kinetic curves of absorbance changes at 394, 414 and 426 nm. These wavelengths correspond to the isosbestic point between compound I and compound II, to the isosbestic point between compound II and the native enzyme, and to the isosbestic point between compound I and the native enzyme, respectively.[20] Integral kinetic curves of compound I, compound II and ferric peroxidase concentration change were derived according to eqns. (2)–(4).

$$[\text{compound I}] = [\text{compound I}]_0 \exp(-k_2 t) \qquad (2)$$

$$[\text{compound II}] = [\text{compound I}]_0 \{(k_2/k_3 - k_2)$$

$$\times [\exp(-k_2 t) - \exp(-k_3 t)]\}$$

$$+ [\text{compound II}]_0 \exp(-k_3 t) \qquad (3)$$

Table 1. Extinction coefficients of radical cations together with oxidation–reduction potentials of *p*-phenylenediamines in buffer solution pH 7.0 and apparent pK_a of protonation of *p*-phenylenediamines.

Mediator	Abbreviation	λ_{max}/nm	ε/mM^{-1}cm^{-1}	E/V vs. NHE	pK_a
N,N,N'N'-Tetramethyl-1,4-phenylenediamine	TMPD	606	11.0	0.27	6.7(1)
N-Methyl-*N*-ethyl-4-(4-morpholinyl)benzylamine	MEMB	611	13.4	0.38	6.5(2)
1-(1-Piperidinyl)-4-(4-morpholinyl)benzene	PMB	606	10.5	0.38	6.7(1)
1-(*N,N*-Dimethylamino)-4-(4-morpholinyl)benzene	AMB	604	9.8	0.39	5.7(1)
1,4-Di(1-piperidinyl)benzene	DPB	603	9.8	0.43	6.5(2)
1,4-Di(4-morpholinyl)benzene	DMB	618	10.1	0.53	4.2(2)

[ferric] = [compound I]$_0$[(k_2/k_3 − k_2)

$$\times \exp(-k_3 t) - (k_3/k_3 - k_2)\exp(-k_2 t) + 1]$$

$$+ [\text{compound II}]_0[1 - \exp(-k_3 t)] + [\text{ferric}]_0$$

(4)

where index '0' indicates the concentrations at initial time, $t = 0$.

The apparent second-order rate constants were calculated as the slope from linear plots of k_2 and k_3 versus AMB concentration. The approximation of kinetic data was made by minimizing the mean squared error using Mathcad 3.1 Windows version (MathSoft Inc., Cambridge, MA).

pK_a values of phenylenediamines were determined by spectrophotometric titration at the same buffer ionic strength and temperature as were used in the kinetic measurements. The absorbance change was measured at 295 nm. pK_a values were calculated by means of data analysis program for non-linear regression ENZFITTER- version 1.05.

All plots were drawn using the data analysis and graphics program GraFit 3.01 (© 1989–1992 Erithacus Software Ltd.).

Results and discussion

The absorption spectrum of the radical cation of one of the new synthesized *p*-phenylenediamines (AMB) is shown in Fig. 1. Strong absorbance of AMB radical cation in UV and visible region was observed. A large single peak in the UV part of the spectrum and two peaks in the visible region were observed at 330 and 560–609 nm, respectively. The absorbances of the radical cation of TMPD[1] and AMB (Fig. 1) are very similar and the extinction coefficients of the new *p*-phenylenediamine radical cations are comparable to TMPD (Table 1).

The redox potential of the one-electron oxidation of the p-phenylenediamines changes from 0.27 to 0.53 V vs. NHE (Table 1). Single protonation of the *p*-phenylenediamines occurs at pH 4.2–6.7 (Table 1).

At pH 7.0 the initial rate of oxidation of *p*-phenylenediamine derivatives by HRP and CiP versus substrate concentration was found to give a hyperbolic curve. The kinetic data were analyzed according to the Michaelis–Menten equation [eqn. (1)]. The calculated values of the
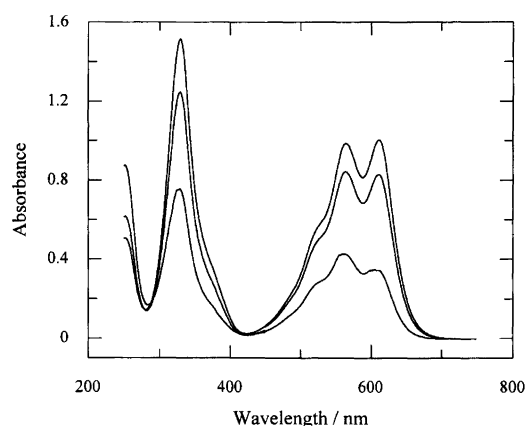

Fig. 1. Absorption spectrum of AMB radical cation at pH 7.0. The reaction mixture contained 0.1 mM AMB, 1 nM CiP, 50 μM H$_2$O$_2$, 50 mM phosphate buffer. The scan rate was 100 nm min^{-1}.

apparent K_m, catalytic constant (k_{cat}) and oxidation constant (k_{ox}) defined as k_{cat}/K_m are listed in Table 2.

