Probing the active site residues in aromatic donor oxidation in horseradish peroxidase: involvement of an arginine and a tyrosine residue in aromatic donor binding

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The plausible role of arginine and tyrosine residues at the active site of horseradish peroxidase (HRP) in aromatic donor (guaiacol) oxidation was probed by chemical modification followed by characterization of the modified enzyme. The arginine-specific reagents phenylglyoxal (PGO), 2,3-butanedione and 1,2-cyclohexanedione all inactivated the enzyme, following pseudo-first-order kinetics with second-order rate constants of $24 \text{ M}^{-1} \cdot \text{min}^{-1}$, $0.8 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $0.54 \text{ M}^{-1} \cdot \text{min}^{-1}$ respectively. Modification with tetranitromethane, a tyrosine-specific reagent, also resulted in 50% loss of activity following pseudo-first-order kinetics with a second-order rate constant of $2.0 \text{ M}^{-1} \cdot \text{min}^{-1}$. The substrate, H_2O_2 , and electron donors such as I^- and SCN^- offered no protection against inactivation by both types of modifier, whereas the enzyme was completely protected by guaiacol or *o*-dianisidine, an aromatic electron donor (second substrate) oxidized by the enzyme. These studies indicate the involvement of arginine and tyrosine residues at the aromatic donor site of HRP. The guaiacol-protected phenylglyoxal-modified enzyme showed almost the same binding parameter (K_d) as the native enzyme, and a similar free energy change (ΔG°) for the binding of the donor. Stoichiometric studies with $[7\text{-}^{14}\text{C}]$ phenylglyoxal showed incorporation of 2 mol of phenylglyoxal per mol of enzyme, indicating modification of one arginine residue for complete inactivation.

The difference absorption spectrum of the tetranitromethane-modified against the native enzyme showed a peak at 428 nm, characteristic of the nitrotyrosyl residue, that was abolished by treatment with sodium dithionite, indicating specific modification of a tyrosine residue. Inactivation stoichiometry showed that modification of one tyrosine residue per enzyme caused 50% inactivation. Binding studies by optical difference spectroscopy indicated that the arginine-modified enzyme could not bind guaiacol at all, whereas the tyrosine-modified enzyme bound it with reduced affinity (K_d 35 mM compared with 10 mM for the native enzyme). Both the modified enzymes, however, retained the property of the formation of compound II (one-electron oxidation state higher than native ferriperoxidase) with H_2O_2 , but reduction of compound II to native enzyme by guaiacol did not occur in the PGO-modified enzyme, owing to lack of binding. No non-specific change in protein structure due to modification was evident from circular dichroism studies. We therefore suggest that the active site of HRP for aromatic donor oxidation is composed of an arginine and an adjacent tyrosine residue, of which the former plays an obligatory role in aromatic donor binding whereas the latter residue plays a facilitatory role, presumably by hydrophobic interaction or hydrogen bonding.

INTRODUCTION

It is well established that oxidation of aromatic donors (AH_2) by horseradish peroxidase (HRP; EC 1.11.1.7; donor- H_2O_2 oxidoreductase) occurs with two one-electron transfer reactions through the intermediate formation of compound I and compound II [1–3] as shown below:

where compound I and compound II are two- and one-electron oxidation states higher than native ferriperoxidase respectively. Compound I is an oxoferryl porphyrin- π -cation radical [4,5] formed by the reaction of ferriperoxidase with H_2O_2 . In cyto-

chrome *c* peroxidase (CCP), compound I is formed by heterolytic cleavage of the O–O bond of H_2O_2 with the help of the distal His-52 and Arg-48 residues [6–9]. Because these residues are highly conserved in several peroxidases [10,11], a similar role for these residues in compound I formation in HRP would be expected [3].

It has been suggested that aromatic donors bind at a hydrophobic site in the vicinity of the haem peripheral 8-methyl group [12–15]. On the basis of optical difference spectroscopy [12,16,17], hyperfine shifted NMR spectroscopy [18], and nuclear magnetic relaxation studies [12,19,20], a number of workers [12–20] have reported that the enzyme binds aromatic donor to form a 1:1 enzyme–donor complex. The binding presumably occurs at a distance of 5.8–11.2 Å from the haem iron centre [12,19]. Sakurada et al. [14] and Thanabal et al. [15] suggested, on the basis of NMR studies and computer-aided calculation, that the most probable binding site is a hydrophobic pocket composed of 8-CH₃, Tyr-185 and Arg-183 at the haem periphery. This is based on the report that the fundamental conformation of HRP is similar to that of CCP [21] and the coordinates of the side chain

Abbreviations used: HRP, horseradish peroxidase; HRP-C*, non-glycosylated recombinant horseradish peroxidase; CCP, cytochrome *c* peroxidase; PGO, phenylglyoxal; BD, 2,3-butanedione; CHD, 1,2-cyclohexanedione; TNM, tetranitromethane; BHA, benzhydroxamic acid; ABTS, 2,2'-azino-bis(3-ethylbenzothiazolinesulphonic acid); PABA, *p*-aminobenzoic acid.

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of the haem peripheral Arg-183, His-42, His-170, Tyr-185 and Arg-183 residues of HRP are similar to Arg-48, His-52, His-175, Tyr-187 and Ser-185 of CCP. It is also proposed, from a model of the HRP active site based on haem alkylation studies, that electrons from substrates are transferred to the exposed haem edge in the vicinity of δ -mesocarbon before reaching the haem ferryl group during oxidation [22]. Although a non-glycosylated recombinant HRP (HRP-C*) is now available [23], very little is known about the role of the active site residues in aromatic donor oxidation by site-directed mutagenesis. Replacement of Phe-41 with valine was shown to cause decrease to one-eighth in the rate constant of compound I formation, with a significant increase in the oxidation of benzhydroxamic acid (BHA) but a decrease in oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-sulphonic acid) (ABTS); in contrast, mutation with tryptophan caused a loss of binding of BHA [24,25]. Mutation of Arg-38 with lysine was also shown to cause a loss of binding of BHA [25]. However, these observations are difficult to interpret in terms of the precise role of these residues in aromatic donor oxidation. Recently Bhattacharyya et al. [26] proposed from chemical modification studies that distal His-42 in HRP is involved in iodide oxidation. This residue is not required for guaiacol binding but is essential for electron transfer from bound donor to compound I [27]. Although preliminary X-ray data on the properties of the crystal of HRP-C* are now available [28], a high-resolution X-ray structure of native glycosylated HRP-C is still lacking. A chemical modification study, with its direct probing of the structure of the active site, is one of the approaches to get information of this sort. In continuing efforts to elucidate the fine structures involved in donor oxidation, we have performed chemical modification experiments with various arginine-specific [29,30] and tyrosine-specific [31,32] modifiers to probe the role of active site arginine and tyrosine residues in aromatic donor oxidation. The evidence presented here indicates for the first time that the active site domain for aromatic donor (guaiacol) oxidation in HRP consists of an arginine residue and a tyrosine residue, of which the former is essential for binding the donor at the active site and the latter plays a facilitatory role, presumably through hydrophobic interaction or hydrogen bonding.

MATERIALS AND METHODS

HRP ($A_{403}/A_{278} = 3$), phenylglyoxal (PGO), 2,3-butanedione (BD), 1,2-cyclohexanedione (CHD), tetranitromethane (TNM), *o*-dianisidine and guaiacol were purchased from Sigma. [7- 14 C]Phenylglyoxal was obtained from Bhabha Atomic Research Centre. Sephadex G-25 was from Pharmacia. All other chemicals used were of analytical grade.

Enzyme assay

Peroxidase-catalysed guaiacol oxidation was monitored at 470 nm as described previously [33]. 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 μ M H_2O_2 added last to start the reaction. All kinetics and spectral studies were carried out in a Shimadzu UV-2201 computerized spectrophotometer at $28 \pm 2^\circ\text{C}$.

Arginine modification

HRP (2 μ M) in 50 mM sodium phosphate buffer, pH 7.4, was incubated with various concentrations of freshly prepared PGO in 100 % ethanol at $28 \pm 2^\circ\text{C}$ [29]. Controls with the same

amount of 100 % ethanol were run simultaneously. Periodically, 2 μ l aliquots of enzyme in the presence or the absence of inhibitor were withdrawn and added to a standard enzyme-assay mixture for activity measurements. Similarly, inactivation studies with various concentrations of CHD or BD were also performed at $28 \pm 2^\circ\text{C}$ in the presence of 50 mM borate buffer, pH 8.0 [30].

Modification of HRP with tetranitromethane

To 1 μ M HRP in 100 mM Tris/HCl buffer, pH 8.0, was added various concentrations of TNM in 95 % ethanol [31] so that the final ethanol concentration did not exceed 10 %. The mixture was incubated at $28 \pm 2^\circ\text{C}$ for various times and 1 μ l aliquots were assayed for enzyme activity.

Substrate protection

HRP (2 μ M) was incubated with 40 mM PGO in 50 mM sodium phosphate buffer, pH 7.4, or 75 mM BD or 125 mM CHD in 50 mM borate buffer, pH 8.0, or 40 mM TNM in 100 mM Tris/HCl buffer, pH 8.0, in the presence or the absence of 100 μ M electron donor such as guaiacol, *o*-dianisidine, iodide or thiocyanate at $28 \pm 2^\circ\text{C}$ for 30 min before assaying an aliquot for enzyme activity.

[7- 14 C]Phenylglyoxal incorporation

HRP (30 μ M) was incubated at 30°C for 30 min with 40 mM PGO in the presence of 1 mM guaiacol in 50 mM sodium phosphate buffer, pH 7.4. The donor-protected enzyme was passed through a Sephadex G-25 column and further modified with 10 mM [7- 14 C]PGO (10 μ Ci) in 50 mM sodium phosphate buffer, pH 7.4. Enzyme activity and [7- 14 C]PGO incorporation were followed with time. For measurement of radioactivity, a 0.1 ml sample precipitated with 5 % trichloroacetic acid was filtered on PGO-soaked GF/C filters (Whatman) and washed several times with 5 % trichloroacetic acid, water and 95 % ethanol. The sample was dried in an oven and counted in a liquid-scintillation counter.

Difference spectrum measurement

Difference optical spectra (enzyme-donor versus enzyme) were recorded in a Shimadzu UV-2201 computerized spectrophotometer as described [13,34–36]. The apparent dissociation constant, K_d , was calculated from the following expression [36]:

$$\frac{1}{[S]} = \frac{[E]\Delta\epsilon}{K_d\Delta A} \frac{1}{K_d} \quad (5)$$

where ΔA is the difference in absorbance between the peak and the trough of the spectrum, $\Delta\epsilon$ is the difference in molar absorptivity [13] 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 earlier [13]. Absorbances were corrected for the dilution (approx. 1 %) caused by donor addition. 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 $1/[S]$ [34]. The free energy change, $\Delta G'$, for binding of donor was calculated from expression [13]:

$$\Delta G' = RT \ln K_d \quad (6)$$

where R is the universal gas constant and T is the temperature in K.

Circular dichroism measurement

The CD spectra were obtained in a Jasco spectropolarimeter J-600. Native, PGO-modified or TNM-modified HRP was purged with nitrogen and CD measurement was conducted from 185 to 250 nm.

RESULTS

Arginine modification

The time courses of inactivation of HRP by various concentrations of PGO, BD and CHD are shown in Figures 1A–1C. In the absence of borate buffer, BD and CHD did not inactivate the enzyme. Semilogarithmic plots of percentage activity remaining against time are linear, suggesting that inactivation follows pseudo-first-order kinetics and is due to a simple chemical event.

Figure 1 Kinetics of the inactivation of HRP by (A) PGO, (B) BD and (C) CHD

Preincubation with the modifier and assay of enzyme activity were as described in the text. The concentration of the modifier used is indicated in the figure. Insets (a) show plots of K_{obs} determined from the slope of the inactivation kinetics, as a function of modifier concentration to determine the second-order rate constant; insets (b) show plots of $\log 1/t_{1/2}$ of inactivation against $\log(\text{modifier concentration})$ to determine the reaction order.

Figure 2 Kinetics of inactivation of HRP by TNM

Preincubation with modifier and assay of enzyme activity were as described in the text. Inset (a) is the plot for determination of the second-order rate constant and inset (b) shows a plot for determination of reaction order.

Figure 3 Optical difference spectra of TNM-modified HRP against native HRP

Both native and TNM-modified HRP were passed through a Sephadex G-25 column and their optical difference spectrum was taken as described in the text. The concentration of enzyme used was $5.0 \mu\text{M}$.

A replot of the first-order rate constants of inactivation against inhibitor concentrations (Figures 1A–1C, inset a) is linear for each reagent, giving second-order rate constants of $24 \text{ M}^{-1} \cdot \text{min}^{-1}$, $0.8 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $0.54 \text{ M}^{-1} \cdot \text{min}^{-1}$ for PGO, BD and CHD respectively. Replots of $\log 1/t_{1/2}$ against $\log [PGO]$, $\log [BD]$ or $\log [CHD]$ [37] resulted in a straight line (Figures 1A–1C, insets b) with slopes (reaction orders) of 1.15, 1.01 or 0.87 respectively, indicating that modification of a single arginine residue per enzyme active site causes a loss of HRP's guaiacol-oxidizing activity.

Tyrosine modification

Treatment of HRP with TNM also resulted in time-dependent loss of enzymic activity to oxidize guaiacol. The inactivation follows pseudo-first-order kinetics (Figure 2). Kinetic analysis of inactivation yielded a second-order rate constant of $2.0 \text{ M}^{-1} \cdot \text{min}^{-1}$ (Figure 2a) and a reaction order of 0.98 (Figure 2b). A difference spectrum of the modified against the native enzyme (Figure 3) showed an absorption maximum at 428 nm, typical of a nitrated tyrosine residue [38]. Reduction of the nitrated HRP with sodium dithionite resulted in the disappearance of the absorption maximum at 428 nm, indicating

Figure 4 Correlation between inactivation and tyrosine modification

HRP ($5.0 \mu\text{M}$) was incubated with 40 mM TNM in 50 mM Tris/HCl buffer, pH 8.0. The percentage residual activity and the increase in absorbance at 428 nm were plotted as a function of time. The inset shows the correlation between the number of tyrosine residues modified per HRP molecule for 50% inactivation.

Table 1 Protection of HRP by electron donors against inactivation by PGO, BD, CHD and TNM

The enzyme, in the presence or absence of various electron donors ($100 \mu\text{M}$), was treated with PGO (40 mM), BD (75 mM), CHD (125 mM), or TNM (40 mM) for 30 min before assay of an aliquot after appropriate dilution

Experiment	Activity (%)			
	PGO	BD	CHD	TNM
PGO	0	—	—	—
BD	—	10	—	—
CHD	—	—	20	—
TNM	—	—	—	50
Guaiaicol	100	81	70	82
<i>o</i> -Dianisidine	80	60	60	70
Iodide	0	10	21	45
Thiocyanate	0	9	20	50

specific nitration of a tyrosine residue. The stoichiometry of the formation of 3-nitrotyrosine was calculated from the increase in absorbance at 428 nm by using the extinction coefficient of $4100 \text{ M}^{-1}\text{cm}^{-1}$ [38]. When 50% activity had been lost, the modification of one tyrosine was almost complete (Figure 4). Further increasing the incubation time or the concentration of TNM did not increase the inactivation. This suggests that a single tyrosine residue is also involved in guaiacol oxidation but the requirement is not obligatory.

Substrate protection against inactivation

In an attempt to locate the site of the essential arginine and tyrosine residues, protection from inhibition by substrate (electron donors) was examined. Guaiacol, *o*-dianisidine (aromatic electron donor), KI and SCN^- are substrates for HRP. However, inactivation by PGO, BD, CHD and TNM was prevented only by aromatic donors (Table 1), suggesting the presence of these residues at the aromatic donor binding site. The primary substrate, H_2O_2 , offered no protection at all.

Figure 5 Correlation between inactivation of HRP and PGO incorporated

The detailed procedure for modification with PGO, assay of enzyme activity and radioactivity incorporated into HRP are described in the text.

Figure 6 Optical difference spectra of (A) HRP-guaiacol complex against HRP, (B) guaiacol-protected PGO-modified HRP-guaiacol complex against guaiacol-protected PGO-modified HRP, (C) PGO-modified HRP-guaiacol complex against PGO-modified HRP and (D) TNM-modified HRP-guaiacol complex against TNM-modified HRP

In (A) to (C) the concentration of HRP used was $7.5 \mu\text{M}$ and those of guaiacol were 3, 6, 9, 12 and 18 mM. In (D), the HRP concentration was $9.0 \mu\text{M}$ and those of guaiacol were 30, 45, 60 and 75 mM.

[7- ^{14}C]phenylglyoxal incorporation

The number of arginine residues modified was measured by [7- ^{14}C]PGO incorporation into the enzyme (Figure 5): 2 mol of PGO are incorporated per mol of enzyme when the enzyme is almost completely inactivated. Because two PGO moieties are required for the formation of a stable complex with the guanidine

Figure 7 Soret spectra of compound II of (A) native, (B) guaiacol-protected PGO-modified HRP and (C) PGO-modified HRP

Enzyme ($0.5 \mu\text{M}$) in 50 mM sodium phosphate buffer, pH 7.4, (tracing a); compound II formed by an H_2O_2 concentration 20 times that of the enzyme (tracing b). Tracing c represents the spectrum obtained after an adding equimolar concentration of guaiacol to the incubation mixture of tracing b. PGO-modified and guaiacol-protected PGO-modified enzymes were prepared as described in the text.

group of arginine [29], modification of a single arginine residue is thus essential for inactivation.

Guaiacol binding in native, modified and protected HRP

The binding of guaiacol to native, modified or donor-protected HRP was studied by optical difference spectroscopy, and its K_d was calculated [34–36]. The difference spectra of native enzyme against that of its guaiacol complex (Figure 6A) are similar to that for the protected enzyme (Figure 6B) having the same peak at 408 nm. In contrast, the PGO-modified enzyme does not bind guaiacol at all, showing no difference spectra (Figure 6C). The K_d for the native HRP–guaiacol complex (10 mM) was almost the same as that for the protected enzyme (9 mM). The TNM-modified enzyme binds guaiacol with characteristic difference spectra (Figure 6D) but with less affinity (K_d 35 mM). The guaiacol-protected enzyme, however, has a similar K_d value (12 mM) to native enzyme. The decreased affinity is due to a decrease in the binding energy ($\Delta G' = -8.3 \text{ kJ/mol}$) compared with the native enzyme ($\Delta G' = -11.4 \text{ kJ/mol}$).

Figure 8 CD spectra of native, PGO-modified and TNM-modified HRP

HRP ($2.0 \mu\text{M}$) was modified with PGO or TNM as described in the text. The modified enzyme was desalted through a Sephadex G-25 column before the CD spectra were taken. (A) Native (tracing a) and PGO-modified (tracing b) HRP; (B) native (tracing a) and TNM-modified (tracing b) HRP.

Formation of catalytic intermediate in native, modified and protected HRP

Because catalytic intermediates are essential for one-electron oxidation of guaiacol, the formation of stable compound II by H_2O_2 was studied in native, PGO-modified and donor-protected HRP (Figure 7). Formation of compound I was not studied because it is not stable for spectrophotometric studies [39]. All these enzymes form compound II with H_2O_2 , with a characteristic Soret peak at 418 nm (Figures 7A–7C) and visible peaks (results not shown) at 527 and 556 nm [39]. Thus the arginine involved in donor binding does not take part in compound II formation. Moreover, both native (Figure 7A) and the guaiacol-protected compound II (Figure 7B) are immediately reduced to the native state (403 nm) by guaiacol, whereas modified compound II (Figure 7C) is not, as the latter does not bind guaiacol (Figure 6C). The TNM-modified enzyme also produced compound II similar to the native enzyme and was reduced to the native state by guaiacol (results not shown) although the latter binds with reduced affinity (Figure 6D).

CD measurement of native and modified HRP

The UV CD spectra, specifically the minima at 221 and 208 nm [40] are characteristic of helical secondary structure [41]. The result (Figure 8) excludes the possibility of inactivation being due to changes in the α -helix, β -sheet or random coil structure of the enzyme after modification with PGO or TNM.

DISCUSSION

Treatment of HRP with three arginine-specific modifiers [29,30,32,42,43] causes irreversible inactivation of the enzyme as regards guaiacol oxidation. Kinetic studies indicate the essential role of only one arginine residue in catalysis. This is established by stoichiometric studies by radiolabelled PGO, where two PGO moieties were incorporated per enzyme molecule for almost

Figure 9 Schematic representation of the distal haem pocket of HRP

This is the computer-simulated secondary structure proposed by Sakurada et al. [14]. AD represents the aromatic donor, which is assumed to bind HRP by a hydrophobic bond with Tyr-185 and a hydrogen bond with Arg-183 and other amino acids.

complete inactivation due to modification of a single arginine out of a total of 21 residues. It is probable that BD and CHD react with several other arginine residues, presumably on the surface of the protein and not related with the activity, but only reaction with the active site residue caused inactivation. The second-order rate constants of inactivation indicate that PGO is more potent than BD and CHD owing to its hydrophobic aromatic ring, which is more accessible to the hydrophobic site where the aromatic donor is proposed to bind [12–15,43]. Specific protection by aromatic donors, not by I^- or SCN^- , indicates that the arginine residue is located at the aromatic donor binding site, away from the I^- or SCN^- binding site [44–47]. Kinetic and stoichiometric studies after TNM modification indicate that 50% inactivation is due to modification of a single tyrosine residue only. Specific protection by aromatic donors further suggests the presence of this residue near the aromatic donor binding site. The increased absorption at 428 nm and its reversal by dithionite indicates specific formation of 3-nitrotyrosine by TNM. The other potential targets are cysteine, lysine and tryptophan residues [48,49]. Because cysteine residues are in a disulphide linkage [50] and nitrolysine and the 7-indole derivative of tryptophan are insensitive to dithionite, TNM modification of these residues is excluded. Almost complete inactivation by arginine-specific modifiers but not by a tyrosine-specific modifier indicates that arginine is obligatory for activity whereas tyrosine is not. The following three possibilities have been proposed to explain the role of these residues in catalysis.

(i) They could control the secondary structure of HRP. The CD analysis indicates that the secondary structure is not perturbed by PGO or TNM.

(ii) They could be involved in compound I/II formation required for donor oxidation. The amino acid sequence of several peroxidases at the haem distal side [11] shows that both histidine and arginine residues are highly conserved. The core of HRP-C and CCP, their active site and substrate access channel, are also conserved [51]. The proposed model structure based on molecular dynamics calculation and energy minimization indicates a resemblance of His-42 and Arg-38 of HRP to the His-52 and Arg-48 of CCP [52]. These residues are therefore implicated in compound I formation in HRP [3]. Our studies indicate that modification of a single arginine residue by PGO does not affect compound II formation and H_2O_2 does not protect the enzyme

against inactivation, suggesting that Arg-38 is not modified by PGO. The alternative possibility, that Arg-38 has no role in compound II formation, goes against the mechanism of compound I formation by distal histidine and arginine residues [3] and hence can be excluded. The TNM-modified enzyme also produces compound II similarly to the native HRP, indicating that there is no role of tyrosine residue in compound II formation. Thus modification of these residues alters the activity other than by blocking compound I/II formation.

(iii) The residues could be involved in the binding of the aromatic donors. Aromatic donors were suggested to bind with some aromatic residues with hydrophobic bonds [13] at a distance of about 5.8–11.2 Å from the haem iron [12,19,20]. Sakurada et al. [14] proposed that aromatic donors bind at a hydrophobic site comprising the haem 8-methyl group and Tyr-185 and Arg-183 (Figure 9) and this has been confirmed by further NMR experiments [15,53,54]. The size of the space in this area is suitable to accommodate the aromatic donor through the hydrophobicity offered by Tyr-185 and hydrogen bonding by Arg-183. Our studies indicate that the PGO-modified enzyme does not bind guaiacol at all whereas TNM-modified enzyme does, although with less affinity. This suggests that this arginine is obligatory in aromatic donor binding, probably for hydrogen bonding, whereas the tyrosine residue seems to facilitate it through hydrophobic interaction or hydrogen bonding. However, steric hindrance caused by nitrotyrosine formation by TNM may also take part in inactivation. As only one arginine and one tyrosine residue are modified by the reagents, we speculate that Arg-183 and Tyr-185 of the proposed aromatic donor binding site [14] have been modified to inactivate the enzyme. This is supported by the fact that these residues are also highly conserved in several peroxidases [11,21]. Although Arg-178 is also present close to Arg-183 in HRP, its involvement is excluded as it is not conserved in several plant peroxidases [11,21] and it is possibly not oriented towards the haem cavity of HRP [14,52]. