Haem propionates control oxidative and reductive activities of horseradish peroxidase by maintaining the correct orientation of the haem

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The role of haem propionates in oxidative and reductive reactions catalysed by horseradish peroxidase (HRP) was studied after successful reconstitution of ferric protoporphyrin IX dimethyl ester (PPDME) into the apoperoxidase. The reconstituted enzyme oxidizes neither guaiacol (aromatic electron donor) nor iodide or thiocyanate (inorganic donor). Although the reconstituted enzyme binds guaiacol with a similar K_d (13 mM) to that of the native enzyme (10 mM), the K_d for SCN^- binding (5 mM) is decreased 20-fold compared with that of the native enzyme (100 mM). This indicates that haem propionates hinder the entry or binding of inorganic anion to the active site of the native HRP. However, the reconstituted enzyme is catalytically inactive as it does not form spectroscopically detectable compound II with H_2O_2 . CD measurements indicate a significant loss of haem CD spectrum of the reconstituted enzyme at 409 nm, suggesting

a loss of asymmetry of the haem–protein interaction. Thus the inability of the reconstituted enzyme to form catalytic intermediates results from the change in orientation of the haem due to loss of interactions via the haem propionates. HRP also catalyses reductive reactions such as reduction of iodine (I^+) in the presence of EDTA and H_2O_2 . The reconstituted enzyme cannot catalyse I^+ reduction because of the loss of I^+ binding to the haem propionate. Since I^+ reduction requires formation of the catalytically active enzyme– I^+ –EDTA ternary complex, the loss of reductive activity is primarily due to the loss of active enzyme formation. Haem propionates thus play a vital role in the oxidative and reductive reactions of HRP by favouring the formation of catalytic intermediates with H_2O_2 by maintaining the correct orientation of the haem with respect to the surrounding residues.

INTRODUCTION

Ferriprotoporphyrin IX plays a vital role in peroxidase catalysis. A knowledge of the nature of its binding to the apoenzyme and its orientation with respect to the residues of the haem pocket is of vital importance in understanding the molecular mechanism of catalysis. Since Theorell's [1] classic work on the reversible cleavage of horseradish peroxidase (HRP; donor– H_2O_2 oxidoreductase; EC 1.11.1.7) into protohaem and apoenzyme, and their successful reconstitution to the active form [1,2], several investigators have used substituted haemins as probes for studying the structure–function relationship in HRP [3–9]. The enzyme reconstituted with proto-, meso- or deuterio-haem or protohaem monomethyl ester can catalyse the formation of compound I, whereas the enzyme containing protohaem dimethyl ester cannot [8]. The enzyme containing meso- or deuterio-haem can catalyse guaiacol oxidation in a similar way to the native enzyme. However, enzyme containing protohaem monomethyl ester shows only 20% of the activity, and the dimethyl ester is inactive. These studies indicate that, although the side chains at the 2 and 4 positions of the porphyrin ring are not essential for catalytic activity, the carboxyl groups at positions 6 and 7 seem to be obligatory [8]. Other studies indicate that haemin 2- and 4-substituents are important for the binding of haem to the apoperoxidase and that they all form active substituted enzyme [9]. As various functional groups of different size can be accommodated at the binding site for 2- and 4-haemin substituents, the binding site appears to have a high degree of conformational flexibility [9]. The role of 6- and 7-haemin substituents in apoperoxidase binding and enzyme activity is more critical and was studied by either blocking the haem propionate carboxyl groups using protohaemin diamide and

dialcohol haemin or increasing the chain length using dibutyric acid haemin [9]. The low activity, atypical spectra and slow binding to apoenzyme by diamide- and dialcohol-substituted enzymes indicate that free carboxylates are essential for rapid generation of the active enzyme. On the other hand, although carboxylates are present in the dibutyric acid derivative, very slow binding and low catalytic activity suggest that the chain length is also a determining factor in the binding of carboxylates probably in a narrow pocket of the apoperoxidase [9]. The role of 2- and 4-haem substituents in the conformation of reconstituted HRP has been studied by CD measurements [10], but studies related to the 6- and 7-substituents are very limited.

