Interpretation of the reactivity of peroxidase compounds I and II with phenols by the Marcus equation

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Abstract The catalytic cycle of heme peroxidases involves two reactive states, compound I and compound II. Although their reduction potentials at pH 7 are similar, compound I is in general more reactive towards organic substrates than compound II. The different reactivities have until now remained unexplained. In this study, the reactions of compounds I and II of peroxidase from horseradish with phenols were analyzed using the Marcus equation of electron-transfer. Both reactions exhibit similar reorganization energies, and the different reactivities of the two enzyme states can be ascribed to a higher apparent rate of activationless electron-transfer in the compound I reactions. This can be attributed to the shorter electron-tunneling distance on electron-transfer to the porphyrin radical cation in compound I, compared to electron-transfer to the iron ion in compound II.

Key words: Heme peroxidase; Electron-transfer; Marcus equation; Phenoxyl radical; Stopped-flow

1. Introduction

Heme peroxidases catalyze the oxidation of organic substrates by hydrogen peroxide or organic peroxides [1]. Their catalytic cycle [2] involves rapid oxygen transfer from a peroxide to the ferric state of the enzyme (E), formally a two-electron oxidation, to yield compound I (cpd I). In this intermediate, the iron is in the ferryl state ($Fe^{IV}=O$) and the second oxidizing equivalent is stored as an organic radical, which can be at an oxidizable amino acid, as in cytochrome *c* peroxidase [3], or as a porphyrin radical cation, as in the peroxidase from horseradish used in this study [4]. Compound I is than reduced back to the ferric enzyme via two consecutive one-electron reductions by the substrate (SH), via compound II (cpd II) as intermediate. In the latter, the iron is still in the ferryl state but the organic radical has been reduced.

$$E + H_2O_2 \rightarrow \text{cpd I} + H_2O \quad (1)$$

$$\text{cpd I} + SH \rightarrow \text{cpd II} + S^{\bullet} \quad (2)$$

$$\text{cpd II} + SH \rightarrow E + S^{\bullet} + H_2O \quad (3)$$

The rates of reduction of cpd I or cpd II by phenols or amines correlate with the oxidizability of the substrate [5,6]. The same is true for the reduction of compound I by indoleacetic acids [7]. Compound I reacts with most substrates several-fold faster than compound II. However, Yamazaki et al. estimated that at pH 7 the reduction potentials of compound I and compound II are very similar, excluding a thermodynamic justification for the higher reactivity of compound I [8].

Recently, we have demonstrated that the application of the Marcus equation of electron-transfer to the reduction of peroxidase intermediates by organic substrates can provide valuable information on the reaction mechanisms [9]. Here, we apply this treatment to new and literature experimental data and show that the lower reactivity of compound II can be explained by the tunneling of the electron from the substrate to the iron ion, across a longer distance than on reduction of compound I, where the porphyrin radical-cation acts as the electron acceptor.

2. Materials and methods

Phenols were purchased from Aldrich or Sigma and were either of >99% purity or were purified by recrystallization. Peroxidase from horseradish (HRP) was from Sigma (type VI-A) and was used as received. The concentration of stocks of HRP was determined from the absorbance at 402 nm assuming $\varepsilon=1.02\times10^5$ M^{-1} cm^{-1} [10]. All solutions were prepared freshly with water purified by a Millipose Milli-Q system.

Stopped-flow measurements used a Hi-Tech SF-61 DX2 sequential-mix instrument equipped with a 100 W Xe lamp and two (detection and reference) photomultipliers (Brandenburg type 4409). The solutions were prepared in 10 mM phosphate buffer at pH 7.0 containing 0.1 M potassium nitrate. The drive syringes and the reaction cell were maintained at 25°C by circulating water.

Sequential mixing was used; HRP was mixed with stoichiometric amounts of hydrogen peroxide in the age-loop and the compound I solution formed this way was mixed with the reducing substrate after 1 s. The final concentration of HRP in the cell was typically ≈0.5 μM and the phenol was in at least 10-fold excess. The formation and decay of compound II were monitored at 426 nm, the isosbestic point between ferric enzyme and compound I. Both processes followed exponential kinetics and the observed rates (k_{obs}) determined by non-linear least-squares fit depended linearly on the concentration of phenol. The rates of reaction of phenols with compound I ($k_{cpd\ I}$) or compound II ($k_{cpd\ II}$) were determined from the slopes of these lines. Up to the fastest rates observed ($k_{obs}\approx500$ s^{-1}) no evidence for saturation kinetics was found.