The results given in Table 2 show that at pH 7.0 in the HRP-catalyzed reaction the apparent Michaelis–Menten constants of the *p*-phenylenediamine vary from 11 to 103 μM and the apparent catalytic constants vary from 211 to 479 s^{-1}. Calculated values of apparent bimolecular rate constants are 2.5–43.6 μM^{-1} s^{-1}. The kinetic parameters of CiP-catalyzed oxidation of *p*-phenylenediamine derivatives are similar to those of the HRP-catalyzed reactions. K_m of these reactions varies in the range

Table 2. Steady-state kinetic parameters of HRP- and CiP-catalyzed oxidation of *p*-phenylenediamines in 0.05 M phosphate buffer pH 7.0 at 25 °C.

Enzyme	Substrate	K_m/μM	k_{cat}/s^{-1}	k_{ox}/μM^{-1} s^{-1}
HRP	TMPD	11(1)	479(14)	44(6)
CiP	TMPD	49(6)	792(41)	16(3)
HRP	MEMB	18(3)	301(14)	17(3)
CiP	MEMB	43(4)	550(20)	13(2)
HRP	PMB	104(11)	260(14)	2.5(4)
CiP	PMB	73(19)	816(165)	11(2)
HRP	AMB	14(1)	427(11)	32(4)
CiP	AMB	7(9)	1030(58)	13(2)
HRP	DMB	66(11)	211(15)	3.2(8)
CiP	DMB	169(22)	304(23)	1.8(3)
HRP	DPB	53(5)	428(17)	8(1)
CiP	DPB	33(6)	500(35)	15(3)

11–169 µM and k_{cat} from 123 to 1030 s^{-1}. Bimolecular reaction rate constants vary in the range 1.8–23.2 µM^{-1} s^{-1}. The results show that the highest reaction rate is obtained with AMB and TMPD and the lowest reaction rate with DMB.

Kinetics of compound I and compound II of CiP reduction by AMB radical cation is shown in Fig. 2. It shows the spectral changes (350–520 nm) that occur during the oxidation of AMB by H_2O_2 catalyzed by CiP up to 50 ms at pH 7.0. As depicted in the inset of Fig. 2, fitting of the data according to eqns. (2)–(4) gives a rather good approximation. The calculated apparent bimolecular rate constant $k_{2,app}$ was 23.1 ± 6 µM^{-1} s^{-1} and $k_{3,app}$ was 11.9 ± 1.5 µM^{-1} s^{-1}. The obtained rate constant obtained, $k_{3,app}$, is in good agreement with the bimolecular rate constant obtained from steady-state measurements (13.4 ± 2.3 µM^{-1} s^{-1}) and it indicates that the reaction of CiP compound II is rate-limiting.

So that the reactivities of HRP and CiP and their abilities to oxidize p-phenylenediamines at various pH could be compared, kinetic measurements were carried out under alkaline conditions. Also, at pH 10 radical cations of p-phenylenediamines are generated by the peroxidases. The initial rates of substrate oxidation show saturation character and the calculated kinetic parameters are listed in Table 3. In the HRP-catalyzed process changing the pH from 7 to 10 caused a small decrease in K_m and a large decrease in the apparent catalytic constants and resulted in a decrease in the bimolecular constants (k_{cat}/K_m) to 1/10 or lower. In the CiP-catalyzed
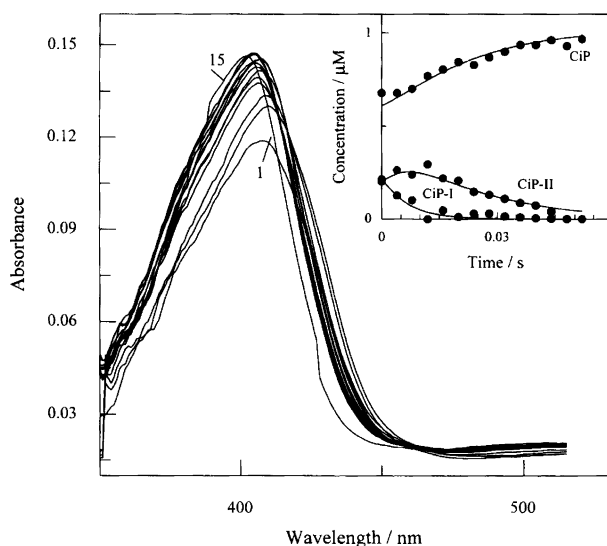
Table 3. Steady-state kinetic parameters of oxidation of reducing substrates by HRP and CiP in 0.05 M sodium carbonate buffer, pH 10.0 at 25 °C.