The possible role of haem propionates in controlling the oxidation of various electron donors by HRP has also been studied. Protonation of an enzyme ionizable group with $\text{p}K_a$ 4.3 appears to induce an 'open' conformer which allows iodide to permeate to the active site for efficient oxidation [11]. This ionizable group is attributed to the haem propionate which may form a salt bridge with the nearby positively charged residue, controlling the binding and oxidation of iodide and thiocyanate [12,13]. This is substantiated by the pH-independent binding of thiocyanate to HRP, reconstituted with apoperoxidase and esterified haem propionic acid [14]. We have further studied the mechanism of HRP-catalysed reductive reaction of iodine with EDTA [15–18] where an ionizable group of $\text{p}K_a$ 4.8, probably contributed by the haem propionate, controls iodine (I^+) binding and its reduction [19]. Thus propionate appears to play an important role in peroxidase catalysis by controlling the binding of some ligands at the active site. It is now believed from the proposed active-site topology of HRP [20,21] that electron donors interact at the haem edge and are oxidized by the flow of electrons through the δ -meso carbon to the haem ferryl group

Abbreviations used: HRP, horseradish peroxidase; PPDME, protoporphyrin IX dimethyl ester.

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either by a single two-electron oxidation by compound I or two one-electron oxidations by compounds I and II [22–24]. Although earlier NMR, mutant and chemical modification studies indicated the possible role of some phenylalanine and arginine residues [25–29] in aromatic donor binding, recent site-directed mutagenesis studies have identified the critical role of Phe-179 in aromatic donor binding [30]. The exact binding site for iodide or thiocyanate has not been identified yet. It may interact near the δ -meso carbon [20,21] at a site equidistant from the 1- and 8-methyl haem groups [12,13]. However, literature on the possible role of haem propionates in the binding of these donors at the haem edge is scant. Although the essential role of haem propionates in compound I formation has previously been suggested [8,9], how they influence it is not yet clear. Moreover, their role in the reductive reactions [15,16,19,31,32] also remains to be established. With this objective, we have studied more critically the precise role played by the haem propionates in HRP-catalysed oxidation of aromatic donors (guaiacol) and inorganic substrates (iodide or thiocyanate) as well as reduction of iodine with EDTA. The result indicates that, on reconstitution of apo-HRP with propionate-modified haem-containing ferric protoporphyrin IX dimethyl ester (Fe^{3+} -PPDME), the reconstituted enzyme (Fe^{3+} -PPDME-HRP) binds guaiacol with the same K_d as native HRP, but the K_d for thiocyanate binding is decreased 20-fold. However, the reconstituted enzyme cannot oxidize these donors because of its inability to form catalytically active enzyme intermediates with H_2O_2 . The complete loss of the haem CD spectrum of the reconstituted enzyme indicates a significant change in the orientation of the haem with respect to the surrounding aromatic amino acids, leading to the loss of its π - π^* interaction. We suggest that, because of the absence of haem carboxylates, the apoperoxidase does not hold the haem in the correct orientation, resulting in the loss of haem asymmetry, which accounts for the loss of active intermediate formation. Other studies indicate that the inhibition of iodine reduction by the propionate-modified enzyme is due to the loss of iodine (I^+) binding [19] in the haem propionate, in addition to the loss of active-intermediate formation. Thus haem propionates control both oxidative and reductive reactions mainly by favouring the formation of catalytic intermediates with H_2O_2 through maintenance of the correct orientation of the haem moiety with respect to the surrounding residues. The recently published crystal structure of HRP-C [33] along with the NMR studies on aromatic donor binding [30] indicate that haem propionates influence haem orientation, H_2O_2 reactivity and donor affinity through the hydrogen-bonding network with the surrounding residues.

MATERIALS AND METHODS

Horseradish peroxidase (HRP type VI; RZ 3.0), guaiacol, PPDME and FeSO_4 were purchased from Sigma, St. Louis, MO, U.S.A. ICl was obtained from Aldrich, Milwaukee, MI, U.S.A. EDTA and KI were the products of Glaxo Laboratories. All other chemicals used were of analytical grade. The concentrations of HRP and Fe^{3+} -PPDME-reconstituted HRP were determined from $\epsilon_{403} = 102 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [34] and $\epsilon_{396} = 65 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [8] respectively.

Measurement of the oxidative activity of HRP using iodide, thiocyanate and guaiacol as electron donor

The rate of iodide oxidation was measured by following the formation of I_3^- at 353 nm [35]. The assay mixture contained in a final volume of 1 ml: 50 mM sodium acetate buffer, pH 5.2, 2 mM KI, 1 nM enzyme and 300 μM H_2O_2 added last to start the

reaction. Peroxidase-catalysed thiocyanate oxidation was determined by the consumption of thiocyanate as described previously [24]. HRP (0.5 μM) was incubated with 1 mM H_2O_2 and 1 mM potassium thiocyanate in 0.1 M sodium acetate buffer, pH 4.5. The reaction was stopped by the addition of a small amount of catalase after a certain interval of time. This was followed by the addition of 1.6 ml of 0.1 M HCl and 0.2 ml of FeCl_3 to 0.2 ml of the reaction mixture, and the A_{450} of the FeSCN^{2+} complex [36] was measured. Peroxidase-catalysed guaiacol oxidation was monitored at 470 nm as described previously [27]. The assay mixture contained in a final volume of 1 ml: 50 mM sodium phosphate buffer, pH 7.4, 20 mM guaiacol, 1 nM enzyme and 300 μM H_2O_2 added last to start the reaction.

Assay of reductive activity of HRP in the presence of EDTA using iodine as electron acceptor

The assay system contained in a final volume of 1 ml: 50 mM sodium acetate buffer, pH 6.0, 0.5 mM freshly prepared ICl, 4 mM EDTA, 300 μM H_2O_2 and 2.5 nM HRP added last to start the reaction. The decrease in A_{460} was recorded at intervals of 30 s to measure the reductive activity [17,19].

Preparation of PPDME-reconstituted HRP

HRP was converted into apoperoxidase by extraction of haem with distilled methyl ethyl ketone at pH 2.0 at 0 °C [37]. Fe^{3+} -PPDME was prepared by insertion of iron into the PPDME using the FeSO_4 /acetic acid method [38]. The PPDME was dissolved in 1 ml of pyridine and 10 ml of pure acetic acid, and immediately afterwards 1 ml of a freshly prepared saturated solution of FeSO_4 was added. A stream of CO_2 or N_2 was passed through the solution while keeping it at 80 °C for 10 min. The mixture was allowed to stand in air for 10 min to allow auto-oxidation to form a ferric complex. The Fe^{3+} -PPDME was then separated by extraction with chloroform and evaporated to dryness. Reconstitution of Fe^{3+} -PPDME with the apo-HRP was carried out by mixing the two together (10:1, v/v) by standard procedures using 5 mM potassium phosphate buffer, pH 6.0, containing 10% (v/v) acetone at 0 °C for 24 h [8]. The Fe^{3+} -PPDME-reconstituted HRP was separated from the free Fe^{3+} -PPDME by passage through a Sephadex G-25 column.

Binding studies by optical difference spectroscopy

Optical difference spectra for the enzyme–ligand complex versus enzyme were recorded as described previously [17,19,24,27,39]. The apparent dissociation constant, K_d , was calculated from the following expression [39]:

$$\frac{1}{[S]} = \frac{[E]\Delta\epsilon}{K_d\Delta A} - \frac{1}{K_d}$$

where ΔA is the difference in absorbance between the peak and the trough of the spectrum, $\Delta\epsilon$ is the difference in molar absorptivity of the free and bound enzyme, $[S]$ is the total substrate concentration and $[E]$ is the total enzyme concentration. The approximations used in this equation were as described previously [27]. Donor was usually added in large excess over HRP and thus free donor concentration approximates the total donor concentration. K_d was calculated from a plot of $1/\Delta A$ against $1/[S]$. All kinetic and spectral measurements were carried out at 28 ± 2 °C using a Shimadzu UV-2201 computerized spectrophotometer.

CD spectroscopy

CD spectra of native and Fe³⁺-PPDME-HRP were recorded in a Jasco J-600 CD spectrometer at 20 °C in the presence of nitrogen, using a scan speed of 50 nm/min and sensitivity of 50 millidegrees.

RESULTS

Reconstitution of apo-HRP with modified haemins

PPDME has a low affinity for the apoenzyme, therefore a 10-fold excess over the apoperoxidase is required for complete reconstitution to Fe³⁺-PPDME-HRP. Optical absorption spectra of the native and propionate-modified enzyme are shown in Figure 1. The spectrum of the native HRP (trace a) has a Soret peak at 403 nm. However, Fe³⁺-PPDME-reconstituted HRP shows a slightly broader Soret peak at 396 nm (trace b), indicating reconstitution to a holoenzyme with an RZ value of 2.0, which is close to the value reported previously [8].

