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Application of Marcus theory of electron transfer for the reactions between HRP compound I and II and 2,4-disubstituted phenols

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Abstract

Reactions between horseradish peroxidase (HRP) compound I and II and some natural phenolic antioxidants were studied at pH 7. The bimolecular rate constants for these reactions were determined using a sequential mixing stopped-flow spectrometer. The rate constants for the reactions of compound I were found to be two orders of magnitude higher than those for compound II. The phenols under study showed a significant difference in their one-electron reduction potential values. As the rate constants also changed systematically with their one-electron potentials, the Marcus theory of electron transfer was applied to the above determined rate constants and the thermodynamic driving force (ΔG°), from which the reorganization energy (λ) for the electron transfer from phenols to both compound I and II was estimated.

Keywords: Horseradish peroxidase; Marcus theory; Electron transfer reactions; Phenols

1. Introduction:

Horseradish peroxidase (HRP) is a plant peroxidase obtained from the roots of horseradish [1–4]. It is a heme enzyme in which the protein is combined with an iron(III) porphyrin group. It catalyses redox reactions involving a variety of organic and inorganic substrates. The redox catalysis uses oxyferrylheme [Fe(IV)=O] formed by the reaction of HRP with hydrogen peroxide and hydroperoxides with two distinct intermediates, compound I (C-I) and compound II (C-II). In C-I, the Fe(III) in native HRP is oxidized to Fe(IV) and the porphyrin is oxidized to its radical cation. The structure, activity and the reactions of C-I

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have been the subject of recent interest [5,6]. It can undergo electron transfer reaction with substrates like phenols and amines in two successive electron transfer steps to finally regenerate the native enzyme [6–10]. Initially, C-I reacts with a substrate by a single electron transfer to give C-II, in which the porphyrin group is reduced; in the second step, C-II undergoes one-electron transfer to reduce the oxyferryl group to ferric heme.

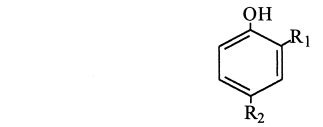
In the present study we employed some phenolic antioxidants as substrates for reaction with C-I and C-II. These phenols (SOH) are natural products and exhibit varying efficacy of antioxidant activity [11–16]. They are electron-rich and are known to undergo one-electron transfer with a number of oxidizing radicals to produce phenoxyl radicals ($SO^{\cdot}$). It has been proposed earlier that these phenols also react with C-I and C-II by electron transfer as given below:

$$HRP + H_2O_2 \rightarrow C\text{-}I + H_2O \quad (1)$$

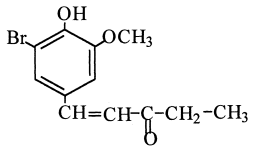
$$C\text{-}I + SOH \rightarrow C\text{-}II + SO^{\cdot} \quad (2)$$

$$C\text{-}II + SOH \rightarrow E + SO^{\cdot} + H_2O \quad (3)$$

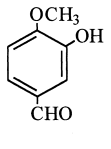
The bimolecular rate constants for these reactions between phenols and C-I and C-II were determined using a stopped-flow technique, by following the formation and decay of C-II as a function of varying phenol concentration. The rate constants thus obtained were compared with their one-electron reduction potentials. The one-electron reduction potential of the phenol, a measure of the energy required to form phenoxyl radical from the phenol, was determined using a pulse radiolysis technique and cyclic voltammetry [11–16]. The value for the reduction potential of the phenol has been found to be essential in assessing the antioxidant activity and ability to react with several oxidants. The difference in the one-electron potential of the two couples involved in the electron transfer reaction is related to the thermodynamic driving force for the reaction. If the electron transfer process is the rate-determining step, the rates of these reactions between C-I and C-II and the phenols should systematically vary with the one-electron reduction potential values of the phenol. To test this hypothesis, the Marcus theory of electron transfer was applied to the above obtained data of electron transfer rate constants, and the thermodynamic driving force and total reorganization energy in the formation of an enzyme–substrate complex prior to electron transfer for C-I and C-II were estimated. The structures of different phenols used in these studies are given below.