Enzyme	Substrate	K_m/µM	k_{cat}/s^{-1}	k_{ox}/µM^{-1} s^{-1}
HRP	TMPD	8(1)	10(1)	1.2(3)
CiP	TMPD	91(10)	570(28)	6(1)
HRP	MEMB	9(1)	9(1)	1.0(2)
CiP	MEMB	83(6)	348(10)	4.2(4)
HRP	PMB	17(2)	5.4(5)	0.32(7)
CiP	PMB	114(5)	526(15)	4.6(3)
HRP	AMB	5.6(9)	8.0(5)	1.4(3)
CiP	AMB	205(10)	570(20)	2.8(2)
HRP	DMB	11(1)	3.6(1)	0.32(5)
CiP	DMB	133(15)	85(5)	0.64(10)
HRP	DPB	19(2)	6.2(2)	0.34(5)
CiP	DPB	271(20)	725(36)	2.7(3)

reaction the oxidation constants of the p-phenylenediamines did not change very much with pH.

Kinetic experiments performed at pH 7 and 10 indicated different behavior for HRP and CiP in the oxidation of p-phenylenediamines. To establish the influence of pH on HRP and CiP activity, we studied the oxidation of AMB in more detail at different pH values. The experimental data were approximated according to a mechanism that involved a single proton dissociation in the active center. In Fig. 3 the fitted curves revealed an ionizable group with pK_a of 7.35 in the HRP reaction and 9.70 in the CiP reaction, respectively. The reaction rate in the HRP-catalyzed oxidation drops very quickly at pH higher than 7. However, the CiP-catalyzed oxidation proceeds nearly unchanged from pH 6 to around pH 8.5 thus having a rather broad pH optimum. The different pK_a values for the two peroxidases indicate that

Fig. 2. Rapid scanning spectra showing the changes in the CiP spectral bands during AMB oxidation by H_2O_2 and peroxidase at pH 7.0 and 25 °C. Scans were recorded every 4 ms. The inset represents the change in concentration of CiP and compounds I and II at the isobestic points: 394, 414 and 426 nm. Experimental data were fitted according to consecutive processes [eqns. (2)–(4)] using rate constants 150 s^{-1} and 48 s^{-1} (solid lines). Conditions after mixing were as follows: CiP, 1 µM; H_2O_2, 1 µM; AMB, 5 µM; phosphate buffer, 20 mM; pH 7.0.
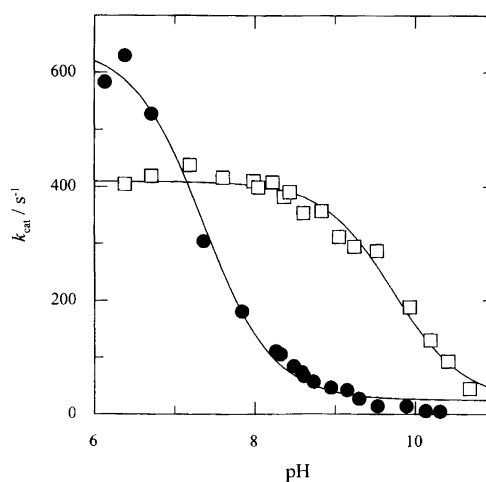

Fig. 3. pH-dependence of the initial rate of peroxidase-catalyzed oxidation of AMB in 33 mM disodium phosphate, 33 mM boric acid and 33 mM sodium carbonate buffer at 25 °C. The squares represent experimental data of the CiP reaction and filled circles the HRP catalysis. Solid curves were calculated using a HRP acid ionization pK_a of 7.35 and CiP of 9.7. Reaction mixtures contained 100 µM AMB, 4 nM HRP, 2 nM CiP, 50 µM H_2O_2.

the activity of HRP and CiP in alkaline solution is determined by deprotonation of different ionizable groups.