3. Results and discussion

In Table 1, we have compiled rates of reaction of HRP compounds I and II with phenols. The one-electron oxidation of phenols causes a large increase of acidity: the pK_a values of the phenoxyl radical cations are of the order of −2 [11–13], several units lower than those of the parent phenols (ca. 10). This implies that at pH 7 the oxidation is accompanied by deprotonation to yield phenoxyl radicals. Under the same conditions, the reduction of compound I leads to protonation of the enzyme [14] so that overall, there is a formal transfer of hydrogen from the phenol to the enzyme. However, the small kinetic isotope effect observed on deuteration and the fact that

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Table 1
Rates of reaction of phenols with HRP compound I ($k_{cpd\ I}$) and compound II ($k_{cpd\ II}$), and the mid-point potential (E) of the respective phenoxyl radicals at pH 7 and 25°C

Substituent	E/V	Ref.	k_2/M^{-1} s^{-1}	Ref.	k_3/M^{-1} s^{-1}	Ref.
p-NH_2	0.42	[26]	$(2.3\pm0.4)\times10^8$	[5]	$(2.8\pm0.8)\times10^7$	This study
p-CH_3O	0.72	[26]	$(1.3\pm0.2)\times10^8$	[5]	$(4.3\pm0.9)\times10^6$	[6]
p-CH_3	0.87	[26]	$(4.2\pm0.2)\times10^7$	[5]	$(1.0\pm0.1)\times10^6$	[6]
m-HO	0.91	[17]	$(1.2\pm0.1)\times10^7$	[9]	$(2.8\pm0.3)\times10^5$	This study
p-F	0.93	[26]	$(1.2\pm0.2)\times10^7$	[9]	$(1.4\pm0.3)\times10^5$	This study
p-Cl	0.94	[26]	$(2.4\pm0.4)\times10^7$	[9]	$(1.2\pm0.2)\times10^6$	This study
p-I	0.96	[26]	$(7.2\pm1.5)\times10^7$	[9]	$(5.7\pm0.3)\times10^6$	This study
p-C_6H_5	0.97	[27]	4.0×10^7	[28]	5.0×10^4	[28]
H-	0.97	[26]	$(2.8\pm0.6)\times10^6$	[5]	$(2.9\pm0.1)\times10^5$	[6]
p-CO_2^-	1.04	[26]	$(4.7\pm0.1)\times10^5$	[9]	$(2.1\pm0.1)\times10^4$	This study
m-CHO	1.05	[9]	$(8.3\pm0.6)\times10^4$	[9]	$(5.9\pm0.2)\times10^3$	This study
p-CN	1.17	[26]	$(1.5\pm0.1)\times10^4$	[9]	$(2.1\pm0.2)\times10^2$	This study

the rates of reaction follow a typical Marcus curve, lead to the conclusion that the rate-determining step is the transfer of an electron [9]. On reduction of compound II, the ferryl oxygen combines with two protons to yield a water molecule. However, the following discussion shows that the rate of reduction of compound II by phenols also follows a typical Marcus curve, parallel to that for reduction of compound I, suggesting that both reactions have electron-transfer as the rate-determining step.

The thermodynamic driving force for each of reactions 2 and 3 ($\Delta E_{cpd\ I}$ and $\Delta E_{cpd\ II}$, respectively) is the difference between the mid-point potentials of the enzyme intermediate (compound I or compound II) and the mid-point potential of the phenoxyl radical formed on oxidation of the phenols. Mid-point potentials of phenoxyl radicals $E(S^{\bullet},H^+/SH)$ have been measured by pulse radiolysis [15] and they are compiled in Table 1. The mid-point potentials for reduction of compound I and compound II have been estimated by equilibration with hexachloroiridate [8]. According to these measurements, E(cpd I/cpd II) = 0.879 V and E(cpd II, $2H^+$/E) = 0.903 V at pH 7, relative to the Standard Hydrogen Electrode (SHE).