Caffeic acid ; $R_1 = OH$, $R_2 = CH{=}CH{-}COOH$
Ferulic acid ; $R_1 = OCH_3$, $R_2 = CH{=}CH{-}COOH$
Eugenol ; $R_1 = OCH_3$, $R_2 = CH_2{-}CH{=}CH_2$
Isoeugenol ; $R_1 = OCH_3$, $R_2 = CH{=}CH{-}CH_3$
Vanillic acid; $R_1 = OCH_3$, $R_2 = COOH$
Vanilline; $R_1 = OCH_3$, $R_2 = CHO$



Bromopentenone



o-Vanilline

2. Experimental

The phenols: ferulic acid; caffeic acid; vanilline; *o*-vanilline; vanillic acid; and eugenol were purchased from Sigma and were of highest purity available. Isoeugenol was from Industrial Perfumes Ltd., Mumbai, and bromopentenone was synthesized and purified as described in [17]. Horseradish peroxidase (HRP) was from Sigma and was used as received. It has a R_z value (A_{403}/A_{280}) of 3.1. Its concentration was calculated by following its absorption at 403 nm ($\varepsilon_{403} = 1.0 \times 10^5\ M^{-1}\ cm^{-1}$). Solutions were prepared in nanopure water from a Millipore system. The concentration of H_2O_2 was determined by an

iodide method as described in [18], where the absorbance of liberated I_3^- was measured at 350 nm using $\varepsilon_{350} = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$. The pH of the solutions was maintained at 7 using 10 mM phosphate buffer. Ionic strength was adjusted using an inert salt 0.1 M KNO_3. All the experiments were carried out at a temperature of 25.0 ± 0.5°C.

Stopped-flow experiments were performed on an Applied Photophysics stopped-flow spectrometer, model SX-18 MV, in sequential mixing mode, where stoichiometric amounts of HRP and H_2O_2 were initially mixed in the ageing loop to produce C-I, which reacted with the phenol substrate after a time delay of ~ 200 ms. The reactions were monitored by the absorption detection method, with an optical path length of 10 mm. The instrument is equipped with a stabilized, ozone-free 150 W xenon lamp with a monochromator and photomultiplier assembly.

The apparent rate constants for the total reaction of phenols with C-I and C-II were studied respectively by following the formation and decay of the absorption due to C-II at 426 nm, the isosbestic point of C-I and the unaltered HRP. The observed rates (k_{obs}) for these individual reactions were determined by non-linear least square fitting of the absorbance vs. time data for formation and decay to a single exponential fit. All the experiments were repeated twice, and at least 3–4 different kinetic traces were analyzed to obtain consistent data at each substrate concentration.

One-electron potentials of the phenoxyl radicals of ferulic acid, caffeic acid, eugenol, isoeugenol, and bromopentenone were determined by pulse radiolysis after reversible electron transfer and redox equilibration between the phenoxyl radical–phenol couple and a standard couple at pH 7. The details are published in [13,15,16]. Briefly, redox equilibrium was established between the two redox couples in a few μs, and by measuring the equilibrium concentrations of the radicals, the equilibrium constant was determined. Using these equilibrium constants in Nernst's equation, the reduction potential of the unknown phenoxyl radical–phenol couple was estimated. For these studies the $N_3^{\bullet}/N_3^-$ couple having a potential of 1.33 V vs. NHE, or phenothiozine couples, like the promethazine di-cation and cation couple ($E^7_{(PMZ^{2+}/PMZ^+)} = 0.90$ V vs. NHE) or the chlorpromazine di-cation and cation couple ($E^7_{(CPZ^{2+}/CPZ^+)} = 0.78$ V vs. NHE) were used as a standard [19].

The one-electron potentials of vanilline, *o*-vanilline and vanillic acid were determined by cyclic voltammetry (Ecochemie Autolab, PG-STAT 20 model) using a glassy carbon electrode as the working electrode and Ag/AgCl, KCl (3M), as the reference electrode. Cyclic voltammograms were recorded uniformly at a scan rate of 50 mV s^{-1} by sweeping the potential between 1.2 and 0 V against the reference electrode. In the case of vanillic acid, the voltammograms were reversible. However, no distinct reversibility was observed with the other compounds tested. In all these cases the peak potential of the voltammogram was taken as the redox potential of the corresponding compound, after correcting for the reference electrode. The results in the case of vanilline were also confirmed by pulse radiolysis.