As in peroxidase catalysis the same steady-state kinetics methods were applied for the evaluation of the kinetic parameters in laccase-catalyzed *p*-phenylenediamine oxidations. The kinetic measurements were performed at pH 5.3 where PpL showed maximal activity (Fig. 4). The activity change at pH 4–5.3 corresponds to single proton transfer with a pK_a of 5.52, and the decrease in activity at pH 5.3–6.6 corresponds to a single proton transfer with a pK_a 5.34. The decrease in PpL activity at pH < 5.3 can be related to substrate protonation as the determined pK_a value of AMB (5.7) is close to pK_a of the decrease in laccase-catalyzed activity during AMB oxidation. However, the activity decrease at pH > 5.5 is probably related to enzyme deprotonation. Evidently protonation of AMB under pH 5.5 blocks its oxidation, whereas deprotonation of enzyme over pH 5.5 reduces its activity. Therefore, a very narrow region of maximal activity for PpL is observed.

In air-saturated buffer solution the dependence of the initial oxidation rate on the substrate concentration was hyperbolic in shape and the kinetic parameters were calculated according to the Michaelis–Menten equation [eqn. (1)]. The results presented in Table 4 show that the apparent Michaelis–Menten constant of substrates varies from 137 to 869 μM and the catalytic constant (k_{cat}) varies from 35 to 270 s^{-1}. Calculated bimolecular rate constants (0.08–1.74 μM^{-1} s^{-1}) are considerably lower than in peroxidase catalysis. As for the peroxidase reactions the highest oxidation rates were observed with AMB and TMPD and lowest with DMB.

The calculated bimolecular rate constants for all of the *p*-phenylenediamine derivatives examined differ by a
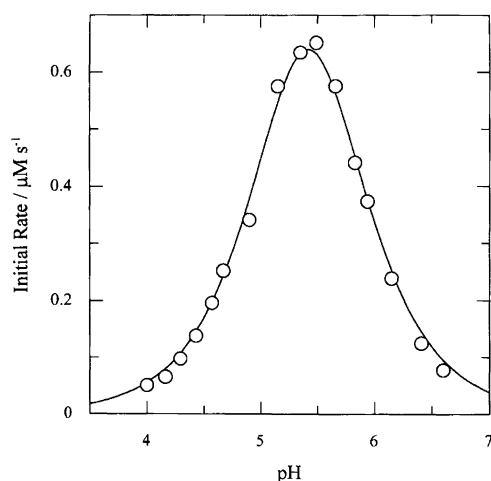
Table 4. Kinetic parameters of PpL-catalyzed oxidation of *p*-phenylenediamines in 0.05 M sodium acetate buffer pH 5.3 at 25 °C.

Substrate	K_m/μM	k_{cat}/s^{-1}	k_{ox}/μM^{-1} s^{-1}
TMPD	155(9)	270(8)	1.74(15)
MEMB	254(11)	190(5)	0.74(5)
PMB	223(37)	132(14)	0.6(1)
AMB	137(17)	218(16)	1.6(3)
DPB	869(275)	70(16)	0.080(4)
DMB	432(82)	35(4)	0.080(3)

factor of ten. These differences can be attributed to the charge of the substrate, redox potential or geometric effects. To clarify which factor determines the reactivity of *p*-phenylenediamines, the logarithm of the apparent bimolecular rate constant was correlated with the redox potential of the substrates. A similar approach was used very recently for the correlation of HRP-I activity with a series of indoleacetic acids.[21] The oxidation of phenols, anilines and benzenethiols by fungal laccases also correlates well with their redox potentials.[22] When the redox rate constants of *p*-phenylenediamines are compared with their redox potentials a decrease in value of the rate constants concomitant with an increase in the redox potential of the mediator was observed at pH 7.0 (Fig. 5). The dependence of log k_{ox} on redox potential for both peroxidases is similar. For the quantitative description of the dependence of k_{ox} on redox potential, the following expression of 'outer sphere' electron transfer rate was used,[23]

$$\log k_{ox} = \log(k_o) - (\lambda - \Delta E_m)^2/4\lambda T k_B \qquad (5)$$

where k_{ox} is experimentally determined and k_o the calculated limiting rate constants, ΔE_m is the difference in redox potential for the reactants or a free energy of

