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# Interaction of aromatic donor molecules with horseradish peroxidase: identification of the binding site and role of heme iron in the binding and activity

Sandeep Modi

*Biological NMR Centre, Medical Science Building, University of Leicester, Leicester, UK*

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**The interaction of aromatic substrates with horseradish peroxidase (HRP) was studied. Chemical modification of HRP was performed using diethylpyrocarbonate (DEPC) and for the first time the amino acid involved in binding with these substrates has been identified. The kinetic parameters for this interaction have been calculated and the role of heme iron in the oxidation of aromatic substrates by HRP has been discussed.**

**Keywords:** aromatic donor molecules, chemical modification, horseradish peroxidase

## Introduction

Horseradish peroxidase (HRP, EC 1.11.1.7, donor, $H_2O_2$, oxidoreductase) is a plant heme protein enzyme that catalyzes primarily the oxidation of a wide variety of oxidizable organic and inorganic donor molecules by $H_2O_2$ (Dunford & Stillman 1976, Frew & Jones 1984, Modi *et al.* 1989a–c). The mechanism of this oxidation reaction implies the binding of both the oxidizable substrate and $H_2O_2$ to the enzyme. Therefore, interaction of oxidizable substrates with HRP has been studied using techniques such as optical difference spectroscopy (Schonbaum 1973, Paul & Ohlsson 1978, Hosoya *et al.* 1989, Modi *et al.* 1989a, Saxena *et al.* 1990, Casella *et al.* 1993), $^1$H-NMR (Morishima & Ogawa 1979, Sakurada *et al.* 1986) and kinetics measurements (Critchlow & Dunford 1972a, b) to obtain information about the relative disposition of the substrate in HRP, the nature of the ionizable group in the heme crevice and the distances of the substrate nuclei relative to the Fe(III) center. Kinetic (Critchlow & Dunford 1972a, b) and NMR (Morishima & Ogawa 1979, Sakurada *et al.* 1986) studies have suggested that aromatic substrate forms a 1:1 complex with HRP and bind near the heme group. The apparent dissociation constant ($K_D$) has been determined by optical spectroscopy. It has been suggested that the distal histidine of HRP is involved in binding with aromatic substrates (Modi *et al.* 1989a, Saxena *et al.* 1990).

Address for correspondence: S. Modi, Biological NMR Centre, Medical Science Building, University of Leicester, Leicester LE1 9HN, UK. Tel: (+44) 533 525586; Fax: (+44) 533 523013.

Despite of the large number of studies in this area, there is no direct evidence of the involvement of distal histidine of HRP in binding with aromatic substrates. There are two possible ways in which the amino acid responsible for binding with aromatic substrates in HRP can be identified. One is to change a particular amino acid by site-directed mutagenesis and other is the chemical modification of an amino acid. Recently, a synthetic gene encoding HRP has been expressed in *Escherichia coli* by Smith *et al.* (1992) and, in addition, distal histidine in HRP has been modified using diethylpyrocarbonate (DEPC) by Bhattacharya *et al.* (1992). In this paper we have also performed chemical modification of HRP using DEPC and for the first time the amino acid residue which is responsible for binding with aromatic substrates has been identified.

Manganese porphyrin reconstituted hemeproteins have long been studied due to the close relationship of manganese with the iron (Waterman & Yonetani, 1970, Modi *et al.* 1990a, 1991a, Saxena *et al.* 1990). However, these studies have largely been confined to spectrophotometric and conformational studies pointing that the protein conformation is probably restored to the native enzyme. It has been shown earlier that aromatic substrates bind at the same binding site to Mn(III)HRP as that of Fe(III)HRP (Saxena *et al.* 1990). In the present study different kinetic parameters has been also calculated for the first time for the interaction of compound II with aromatic substrates for Mn(III)HRP and have been compared with Fe(III)HRP. In this paper, it has also been shown that the iron atom in HRP plays no role in the oxidation of substrates once compound II has been formed. These parameters are also

calculated for DEPC-modified Fe(III)HRP and Mn(III) HRP, and have been compared with native Fe(III)HRP and Mn(III)HRP.