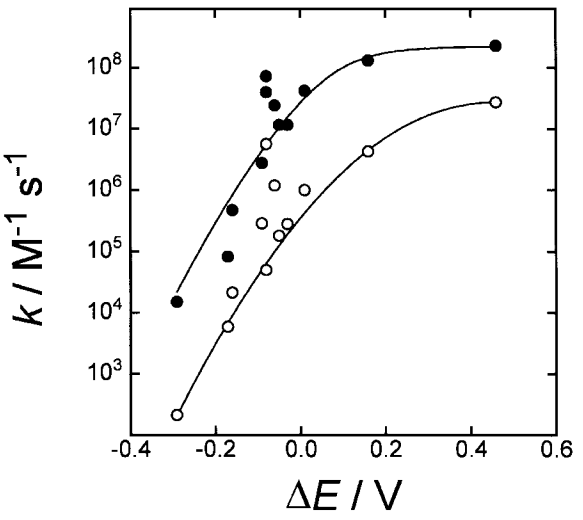

Fig. 1. Rates of reaction of compound I (solid symbols) and compound II (open symbols) with phenols at pH 7.0 and 25°C plotted against the thermodynamic driving force for the reaction. The lines are non-linear least-squares fits to a modified Marcus equation described in the text and with parameters listed in Table 2.

$$\Delta E_{cpd\ I} = E(\text{cpd I/cpd II}) - E(S^{\bullet},H^+/SH) \quad (4)$$

$$\Delta E_{cpd\ II} = E(\text{cpd II, } 2H^+/E) - E(S^{\bullet},H^+/SH) \quad (5)$$

Those values revealed that compound II is a slightly stronger oxidant than compound I, despite its lower reactivity. Yamazaki and colleagues attributed this apparent contradiction to 'a higher mobility of a porphyrin π-electron than an iron valence electron' [16].

In Fig. 1, we have plotted the rates of reduction of compound I and compound II against the thermodynamic driving force calculated according to Eqs. 4 and 5. As expected from numerous previous reports, compound I reacted several-fold faster with each phenol than compound II. Also in agreement with previous results, electron-richer phenols are more reactive because they yield phenoxyl radicals with lower reduction potentials and therefore the thermodynamic driving force for their oxidation (ΔE) is higher. In the endothermic region, the reaction rates plotted on a logarithmic scale varied approximately linearly with (ΔE); however, in the exothermic region a curvature of the plot is noticeable.

In a previous report [9], we have proposed an interpretation of the rate of compound I with reducing substrates based on the Marcus equation of electron-transfer. In this treatment, the rate of reaction is controlled by the dissociation constant K_D of the complex between the enzyme reactive intermediate (cpd I or cpd II) enzyme-substrate complex and the rate of electron-transfer within this complex (k_{ET})[(1)]:

$$k = K_D^{-1}\, k_{ET} \quad (6)$$

where k is the rate of reaction ($k_{cpd\ I}$ or $k_{cpd\ II}$). The rate of electron-transfer depends on the thermodynamic driving force ΔE according to Eqs. 7 and 8:

$$k_{ET} = k_0 \exp(-\Delta G^{\ddagger}/RT) \quad (7)$$

$$\Delta G^{\ddagger} = \lambda(1 - F\,\Delta E/\lambda)^2/4 \quad (8)$$

Here, $\Delta G^{\ddagger}$ is the activation free energy, k_0 is the rate of activationless electron-transfer in the enzyme-substrate complex,

(1) In the previous study we have used the Michaelis-Menten constant K_m instead of K_D. However, this is not strictly correct. We are grateful to Dr. Andy Smith for pointing this out.

Table 2
Reorganization energies (λ) and pre-exponential factors estimated for the oxidation of phenols by compound I or compound II at pH 7 and 25°C

	λ/eV[a]	$(k_0/K_D)/10^9$ M^{-1} s^{-1}
Compound I	0.52 ± 0.33	4.7 ± 1.8
Compound II	0.46 ± 0.05	0.031 ± 0.003

[a]1 eV = 96.5 kJ mol^{-1}.

