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Kinetic studies on oxidation of aromatic donor molecules by horseradish peroxidase and lactoperoxidase

Sandeep Modi & Digamber V. Behere†

Centre for Mechanisms of Human Toxicity, University of Leicester, UK and †Chemical Physics Group TIFR, Bombay, India

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Based on kinetic evidence, it has been shown for the first time that the mode of binding of aromatic donor molecules is similar in horseradish peroxidase and lactoperoxidase; also that the nature of the heme plays an important role in the reaction with hydrogen peroxide, and has no effect on the reaction of the intermediate compound II with aromatic substrates.

Keywords: aromatic donor molecules, horseradish peroxidase, kinetics, lactoperoxidase

Introduction

Horseradish peroxidase (HRP) and lactoperoxidase (LPO) (EC 1.11.1.7, donor, H_2O_2, oxidoreductase) are heme protein enzymes that catalyse primarily the oxidation of a wide variety of organic and inorganic donor molecules by hydrogen peroxide (Dunford & Stillman 1976, Morishima & Ogawa 1979, Frew & Jones 1984, Sakurada *et al*. 1986, Hosoya *et al*. 1989, Modi *et al*. 1989a,b,c, Saxena *et al*. 1990). They are members of different peroxidase superfamilies (Welinder 1979, 1985, Kaput *et al*. 1982). The mechanism of oxidation of substrates is well established; HRP and LPO go through two intermediates (compound I and compound II) during the reaction (Dunford & Stillman 1976, Frew & Jones 1984):

$$\text{HRP/LPO} + H_2O_2 \xrightarrow{k_1} \text{Compound I}$$

$$\text{Compound I} + AH_2 \xrightarrow{k_7} \text{Compound II} + AH^{\cdot}$$

$$\text{Compound II} + AH_2 \xrightarrow{k_4} \text{HRP/LPO} + AH^{\cdot}$$

Despite a large number of studies on HRP, however, little is known about the mechanism of electron transfer from the substrate to compounds I and II, and it is still not clear how electrons are transferred. It has previously been suggested, on the basis of overall oxidation reaction of aniline derivatives, that the electron density on the nitrogen atoms of the anilines is important (Bordeleau & Bartha 1972), but this was questioned in the HOMO energy level calculations (Hosoya *et al*. 1983, Sakurada, *et al*. 1990). The analysis of the interaction of HRP–aromatic donor molecules using computer modelling suggests that the aromatic donors bind to the HRP near heme peripheral 8-CH_3, Tyr-185, His-42 and Arg-183 residues. Involvement of His-42 of HRP in interaction with aromatic donor substrates has been clearly shown by its chemical modification (Modi 1995). Such computer modelling has not so far been done for LPO, and there are no kinetic studies for the oxidation of organic substrates by LPO; consequently, it is not known if the mode of binding in these two peroxidases is similar or not. In this peroxidase catalytic cycle, the role of heme is unclear.

In this study, we have examined the oxidation of phenols with different substituents by HRP and

Address for correspondence: S. Modi, Centre for Mechanisms of Human Toxicity, Hodgkin Building, Lancaster Road, University of Leicester, LE1 9HN, UK. Tel: (+44)116 2525586; Fax: (+44)116 2525616; E-mail: sm32@leicester.ac.uk.

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LPO. We have also replaced protoporphyrin IX of HRP with two other hemes (mesoporphyrin and protoporphyrin dimethyl ester) to study the effects of heme substituents on the rate of oxidation with substrate and H_2O_2. The effect of variation of the heme on the peroxidase catalytic reaction is discussed for the first time. We have also calculated the binding constant for each substrate with different HRPs and LPO. All the results are compared with each other, and are discussed in the light of the electron density of the oxygen atom of substituted phenols.

Experimental

Horseradish peroxidase was purified from crude HRP (Sigma, Dorset, UK; Rz* = A_{403}/A_{280} = 0.8) by DEAE and CM-cellulose column chromatography (Modi *et al*. 1989b). Lactoperoxidase was isolated and purified from fresh unskimmed cow's milk by a procedure described previously (Modi *et al*. 1989a). All other reagents were of analytical grade (Fissons, Loughborough, UK). Preparation of mesoporphyrin–horseradish peroxidase (Fe(III)MP–HRP) and protoporphyrin dimethyl ester horseradish peroxidase (Fe(III)PPDME–HRP) was done by a similar procedure to that described previously (Modi *et al*. 1990, 1994, Saxena *et al*. 1990). Difference optical spectra (enzyme–substrate complex versus enzyme) were obtained on a Beckman DU 650 spectrophotometer at 23°C. Titrations were carried out by addition of 1.5–100 mM of the substrates to the enzyme (10 μM) in 0.1 M phosphate buffer, pH 6.1.