Fig. 4. pH–rate profile for the reaction of PpL with AMB in 50 mM sodium acetate (pH 3.6–5.8) and 50 mM sodium phosphate buffer (pH 5.8–7.0) at 25 °C. The circles represent experimental data in the presence of 100 μM AMB and 6 nM enzyme. The solid curve was calculated using an enzyme acid dissociation pK_a of 5.34 and substrate pK_a of 5.52.
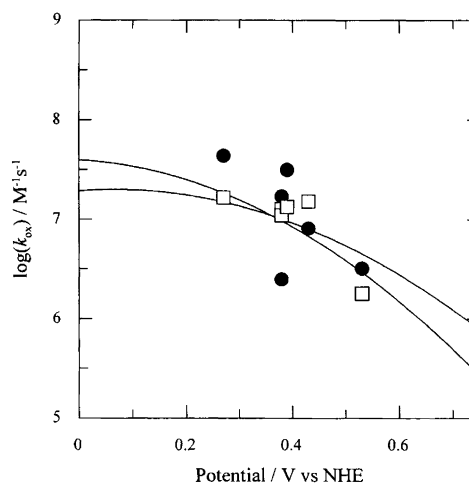

Fig. 5. The dependence of the rate of HRP- and CiP-catalyzed oxidation of *p*-phenylenediamines on their redox potentials at pH 7.0. Filled circles represent experimentally determined bimolecular constants for HRP and squares the same for CiP. Solid curves were plotted according to eqn. (5).
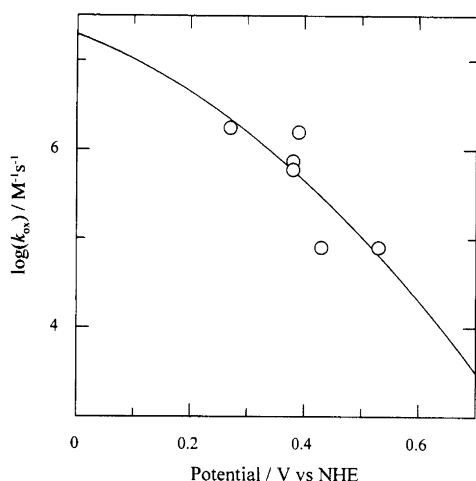
Fig. 6. The dependence of rate of PpL-catalyzed oxidation of p-phenylenediamines on their redox potentials at pH 5.3. Empty circles represent experimentally determined bimolecular constants. The solid curve was plotted according to eqn. (5) and using $k_0 = 2.5 \times 10^7\,M^{-1}\,s^{-1}$ and $\lambda = 1.04$ eV.

reaction expressed in eV, λ is the solvent reorganization energy and k_B the Boltzmann constant.

For an approximation of the experimental data for a peroxidase-catalyzed p-phenylenediamine oxidation according to the 'outer sphere' electron transfer model,[23] an oxidation–reduction potential of 0.900 V was used for HRP[24] and 0.982 V for CiP,[17] the same as for the peroxidase from *Arthromyces ramosus* at pH 7.0. When these values were used in eqn. (5) the following parameters $k_0 = 4.0 \times 10^7\,M^{-1}\,s^{-1}$ and $\lambda = 0.85$ eV for HRP and $k_0 = -2 \times 10^7\,M^{-1}\,s^{-1}$ and $\lambda = 0.75$ eV for CiP were calculated. The estimated maximal rate is close to the highest value of HRP- and CiP-catalyzed p-phenylenediamine oxidation (Table 2). These values are larger than those determined for glucose oxidase–phenylenediamine–quinones complexes.[3]

At pH 10 the approximated redox potential of HRP and CiP was 0.647 V and 0.789 V, respectively.[17,24] Therefore it is likely that the decrease in the rate of p-phenylenediamines oxidation at pH 10 is related to a reduction in the driving force (free energy) of the reaction following the decrease in redox potential of the enzymes.

In the *Polyporus pinsitus* laccase-catalyzed oxidation of p-phenylenediamines the bimolecular rate constant varied by a factor of 20 when considering the entire range of p-phenylenediamines. A correlation between substrate redox potential and enzyme activity was found at pH 5.3 (Fig. 6). When an oxidation–reduction potential[25] of 0.79 V for PpL was used at pH 5.3, the determined electron transfer parameters were $k_0 = 2.5 \times 10^7\,M^{-1}\,s^{-1}$ and $\lambda = 1.04$ eV.

In summary, this work demonstrates the applicability of p-phenylenediamine derivatives as reducing substrates for heme-containing peroxidases and fungal laccases. Changes of the substituents influenced the compounds'

redox properties, which in turn affected their kinetic parameters. The rate constants for *Polyporus pinsitus* laccase are smaller than those of horseradish peroxidase and fungal *Coprinus cinereus* peroxidase.

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