F and R are the Faraday and gas constants, respectively, and T is the absolute temperature. Eqs. 6 and 8 can be combined to yield a general expression for the rate of reaction of the enzyme intermediates with the a reducing substrate (Eq. 9):

$$\log k = \log(k_0/K_D) - (\lambda/4)(1-\Delta E/\lambda)^2/0.059 \quad (9)$$

with ΔE in V and λ in eV. The rate of reaction of compound I with phenols has been suggested to approach a diffusion limit of $k_{diff} = 2.3 \times 10^8$ M^{-1} s^{-1}; this can be taken into account by calculating a corrected k' (Eq. 10):

$$1/k' = 1/k + 1/k_{diff} \quad (10)$$

Presumably, the diffusion limit k_{diff} is the same for compound I and compound II. The expression for k has only two adjustable parameters: λ and (k_0/K_D). Fig. 1 shows a non-linear least-squares fit of this expression to the rates of reaction of phenols with compound I and compound II, $k_{cpd\ I}$ and $k_{cpd\ II}$. (Note that in order to include the diffusion correction, the fit is done on k rather than log k.) The parameters derived from the fitting are shown in Table 2.

In the endothermic region, the lines fitted to $k_{cpd\ I}$ and $k_{cpd\ II}$ are parallel, which means that the two reactions have similar reorganization energies. The values of λ estimated from the fit are comparable to those for electron-transfer reactions between organic compounds in aqueous solution. For example, the reorganization energy associated with the electron-transfer between phenoxyl radicals and phenylthiols has recently been estimated as λ = 0.6 eV (≈60 kJ mol^{-1}) [17].

The main difference between the curves for $k_{cpd\ I}$ and $k_{cpd\ II}$ is a vertical shift that reflects a pre-exponential factor (k_0/K_D) ca. two orders of magnitude lower in the latter case. This can be due to (a) a larger affinity (lower K_D) of compound I for reducing substrates or (b) a larger k_0 for the reduction of compound I.

The values of K_D for the complexes between phenols and peroxidase intermediates are difficult to determine. These enzymes do not usually show saturation kinetics (which can be taken as indication for large K_D values), although saturation kinetics can sometimes be observed [18]. Some K_D values for complexes between ferric enzyme and some substrates are available from NMR measurements; they span the nanomolar to the millimolar range [19].

The rate of activationless electron-transfer k_0 depends on the electronic coupling between the electron donor (substrate) and acceptor (heme). In proteins, the coupling depends on the distance (d) between donor and acceptor [20], such that k_0 can be predicted to vary with distance according to the approximate relation:

$$k_0 \approx k_0^c \exp(-\beta d) \quad (11)$$

where k_0^c is the activationless rate at contact distance and β is the distance factor [20–24]. The latter was found from the analysis of a series of electron-transfer reactions to be usually of the order of $\beta \approx 1.4$ $Å^{-1}$ [20]. However, it depends on the secondary structure of the protein between donor and acceptor, with values varying in the range 1–2 $Å^{-1}$ [24].

Structurally, the most important difference between reduction of compound I and compound II is that in the former case the electron is transferred to the porphyrin radical cation, whereas in the latter it is the iron that changes the oxidation state. In peroxidase from horseradish, it has been shown that reaction takes place at the heme edge [25]. This implies that for reduction of compound II the electron has to be transferred across the additional distance of the heme radius, which is of the order of ≈3.5 Å. Eq. 11 with $\beta = 1.4$ $Å^{-1}$ predicts that, all other factors constant, the reduction of compound II should be ≈130-fold slower than the reduction of compound I. The extreme values of the distance factor β (1–2 $Å^{-1}$) would make compound II 33- to 1100-fold less reactive than compound I. These figures are consistent with the ratio of the (k_0/K_D) values for reduction of compound I and compound II derived from fitting Marcus curves to the experimental data (152 ± 58). On this basis, we suggest that the lower reactivity of compound II compared to compound I of heme peroxidases may be due to the longer electron-transfer distance in the former case.

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