## Materials and methods

HRP was purified from crude HRP (Sigma, $R_z = A_{403}/A_{280} = 0.8$) by DEAE and CM-cellulose column chromatography (Aibara *et al.* 1982, Modi *et al.* 1989b). The B and C HRP isoenzymes were collected ($R_z = 3.2$) after elution with acetate buffer (100 mM, pH 4.4). The concentration of the enzyme was determined spectrophotometrically using a molar extinction coefficient of $1.02 \times 10^5$ cm$^{-1}$ mM$^{-1}$ at 403 nm for HRP (Aibara *et al.* 1982). All other reagents were of analytical grade (Fissions, Loughborough, UK).

HRP was modified using DEPC according to recent reports (Blanke & Hager 1990, Bhattacharya *et al.* 1992). Fresh solutions of DEPC were prepared by dilution of the original DEPC stock solution in absolute ethanol. HRP was incubated with a large excess (> 10 000 times) of DEPC in 0.1 mM phosphate buffer (pH 7.5) for about 1 h. Excess DEPC was removed from HRP using a small G-25 Sephadex column (1.5 × 6 cm).

Preparation of Mn(III)HRP was done as described before (Modi *et al.* 1990a, Saxena *et al.* 1990). The apoprotein of HRP was prepared by lowering the pH of the aqueous solution of Fe(III)HRP to 2.0 and then heme was removed by extraction with ice-cold 2-butanone several times. Mn(III) protoporphyrin was prepared and purified by the method described previously (Yonetani & Asakura 1969). Reconstitution of apo HRP with Mn(III) protoporphyrin IX was performed in 10 mM Tris–HCl (pH 8.0) at 2°C and was purified using a DEAE cellulose column.

Difference optical spectra (enzyme–substrate complex versus enzyme) were obtained using a Beckman DU 650 spectrophotometer with 3 ml quartz cells (10 mm path length) at 23°C. Titrations were performed by the addition of 1.5–100 mM of the substrates to the enzyme (10 μM) in 0.1 M phosphate buffer (pH 6.1).

Kinetics studies on HRP using hydroquinone and *p*-hydroxybenzoic acid as donor substrates were carried out at 23°C in 0.1 M phosphate buffer (pH 6.1). Reactions were performed under $H_2O_2$ saturating conditions and, assuming the reaction of compound II as the slow step, it is possible to treat the kinetic data as the Michaelis–Menten scheme (Dunford & Stillman 1976, Casella *et al.* 1993). The reaction mixture consists of ($2–1000 \times 10^{-6}$ M) donor substrate, $H_2O_2$ ($4 \times 10^{-4}$ M) and HRP ($1.8 \times 10^{-6}$ M). The reaction was followed by a decrease in absorption of the substrates. For hydroquinone, the monitored wavelength and the molar extinction coefficient were 288 nm and 2680 M$^{-1}$ cm$^{-1}$, respectively; for *p*-hydroxybenzoic acid, the monitored wavelength and the molar extinction coefficient were 246 nm and 11 500 M$^{-1}$ cm$^{-1}$, respectively. The kinetic data obtained was fitted to a Michaelis–Menten equation, and kinetics parameters $K_m$ and $V_{max}$ were calculated.

## Results and discussion

The absorption spectrum of Fe(III)HRP in the Soret region alters only slightly in the presence of even a large excess of the aromatic donor molecules (resorcinol, hydroquinone and *p*-hydroxybenzoic acid). This suggests that aromatic donors do not bind directly to the metal center (Modi *et al.* 1989a). The binding constant can be calculated by the use of the double reciprocal plot (Sakurada *et al.* 1986, Saxena *et al.* 1990; $1/\Delta A$ versus $1/S_0$) for these aromatic donor molecules (Figure 1 and Table 1).

Similarly, binding constants for these aromatic donor molecules to Mn(III)HRP were calculated (Table 1). The values of $K_A$ so obtained are comparable to Fe(III)HRP as expected. The same was observed previously (Saxena *et al.* 1990) and from this result it was suggested that the secondary structure in Mn(III)HRP is restored to the same extent as Fe(III)HRP and also that the porphyrin rather than the metal ion is responsible for the conformational integrity of the protein.

HRP was modified by incubation with excess (> 10 000 times) DEPC for 1 h. The modification of HRP by DEPC requires a large excess of the reagent in order to counteract its rapid hydrolysis in aqueous solution. Excess of DEPC was removed from HRP using a small G-25 Sephadex column (see Materials and methods). Figure 2 shows optical spectra of native Fe(III)HRP and DEPC-modified Fe(III)HRP at pH 6.1. At pH 6.1, optical spectra for both the native Fe(III)HRP and DEPC-modified Fe(III)HRP are very similar, except for the region around 240 nm

**Table 1.** Apparent dissociation constants (mM) and $\Delta G$ (kcal mol$^{-1}$; value in parentheses) for binding of resorcinol, hydroquinone and *p*-hydroxybenzoic acid to enzymes in 0.1 M phosphate buffer (pH 6.1) at 23°C

| Enzyme | Resorcinol | Hydroquinone | *p*-Hydroxybenzoic acid |
|---|---|---|---|
| Native Fe(III)HRP | 5.0 ± 0.5 | 7.8 ± 0.9 | 14 ± 2 |
| | (−3.1) | (−2.8) | (−2.5) |
| Native Mn(III)HRP | 4.3 ± 0.5 | 7.1 ± 0.8 | 16 ± 2 |
| | (−3.2) | (−2.9) | (−2.4) |
| DEPC-modified Fe(III)HRP | 150 ± 30 | 210 ± 50 | 280 ± 60 |
| | (−1.1) | (−0.92) | (−0.75) |
| DEPC-modified Mn(III)HRP | 160 ± 20 | 200 ± 40 | 300 ± 70 |
| | (−1.1) | (−0.95) | (−0.71) |

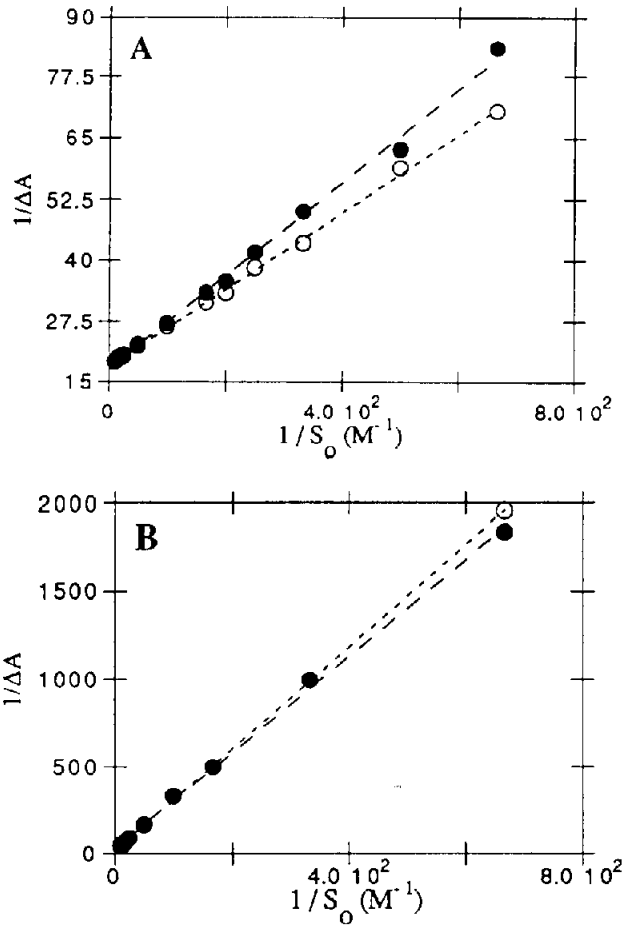


**Figure 1.** (**A**) Variation of change in absorbance ($\Delta A$) of the Soret band with resorcinol concentration for Fe(III)HRP (○) and Mn(III)HRP (●). (**B**) Variation of change in absorbance ($\Delta A$) of the Soret band with resorcinol concentration for DEPC-modified Fe(III)HRP (○) and DEPC-modified Mn(III)HRP (●).

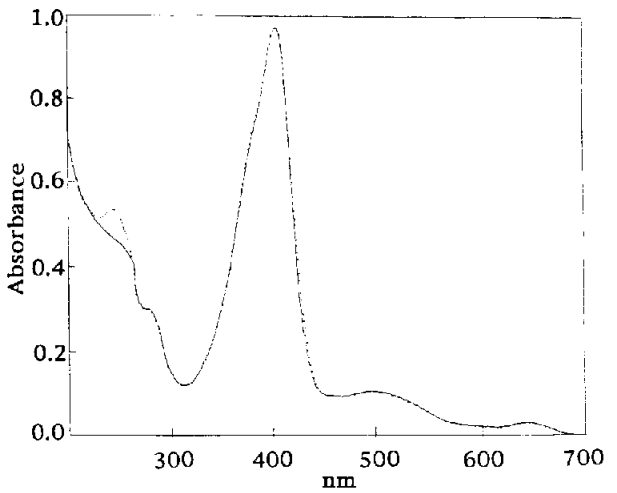


**Figure 2.** Electronic UV/visible absorption spectrum of native HRP (9.5 μM) at pH 7.5 (——) and DEPC-modified HRP at pH 7.5 (···). For modification, HRP (9.5 M) was incubated with DEPC (60 mM) in 0.1 M phosphate buffer (pH 7.5) for about 1 h at 20°C.

(Figure 2). The modification by DEPC is followed by an increase in absorbance at 240 nm and the stoichiometry of the formation of *N*-carbethoxyhistidine residue was calculated from this increase in absorbance at 240 nm using the extinction coefficient of 3200 $M^{-1}$ $cm^{-1}$ (Church *et al.* 1985, Blanke & Hager 1990, Bhattacharya *et al.* 1992). The number of histidine residues modified in HRP upon addition of DEPC was found to be two per molecule of HRP (Bhattacharya *et al.* 1992). On addition of excess hydroxyl amine (300 mM) to DEPC-modified HRP at pH 6.1, the optical spectra (region around 240 nm) of DEPC-modified HRP is the same as native HRP at pH 6.1 (data not shown). The increase in absorption at 240 nm following incubation with DEPC and its reversal by hydroxyl amine gives strong evidence for the formation of carbethoxyhistidine (Miles 1977), i.e. modification of the histidine residues of HRP.

The primary amino acid sequence of HRP revealed the presence of three histidine amino acids (His-40, His-42 and His-170) at the heme periphery (Welinder 1979). Computer modeling of histidine molecules around the heme cavity shows that His-40 and His-42 are located at the distal pocket of the heme in HRP, whereas His-170 is ligated to heme iron at the fifth coordination site (Sakurada *et al.* 1986). This histidine (His-170) is also known as proximal histidine. Therefore His-170 will resist modification by DEPC due to the ligation of this residue to heme iron through one of its nitrogens. His-40 and His-42 are therefore likely candidates for modification by DEPC. The X-ray crystallographic structural analysis of yeast cytochrome *c* peroxidase. (Poulos & Kraut 1980) and beef liver catalase (Fita & Rossman 1985) has revealed the presence of a histidine residue at the heme distal pocket. From computer modeling of HRP (Sakurada *et al.* 1986), it has been shown that it contains a distal histidine residue, which is His-42. Sequence analysis of cytochrome *c* peroxidase (Kaput *et al.* 1982) and HRP (Welinder 1979) shows a strong homology in the amino acid sequence of the two enzymes near the heme distal pocket. Amino acid residues 48–52, i.e. Arg-48–Leu–Ala–Trp–His-52, of cytochrome *c* peroxidase are comparable with similar residues 38–42, i.e. Arg-38–Leu–His–Phe–His-42 of HRP. His-52 of cytochrome *c* peroxidase appears to be the same residue as His-42 of HRP, i.e. in both cases distal histidine. His-40 in HRP is replaced by Ala-50 in cytochrome *c* peroxidase and is not conserved in any peroxidase known so far (Tien & Tu 1987).

A similarly modification of the distal histidine was carried out for Mn(III)HRP (spectra not shown). This modification reaction is specific for histidines. The number of histidine residues modified in Mn(III)HRP upon addition of DEPC was again found to be two per molecule of Mn(III)HRP.

By the use of the double reciprocal plot ($1/\Delta A$ versus $1/S_0$), the binding constant for the binding of these aromatic donor molecules to DEPC-modified Fe(III)HRP and Mn(III)HRP can be calculated (Figure 1 and Table 1). This data show that for every aromatic donor molecule studied here, the binding constant for DEPC-modified HRP is much lower than that of native HRP. From the above it is very clear that DEPC modifies His-42 and His-40. From computer modeling it is shown that His-42 hangs in the heme cavity

perpendicular to the heme plane and is very close to the heme iron (Sakurada *et al.* 1986). Therefore, in native HRP, distal histidine (His-42) becomes unavailable for binding to aromatic donor molecules following modification by DEPC. The His-40 in HRP appears to be away from the heme plane. Therefore, it may not have any role in the binding of aromatic donor molecules with HRP. It is to be noted that His-40 is not conserved in any peroxidase (e.g. His-40 in HRP is replaced by Ala-50 in cytochrome *c* peroxidase).

It is also interesting to note that the difference in $\Delta G$ values (Table 1) between native HRP and DEPC-modified HRP is about 1–2 kcal $mol^{-1}$, which can be accounted for by the hydrogen bond between the substrate molecule and His-42 of HRP. On modification of distal histidine (His-42), the hydrogen bond between the distal histidine and aromatic compound is lost, therefore $\Delta G$ values are less negative for DEPC-modified HRP (Table 1). This result supports the earlier theory that an aromatic donor binds to distal histidine in the cavity which is near the heme peripheral 8-$CH_3$, Tyr-185 and Arg-183 residues. On modification of distal histidine, the hydrogen bond is lost, but aromatic donor molecules are still able to bind because of hydrophobic interactions and $\pi$–$\pi$ interactions between the benzene ring of Tyr-185 and the aromatic donor compound. Previous computer graphics studies have also suggested that Tyr-185 may be involved in the interaction with the aromatic substrates (Sakurada *et al.* 1986). Since tyrosine is also present in a similar position in turnip peroxidase and cytochrome *c* peroxidase, it seems possible that tyrosine may have an essential role in the peroxidase action.

Therefore, these result also support the earlier assumption that an aromatic donor is oriented by a hydrogen bond to distal histidine (His-42) and, as an electron is transferred from substrate to the porphyrin cation radical, a proton is transferred along the hydrogen bond to His-42 (Oertling *et al.* 1988, Modi *et al.* 1990b). This results in homolytic cleavage of the H–O bond with the formation of a free radical of the oxidized substrate.

It will be interesting to see how kinetic parameters compare among themselves for oxidation of substrate with native and DEPC-modified Mn(III)HRP and Fe(III)HRP. As discussed in Materials and methods, the reaction of compound II with aromatic substrates can be treated as a Michaelis–Menten scheme, when the concentration of $H_2O_2$ is very high. Under saturating $H_2O_2$ concentration, the reaction of compound II with substrate is much slower than the reaction of compound I with substrate. Table 2 lists all of the kinetic parameters for hydroquinone and *p*-hydroxybenzoic acid. For Fe(III)HRP, the parameters calculated here are different to those reported earlier, especially the values of $K_m$ (Casella *et al.* 1993). These differences may be because of different conditions. For resorcinol it was not possible to measure kinetics parameters as the substrate absorption was superimposed with that of the oxidized product.

From Table 2, it can also be seen that kinetics parameters for oxidation of substrate are very similar for Fe(III)HRP and Mn(III)HRP, which means that the reactivity of compound II intermediate with the substrate is the same in both cases. This suggests that the metal ion does not play any role in oxidation once compound II is formed. After formation of compound II, protein structure and binding with the aromatic substrate plays an important role. It should be noted that the coordination geometry of metal ion plays an important role in the rate of formation of compound II (Modi *et al.* 1990b, 1991b). It can also be seen that DEPC-modified HRP has low activity. In DEPC-modified HRP, $K_m$ values are much higher as compared with native HRP. This is as expected, since DEPC-modified HRPs have a lower affinity for substrated as compared with native HRP (Table 1).

**Table 2.** Catalytic and kinetic parameters of enzymes for the oxidation of hydroquinone and *p*-hydroxybenzoic acid (values in parentheses) by $H_2O_2$ in 0.1 M phosphate buffer (pH 6.1) at 23°C

| Enzyme | $K_m$ (mM) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}$ $min^{-1}$) |
|---|---|---|---|
| Native Fe(III)HRP | 6.6 | 1100 | 167 |
| | (12) | (25) | (2.1) |
| Native Mn(III)HRP | 6.2 | 800 | 129 |
| | (14) | (16) | (1.1) |
| DEPC-modified Fe(III)HRP | 210 | 400 | 1.9 |
| | (220) | (5) | (0.02) |
| DEPC-modified Mn(III)HRP | 230 | 380 | 1.7 |
| | (320) | (4) | (0.01) |

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