The kinetic studies on LPO and different HRPs were carried out at 23°C in 0.1M phosphate buffer (pH 6.1) on an Applied Photophysics stopped-flow spectrophotometer (SF.17MV). The fresh compound II of LPO and HRPs was generated by addition of the equivalent of H_2O_2 and $K_4Fe(CN)_6$. The reaction was followed by an increase in absorption of the Soret band of LPO (412 nm) and HRP (403 nm). Usually more than 10-fold excess of substrate concentra-tion (10–100 μM) was used over the compound II concentration (1–4 μM). For each substrate, the observed rate (k_{obs}) was recorded for different concentrations of the substrate. From this data, the rate of oxidation of substrate by compound II (k_4) was calculated for each substrate. Similarly the reaction of H_2O_2 with different enzymes (k_1) was calculated by monitoring the decrease in absorbance of the Soret band (412 nm for LPO and 403 nm for HRP) (Modi *et al*. 1991).

Results and discussion

The essential features of the absorption spectra of HRP and LPO remain unaltered (except for a small decrease/increase in Soret band intensity) even after addition of excess aromatic donor molecules (data not shown). This suggests that aromatic donor molecules do not bind to the heme iron of the enzyme, and also that the electronic structure of the heme iron (S = 5/2) remains unchanged on binding of aromatic donor molecules. From optical-difference spectroscopy (Type I (Saxena *et al*. 1990)), the binding constants of various phenols to the enzyme can be calculated, and these are listed in Table I. The trend of the binding constant fits well with the electron density on the oxygen atom of the phenol, and with the order of hydrophobicity (Modi *et al*. 1989c, Saxena *et al*. 1990). It has been suggested earlier that the substrate may form a hydrogen bond with the NH group of distal histidine in both cases (HRP and LPO) (Modi *et al*. 1989c, Saxena *et al*. 1990).

It is interesting to note that in the case of HRP, change of protoporphyrin IX to mesoporphyrin (MP) and protoporphyrin dimethyl ester (PPDME) does not affect the binding of aromatic donor molecules, which is consistent with the earlier observation that aromatic donors bind to HRP at least 7–8 Å away from the heme iron. The K_D values for binding of these substrates to LPO are an order of magnitude higher compared to that of HRP, which suggests that in case of LPO, the binding pocket may be more compact (Modi *et al*. 1989c). This is consistent with earlier distance calculations which show that these substrates bind to HRP and LPO at distances of 7–8 Å and 10–11 Å, respectively, away from the heme iron (Modi *et al*. 1989c, Saxena *et al*. 1990).

The rate constants for the reaction of the enzymes with H_2O_2 (k_1) were calculated as discussed in Experimental. The k_1 value for MP–HRP [(5.1 ± 0.5) $\times 10^7$ M^{-1} s^{-1}] is about three times greater than that for the native HRP [(1.8 ± 0.2) $\times 10^{-1}$ M^{-1} s^{-1}], which suggests that for the formation of compound I, basicity of the heme plays a crucial role. By changing the vinyl group of the protoporphyrin IX of native HRP to an ethyl group (MP), the electron density on the heme iron is increased, so the reaction rate

* Purity of hemeprotein is commonly expressed by purity number, called Reinheitzahl number.

Table 1. Apparent dissociation constants (K_D, mM) and k_4 values ($M^{-1}\,s^{-1}$) for the binding of substrates to the enzyme in 0.1 M phosphate buffer (pH 6.1) at 23°C