Enzymic activity of the reconstituted HRP

Table 1 shows the oxidative and reductive reactions catalysed by the reconstituted HRP. Unlike haemin-substituted HRP, the reconstituted enzyme has very low oxidative activity with iodide, thiocyanate or guaiacol in the presence of H₂O₂. The reconstituted enzyme has no significant ability to reduce iodine to iodide in the presence of EDTA and H₂O₂. The results indicate that free propionate is essential for both the oxidative and reductive reactions catalysed by HRP. In order to check whether Fe³⁺-PPDME is reconstituted with the apo-HRP at the appropriate haemin-binding site, the activity of the reconstituted HRP was studied by adding haemin, which will interact at the correct place to produce fully active enzyme if Fe³⁺-PPDME is not bound to the specific site. Table 2 shows that, unlike haemin-

Figure 1 Soret spectrum of the Fe³⁺-PPDME-HRP

Trace a, HRP (1.5 μM); trace b, Fe³⁺-PPDME-reconstituted HRP (1.5 μM).

Table 1 Oxidative and reductive activities of Fe³⁺-PPDME-HRP

Apo-HRP was reconstituted separately with protohaemin or Fe³⁺-PPDME and the activities of the reconstituted enzyme were measured as described in the Materials and methods section. The values represent the activity after correction for the non-enzymic part, if any.

Experiment	Oxidative activity ($k_{\text{cat}} \cdot \text{min}^{-1}$)			I ⁺ reductive activity ($k_{\text{cat}} \cdot \text{min}^{-1}$)
	Iodide	Thiocyanate	Guaiacol	
Protohaemin-reconstituted HRP	38 500	270	30 760	80 000
Ferric-PPDME-reconstituted HRP	2 690	30	2 150	0

Table 2 Binding of haemin to the specific site of apo-HRP in the presence and absence of Fe³⁺-PPDME as indicated by the activity test

Ten equivalents of Fe³⁺-PPDME were added before the addition of haemin to the preincubation system containing apo-HRP (1.5 μM) in 10 mM phosphate buffer, pH 6.0, at 0 °C. The enzyme activity was measured by iodide oxidation.

Experiment	Iodide oxidation ($k_{\text{cat}} \cdot \text{min}^{-1}$)
Native HRP	38 460
Apo-HRP	380
Apo-HRP + haemin	38 500
Apo-HRP + Fe ³⁺ -PPDME	2 690
Apo-HRP + Fe ³⁺ -PPDME + haemin	2 660

reconstituted HRP which has the full activity of the native enzyme, Fe³⁺-PPDME-reconstituted enzyme does not show any further increase in activity after the addition of haemin, indicating that haemin cannot displace already bound Fe³⁺-PPDME from its natural binding site. In other words, propionate-modified haem has interacted with the apo-enzyme at the right place (His-170) under the conditions used. Thus the loss of activity is not due to faulty reconstitution of the Fe³⁺-PPDME at a non-specific site. Since oxidative or reductive activity depends on the formation of catalytically active enzyme intermediates and the enzyme-donor complex, these two parameters were therefore checked (as shown below) to investigate why the reconstituted enzyme is unable to catalyse significant oxidative or reductive reaction.

Reactivity with haem-iron ligands: spectral evidence for formation of compound II and enzyme-CN⁻ complex

The reactivity of haem iron with H₂O₂ or CN⁻ to form spectroscopically demonstrable compound II or cyanide complex [40] was studied by adding H₂O₂ or CN⁻ to the Fe³⁺-PPDME-reconstituted enzyme. Unlike native HRP, the propionate-modified HRP does not show compound II formation (not shown), indicating its inability to react with H₂O₂. Whereas five equivalents of H₂O₂ can produce spectroscopically detectable stable compound II at 418 nm with native HRP [40], the addition of about 40 equivalents of H₂O₂ to the reconstituted enzyme produced very little compound II. The reconstituted enzyme also shows very poor reactivity with CN⁻. It does not show the characteristic peak of HRP-CN⁻ complex at 425 nm [40], except that a shoulder appears at 418 nm due to formation of some enzyme-CN⁻ complex (not shown). When the K_d value of the complex-formation was calculated from the optical difference spectra of the reconstituted enzyme-CN⁻ complex versus reconstituted enzyme, the reactivity of the haem iron towards CN⁻ was found to be significantly reduced, as shown by the increased K_d value (125 μM instead of 2 μM) for the native HRP-CN⁻ complex (Table 3).