3. Results and discussion

The observed rates of the reactions, as determined either by exponential formation or decay of C-II at 426 nm, were found to depend linearly on the concentration of the phenolic substrate. The slopes of these plots correspond to the apparent bimolecular rate constants k_I and k_{II}, respectively, for the individual reactions of C-I and C-II with the substrates. Table 1 lists these values for all the above-mentioned phenols. From Table 1 it can be seen that the rate constant for the reaction of C-I with highly electron-rich phenols having a very low oxidation potential (e.g. caffeic acid) approached the diffusion limit of $\sim 2 \times 10^8$ as suggested by Dunford et al. [10]. However, the rate constants for C-II were found to be much slower, by almost two orders of magnitude.

The overall reaction between C-I, C-II and the phenol is not a single-step process, but involves multiple reactions with the formation of a reversible enzyme–substrate complex followed by the electron transfer. The sequence of reactions

Table 1
Rate constants for the reaction of HRP compound I and II with phenols and the reduction potentials of the respective radicals at pH 7 and 25°C

Phenol	E^7 vs. NHE (mV)	k (C-I) ($M^{-1}\ s^{-1}$)	k (C-II) ($M^{-1}\ s^{-1}$)
Caffeic acid	540 [14]	$2.2 \pm 0.2 \times 10^8$	$4.6 \pm 0.3 \times 10^6$
Ferulic acid	689 [11]	$1.0 \pm 0.1 \times 10^8$	$3.4 \pm 0.1 \times 10^6$
Isoeugenol	657 [12]	$1.2 \pm 0.1 \times 10^8$	$1.6 \pm 0.1 \times 10^6$
Eugenol	751 [12]	$2.5 \pm 0.1 \times 10^7$	$6.4 \pm 0.3 \times 10^5$
Bromopentenone	851 [13]	$4.2 \pm 0.4 \times 10^6$	$2.3 \pm 0.1 \times 10^5$
Vanilline	942[a]	$2.2 \pm 0.1 \times 10^7$	$1.6 \pm 0.4 \times 10^6$
Vanillic acid	952[a]	$5.6 \pm 0.1 \times 10^5$	$2.5 \pm 0.3 \times 10^4$
o-Vanilline	821[a]	$3.4 \pm 0.1 \times 10^5$	$2.7 \pm 0.2 \times 10^3$

[a] Cyclic voltammetry.

between C-I and SOH can be represented by the following equation:

$$\text{C-I} + \text{SOH} \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} (\text{C-I} \cdots \text{SOH}) \underset{k_{-et}}{\overset{k_{et}}{\rightleftharpoons}} \begin{matrix} (\text{C-I}^- \cdots \text{SOH}^+) \\ \downarrow k_p \\ \text{C-II} + \text{SO}^{\cdot} \end{matrix} \quad (4)$$

Similarly, reaction of a phenol with C-II also involves an enzyme–substrate complex formation followed by an electron transfer reaction. In the case of phenols, immediately after electron transfer, the oxidized phenol loses a proton quickly to give its corresponding phenoxyl radical, as the phenoxyl radicals have a pK_a of < 2. The distal histidine in C-I subsequently gets protonated without any net release of proton. As reverse electron transfer (k_{-et}) is an improbable process from an energy point of view, it is therefore much slower than the rate of proton loss (k_p). Hence, we assumed that the apparent bimolecular rate constants k_I and k_{II} for C-I and C-II are the result of the overall reaction involving a phenol and either C-I or C-II, respectively. They can be expressed by applying steady state treatment to the above reaction in terms of k_d and k_{et} as in [20,21]:

$$k_I = \frac{k_d^I}{1 + (k_d^I / K^I k_{et}^I)} \quad (5)$$

$$k_{II} = \frac{k_d^{II}}{1 + (k_d^{II} / K^{II} k_{et}^{II})} \quad (6)$$

where k_d^I and k_d^{II} are the diffusion controlled rate constants for the formation of the enzyme–substrate complex. K^I and K^{II} are the diffusional equilibrium constant and defined as ($K = k_d/k_{-d}$). k_{et}^I and k_{et}^{II} are the true electron transfer rate constants.

Furthermore, we argued that if the electron transfer step is rate determining, the above determined rate constants should be related to the one-electron potentials of the individual couples involved, similar to any electron transfer reaction taking place in the normal Marcus region. Table 1 endorses this fact, provoking us to apply the Marcus theory of electron transfer to these reactions [20–22]. According to this theory, the rate of electron transfer depends on the thermodynamic driving force (ΔG°), which in turn is related to the potential difference (ΔE) between the two couples involved as discussed below.

According to classical transition-state theory the electron transfer rate constant (k_{et}) is given as [20]:

$$k_{et} = \nu \exp\left(\frac{-\Delta G^*}{RT}\right) \quad (7)$$

In Eq. (7), ν is the frequency factor and ΔG^* is the free energy of activation for the electron transfer process. R is the gas constant and T is

the absolute temperature. After substituting this in Eqs. (5) and (6) we get:

$$k_{\mathrm{I}} = \frac{k_{\mathrm{d}}^{\mathrm{I}}}{1 + (k_{\mathrm{d}}^{\mathrm{I}}/\nu K^{\mathrm{I}})\exp\left(\frac{\Delta G_{\mathrm{I}}^{*}}{RT}\right)} \quad (8)$$

$$k_{\mathrm{II}} = \frac{k_{\mathrm{d}}^{\mathrm{II}}}{1 + (k_{\mathrm{d}}^{\mathrm{II}}/\nu K^{\mathrm{II}})\exp\left(\frac{\Delta G_{\mathrm{II}}^{*}}{RT}\right)} \quad (9)$$

The free energy of activation ΔG^* and driving force ΔG° are related by quadratic equation, derived by Marcus as [20]:

$$\Delta G^{*} = \frac{(\Delta G^{\mathrm{o}} + \lambda)^2}{4\lambda} \quad (10)$$

where ΔG° is the free energy change for the electron transfer reaction and λ is the total reorganization energy, composed of intramolecular and solvent reorganization energy, related to the change in Gibbs energy required for the reactant state to distort to the configuration of the product state prior to electron transfer.

ΔG° for the reaction of C-I and C-II with phenols can be written as:

$$\Delta G_{\mathrm{I}}^{\mathrm{o}} = E_{(\mathrm{SOH/SO^{\cdot},H^{+}})} - E_{(\mathrm{C\text{-}I/C\text{-}II})} \quad (11)$$

$$\Delta G_{\mathrm{II}}^{\mathrm{o}} = E_{(\mathrm{SOH/SO^{\cdot},H^{+}})} - E_{(\mathrm{C\text{-}II/HRP})} \quad (12)$$

Here ΔG° is in eV, and E is the redox potential in volts (V) for the donor and acceptor, with respect to the same standard.

$E_{(\mathrm{C\text{-}I/C\text{-}II})}$ and $E_{(\mathrm{C\text{-}II/HRP})}$ correspond to the mid-point potentials at pH 7 and have been evaluated by polarography as 0.879 and 0.903 V vs. NHE, respectively [23,24]. The one-electron reduction potentials of the phenols [$E_{(\mathrm{SO^{\cdot},H^{+}/SOH})}$] determined by pulse radiolysis and cyclic voltammetry are listed in Table 1. Using these two values, ΔG° for all the individual reactions were calculated.

The rate constants (k_{I}, k_{II}) for the reaction of C-I and C-II with the phenols were plotted against

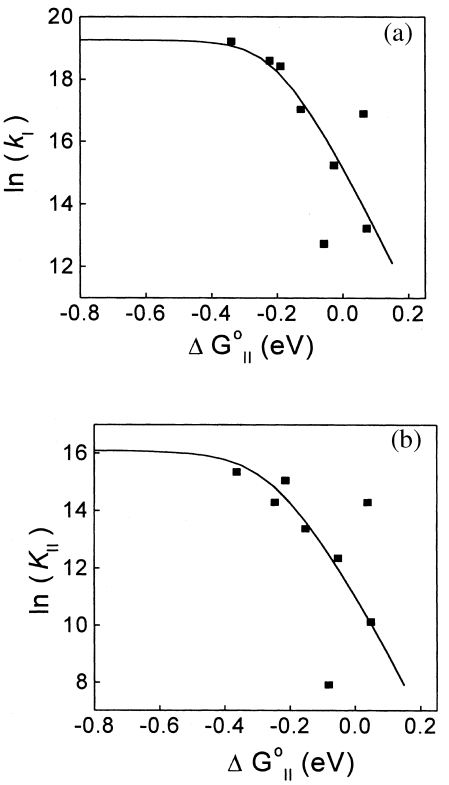


Fig. 1. Variation of apparent bimolecular rate constants for reaction of C-I (a) and C-II (b) with phenols on the thermodynamic driving force ΔG°. Symbols show the experimental values and solid lines indicate the best-fit curve to Eqs. (8)–(10).

the corresponding ΔG° values (Fig. 1a,b). A number of iterative methods have been employed varying the values of k_{d}, νK and λ to get best correlation for the $k_{\mathrm{I}}/k_{\mathrm{II}}$ and ΔG° values. The best correlation thus obtained is presented in Fig. 1a,b for C-I and C-II separately. Here the symbols indicate the experimental values and solid lines the calculated ones according to Eqs. (8)–(10). The values for λ, k_{d} and νK as obtained from the best correlation for C-I and C-II are given in Table 2. The values of λ have been evaluated to be $\sim$1.26 and 0.9 eV for the electron transfer reaction between phenols and C-I and C-II, respectively. The quantity λ describes the energy required for the total nuclear and solvent reorientations taking place prior to the

Table 2
Reorganization energies (λ), pre-exponential factors (νK) and k_d values calculated for the reaction of phenols with compound I and compound II

	λ (eV)	νK ($M^{-1}\ s^{-1}$)	k_d ($M^{-1}\ s^{-1}$)
Compound I	1.26 ± 0.02	$7.0 \pm 0.3 \times 10^{11}$	2.3×10^{8}
Compound II	0.90 ± 0.01	$3.8 \pm 0.3 \times 10^{8}$	0.1×10^{8}

electron transfer. Earlier studies on electron transfer between proteins and protein–substrates showed largely varying reorganization energies of 1.5–0.1 eV [25–28]. Candeias et al. obtained reorganization energies of ~ 0.5 eV for electron transfer between C-I and C-II and some mono-substituted phenols, while Zahida et al. obtained a lower value of 0.2 eV for C-II and similar phenols. An independent study on substituted indoles with C-I showed much smaller values of λ [26]. Our studies using some natural *ortho*-methoxy phenols differing largely in *para* substitution showed an overall reorganization energy that is much higher than these studies. The large values of λ obtained in our case may be due to the fact that the substrates in this study have all got bulky *ortho* substitution (either methoxy or hydroxy group, etc.). This can cause steric hindrance for the formation of enzyme–substrate complex, and therefore a major reorganization could be required before electron transfer takes place.

The diffusional rate constant k_d and νK values are lower by an order of magnitude for C-II as compared to C-I. The k_d value for C-I and phenol matches with the diffusion limited rate constant estimated by Dunford et al. [10] and verified by many others. This indicates the formation of a strong enzyme–substrate complex in the case of C-I, and its formation is probably controlled by the diffusion of C-I and the phenolic substrate. On the other hand, in case of C-II, the k_d value is much less than the diffusion limit. Low values for k_d and νK in the case of the reaction of C-II may also be due either to the formation of a weak enzyme–substrate complex, or due to lower mobility of the iron valence electron [23–26].

Thus, our studies give direct evidence that the Marcus theory of electron transfer can be applied to the reactions of HRP compound-I and II with phenols. This also confirms that the electron transfer reaction is the rate-determining step for these studies.

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