Substrates	Enzyme	K_D (mM)	k_4 ($M^{-1}\,s^{-1}$)
Phenol	Fe(III)HRP	8.2 ± 0.7	$(3.3 \pm 0.2) \times 10^5$
	Fe(III)MP–HRP	8.8 ± 0.9	$(3.2 \pm 0.1) \times 10^5$
	Fe(III)PPDME–HRP	7.4 ± 0.7	$(2.8 \pm 0.2) \times 10^5$
	Fe(III)LPO	95 ± 12	$(3.1 \pm 0.3) \times 10^5$
p-Cresol	Fe(III)HRP	2.4 ± 0.2	$(1.2 \pm 0.1) \times 10^6$
	Fe(III)MP–HRP	2.3 ± 0.3	$(1.4 \pm 0.2) \times 10^6$
	Fe(III)PPDME–HRP	2.6 ± 0.3	$(0.9 \pm 0.1) \times 10^6$
	Fe(III)LPO	60 ± 10	$(1.0 \pm 0.2) \times 10^6$
o-Cresol	Fe(III)HRP	4.5 ± 0.5	$(8.3 \pm 0.2) \times 10^4$
	Fe(III)MP–HRP	4.9 ± 0.6	$(8.2 \pm 0.2) \times 10^4$
	Fe(IIII)PPDME–HRP	4.2 ± 0.6	$(7.6 \pm 0.5) \times 10^4$
	Fe(III)LPO	80 ± 8	$(8.0 \pm 0.6) \times 10^4$
m-Cresol	Fe(III)HRP	5.1 ± 0.7	$(4.1 \pm 0.4) \times 10^5$
	Fe(III)MP–HRP	5.8 ± 0.9	$(4.3 \pm 0.2) \times 10^5$
	Fe(III)PPDME–HRP	5.4 ± 0.7	$(3.7 \pm 0.4) \times 10^5$
	Fe(III)LPO	87 ± 9	$(3.8 \pm 0.4) \times 10^5$
p-Chloro-phenol	Fe(III)HRP	11 ± 1	$(1.2 \pm 0.1) \times 10^6$
	Fe(III)MP–HRP	10.6 ± 0.9	$(1.4 \pm 0.2) \times 10^6$
	Fe(III)PPDME–HRP	10 ± 1	$(1.0 \pm 0.1) \times 10^6$
	Fe(III)LPO	120 ± 16	$(1.0 \pm 0.2) \times 10^6$
Resorcinol	Fe(III)HRP	5.0 ± 0.6	$(3.5 \pm 0.4) \times 10^5$
	Fe(III)MP–HRP	5.6 ± 0.7	$(3.2 \pm 0.3) \times 10^5$
	Fe(III)PPDME–HRP	5.3 ± 0.5	$(3.1 \pm 0.4) \times 10^5$
	Fe(III)LPO	80 ± 10	$(2.9 \pm 0.4) \times 10^5$

is higher in the case of MP–HRP. For PPDME–HRP, the reaction rate [$(2.1 \pm 0.3) \times 10^7$ $M^{-1}\,s^{-1}$] is very similar to that of the native HRP. This contrasts with the earlier suggestion that the propionic acid group of heme in HRP plays an important role in the reaction with H_2O_2 (Ugarova *et al*. 1981). LPO cannot be reconstituted with different hemes, as the heme is covalently bound to the protein (Nichol *et al*. 1987), but the k_1 value [$(1.2 \pm 0.2) \times 10^7$ $M^{-1}\,s^{-1}$] was similar to that of native HRP.

Table I lists k_4 values of substituted phenols determined as described in Experimental. It is interesting to see that no correlation between k_4 values and the net charge on the oxygen atom or carbon atom of substituted phenols was observed. This contrasts with the earlier suggestion, based on the net rate of oxidation of aromatic substrates, that the rate of oxidation depends on the electron density of the oxygen atom of substituted phenols (Bordeleau & Bartha 1972). Our results support the HOMO calculations and the hypothesis that the rate of oxidation by compound II depends on the ease with which a π electron is released from the substrate (Hosoya *et al*. 1983, Sakurada *et al*. 1990). It is also interesting to note that the same trend of binding constant (K_D) and oxidation by compound II (k_4) is observed with different substrates in both cases (LPO and HRP), which suggests that LPO and HRP may have a similar mode of binding with these substrates. It is again interesting to note that k_4 is almost unchanged for MP–HRP, PPDME–HRP and LPO. This suggests that heme plays no role in determining the rate of oxidation of aromatic substrates by compound II, and also supports the earlier observation that the mode of binding of these substrates in LPO and HRP is similar. The net rate of oxidation of these substrates by LPO and HRP is different because of differences in binding, which suggests that the initial docking of substrate by the enzyme is very important in its oxidation. Recently, by superimposing crystal structures for lignin peroxidase and cytochrome c peroxidase, it has been

shown that the overall structures are same despite the low level of sequence identity (15%) (Li & Poulous 1994). The structure of the mammalian peroxidase has been reported to be very different from the plant peroxidase family (Zeng & Fenna 1992), therefore it is surprising that the k_4 values for LPO and HRP are very similar. In the case of most peroxidases, electron transfer reactions occur at the heme edge, as substrates have restricted access to the peroxide binding pocket.

These results clearly show that the peroxidases are extremely accommodating with respect to a range of substrates. The ability of a molecule to serve as an electron donor substrate appears to be dependent mainly on its redox potential, and on the electronic properties of the substrates.

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