Binding studies with electron donors and acceptor by optical difference spectroscopy

As binding of electron donor or acceptor is a prerequisite for oxidative and reductive reactions, the binding of various electron donors, such as guaiacol, thiocyanate and EDTA, and electron acceptors, such as iodine (I⁺), to the native and Fe³⁺-PPDME-reconstituted HRP was studied by optical difference spectroscopy, and the K_d value for the enzyme-ligand complex-

Table 3 Difference spectral characterization and apparent dissociation constant (K_d) of HRP–ligand complexes

Enzyme	Ligand	Spectrum of complex (nm)		$\Delta \epsilon$ Peak–trough ($\text{mM}^{-1} \cdot \text{cm}^{-1}$)	K_d
		Peak	Trough		
HRP	CN^-	423	395	96	$2 \mu\text{M}$
Fe^{3+} -PPDME–HRP	CN^-	421	400	10	$125 \mu\text{M}$
HRP	Guaiacol	408	—	4.0	10 mM
Fe^{3+} -PPDME–HRP	Guaiacol	400, 525, 625	450	5.0	13 mM
HRP	SCN^-	416	395	16.0	100 mM
Fe^{3+} -PPDME–HRP	SCN^-	377, 424, 525, 663	404, 600	7.4	5 mM
HRP	EDTA	420	395	6.2	5 mM
Fe^{3+} -PPDME–HRP	EDTA	397	425	2.2	5 mM
HRP	I^+	400	423	7.0	$20 \mu\text{M}$
Fe^{3+} -PPDME–HRP	I^+	—	—	—	—

Figure 2 Guaiacol and thiocyanate binding to Fe^{3+} -PPDME-reconstituted HRP by optical difference spectroscopy

(A) Difference spectra of Fe^{3+} -PPDME–HRP–guaiacol complex versus Fe^{3+} -PPDME–HRP in 50 mM phosphate buffer, pH 7.0. The concentration of Fe^{3+} -PPDME–HRP was $10.0 \mu\text{M}$ and guaiacol concentrations used were 4.5 mM (i) to 25.0 mM (f). (B) Difference spectra of Fe^{3+} -PPDME–HRP– SCN^- complex versus Fe^{3+} -PPDME–HRP in 50 mM phosphate buffer, pH 4.5, were obtained with $5.0 \mu\text{M}$ Fe^{3+} -PPDME–HRP using SCN^- concentrations from 5 mM (i) to 100 mM (f). Inset represents the plot of $1/\Delta A$ against $1/[\text{donor}]$ to determine the K_d value of the complex-formation.

formation was calculated from the plot of $1/\Delta A$ against $1/[\text{S}]$. The difference spectrum of the enzyme–guaiacol complex for the Fe^{3+} -PPDME-reconstituted HRP (Figure 2A) is somewhat different from that of the native HRP [27]. The former shows a peak at 400 nm instead of 408 nm [27] and a trough at 450 nm. Two more broad peaks at about 525 nm and 625 nm (not shown) are also observed. However, the modified HRP binds guaiacol with a similar K_d (13 mM) to that of the native HRP (10 mM) (Table 3). SCN^- also binds to the propionate-modified HRP giving a difference spectrum (Figure 2B) that is completely different from that of the native HRP [24]. The reconstituted enzyme shows peaks at 377 nm and 424 nm and a trough at 404 nm (Figure 2B). However, an additional broad peak at 525 nm and a small peak at 663 nm with a broad trough at 600 nm (not shown) are also evident. This is in contrast with the native HRP which shows a peak at 416 nm and a trough at 395 nm [24]. Surprisingly, the K_d value for SCN^- binding (Table 3) to the reconstituted enzyme is significantly lower (5 mM) than that of the native HRP (100 mM). Thus, although a propionate-modified HRP can bind aromatic donors such as guaiacol with similar K_d to that of the native enzyme, the binding of inorganic donors such as SCN^- is increased 20-fold over the native HRP. For the reduction of I^+ by EDTA, both EDTA and I^+ have been shown to bind HRP with characteristic difference spectra [17,19]. The propionate-modified HRP can bind EDTA, showing a difference spectrum different from that of the native enzyme [17]. It shows a peak at 397 nm and a trough at 425 nm (not shown) instead of a peak at 420 nm and a trough at 395 nm for the native HRP [17]. The K_d value for this reconstituted HRP–EDTA complex was found to be 5 mM which is the same as that for the native HRP (Table 3). However, Fe^{3+} -PPDME-reconstituted HRP does not bind I^+ at all, as shown by no significant difference spectrum (not shown) compared with the native HRP, which shows a peak at 400 nm and a trough at 423 nm [19]. It is interesting to note that, although the donors bind to the Fe^{3+} -PPDME-reconstituted enzyme with the same or lower K_d , the spectral nature of the reconstituted HRP–donor complexes is significantly different from that of the native HRP–donor complexes, presumably because of esterified haem propionates.

Figure 3 CD spectra of the native and Fe^{3+} -PPDME–HRP

(A) UV CD spectrum of the Fe^{3+} -PPDME–HRP (trace a) and native HRP (trace b). The concentration of both the enzymes was $1.1 \mu\text{M}$. (B) Soret CD spectrum of Fe^{3+} -PPDME–HRP (trace a) and native HRP (trace b). The concentration of both the enzymes was $3.3 \mu\text{M}$.

CD measurements of the native and modified HRP

The UV CD spectra, specifically the minima at 221 nm and 208 nm, are characteristic of the helical secondary structure [41]. The CD measurements of the enzymes (Figure 3A) indicate that

the secondary structure of propionate-modified HRP (trace a) is similar to that of the native HRP (trace b). Computer analysis of the secondary structure [42] indicates that both enzymes have 32.4% α -helix, 17.6% β -helix and 50.0% random coil. However, when haem CD spectra were recorded (Figure 3B), Fe³⁺-PPDME-reconstituted HRP did not show any significant haem CD band at 409 nm (trace a), as shown by the native HRP (trace b). This indicates a significant change in the orientation of the haem with respect to the residues of the haem pocket in the reconstituted enzyme.

DISCUSSION

To examine the role of haem propionates in the oxidative and reductive reactions of HRP, emphasis was placed on (a) formation of catalytic intermediates with H₂O₂ and (b) binding of electron donor or acceptor after reconstitution of Fe³⁺-PPDME with the apo-HRP. The reconstituted enzyme has an RZ value of 2.0, which is comparable with that obtained previously [8]. This apparently lower RZ value compared with that of native HRP may be due to a lower absorption coefficient (ϵ_{396} 65 mM⁻¹·cm⁻¹) of the Fe³⁺-PPDME-reconstituted enzyme [8]. However, the propionate-modified enzyme shows a dramatic decrease in oxidation of guaiacol, iodide and SCN⁻. Although the difference spectra for the binding of guaiacol [27,39] change considerably in Fe³⁺-PPDME-HRP, the K_d value does not alter significantly. However, in the case of SCN⁻ binding, not only was the change in difference spectra observed but the K_d value was also decreased 20-fold, indicating favourable binding. This favoured binding indicates that haem carboxylates have a significant inhibitory effect on the binding of the inorganic anions in the native enzyme, presumably because of formation of a salt bridge between the haem propionate and the nearby positively charged residue, hindering the entry of anion to the binding site [12]. This view was supported by NMR studies showing pH-independent binding of SCN⁻ to the propionate-modified HRP with some decrease in K_d [14]. The favoured binding in Fe³⁺-PPDME-HRP may thus be explained by loss of the salt bridge following esterification of the haem propionates. Apart from donor binding, the enzyme activity is also dependent on the formation of catalytic intermediates with H₂O₂. Although distal His-42 and Arg-38 are involved in the heterolytic cleavage of H₂O₂ to form compound I [28,43], haem carboxylates have a definite effect on it. Although Fe³⁺-PPDME binds apo-HRP at the correct binding site (proximal His-170), it cannot form catalytic intermediates because of the change in orientation of the haem with respect to the surrounding residues, especially His-42 and Arg-38, which are required for compound I formation [28,43]. Our CD measurements show no significant change in conformation of the protein of the reconstituted enzyme, but the haem CD spectrum is almost completely lost. Theoretically, the haem CD bands can be related to the structural basis for the catalytic properties of the peroxidases. The haem CD band intensity is determined by the conformation of the haem with respect to the residues surrounding it [44]. The haem CD spectrum of Fe³⁺-PPDME-HRP is lost because of the change in orientation of the haem, resulting in the loss of the intense Cotton effect due to haem π - π^* transition with the surrounding aromatic amino acids [45]. The interaction of the haem propionates with the nearby positively charged amino acid residues [46] seems to be vital for the correct orientation of the haem to offer asymmetry of haem-protein interaction [44,47]. This plays an important role in the formation of the catalytic intermediates and associated catalytic activity. Presumably because of the change in the orientation of the Fe³⁺-

PPDME-haem, the distance between the haem iron and the distal histidine and arginine residues is too great for catalysis of heterolytic cleavage of H₂O₂ to occur.

In addition to its oxidative activity, HRP catalyses various reductive reactions with pseudocatalytic decomposition of H₂O₂ to O₂ [31,32]. We have shown previously that HRP catalyses reduction of iodine (I⁺) by EDTA in the presence of H₂O₂ through the formation of an active enzyme-I⁺-EDTA ternary complex [15-19]. For this reductive activity, EDTA binds at or near the iodide-binding site of the enzyme [17,18,48]. However, I⁺ binding to the enzyme is facilitated by deprotonation of an ionizable residue of pK_a 4.8, which is presumably contributed by the haem propionate [19]. The present study indicates that the Fe³⁺-PPDME-HRP does not bind I⁺ at all, although it binds EDTA with the same K_d as the native enzyme [17]. Thus haem propionate is involved in the binding of I⁺ during the reductive reaction. At pH above 4.8 (pK_a of propionic acid), the negatively charged propionate facilitates I⁺ binding, probably through electrostatic interaction. However, as compound I and II formation is required for reductive activity [19], the loss of intermediate formation in Fe³⁺-PPDME-reconstituted HRP is the major cause for the blockade of this reductive activity.

It is intriguing how propionates influence binding affinity of the donors, reactivity with H₂O₂ and orientation of the haem moiety. The recently published crystal structure of HRP-C [33] and ¹H-NMR studies on aromatic donor binding using wild-type and mutant enzymes [30] have provided valuable information on the structure-function relationship of HRP. The stability of the haem and surrounding protein structure, which governs catalytic activity by regulating the entry and binding of electron donors and formation of catalytic intermediates, is greatly influenced by the hydrogen-bonding network around the haem where haem propionates play a significant role [33]. Both mutant studies and crystal structure have identified a critical role for Phe-179 in aromatic donor (benzhydroxamic acid) binding [30,33]. Our studies indicate that propionate-modified enzyme binds guaiacol with similar K_d to that of the native enzyme, indicating that guaiacol binding at this site is not influenced by the haem propionate, although C-17 propionate is close to this site [30]. Perhaps guaiacol binding is mainly influenced by hydrophobic interaction with the Phe-179 residue. However, the hydrogen-bonding network, particularly through the haem propionate, appears to control the entry and binding of inorganic anionic substrates (I⁻, SCN⁻) to the active site. The crystal structure shows the existence of direct hydrogen bonds between the protein and the haem propionates through Gln-176, Ser-73, Ser-35 and Arg-31. The distal pocket is connected to the proximal side by a hydrogen-bonding network involving Arg-38, which is hydrogen-bonded through water-41 to a haem propionate. On esterification of this propionate, this hydrogen-bonding network is disrupted, which perhaps favours the entry and binding of anions such as SCN⁻ to the active site, as observed in this study. When both the propionates are esterified, the hydrogen-bonding network in the haem cavity is seriously perturbed, resulting in destabilization of the haem and the surrounding protein structure, as evidenced by the loss of the haem CD spectrum. This destabilization prevents haem reactivity with H₂O₂ for compound I formation [28,43] and blocks catalytic activity. As the co-ordinates of HRP-C and its donor complex are now available from crystallographic data at Brookhaven, it will be interesting to reanalyse our data and our proposition with the help of computer graphics, which will be attempted soon. A crystal structure of Fe³⁺-PPDME-HRP is also expected to be produced to establish the critical role of haem propionates in controlling the catalytic activities of HRP.

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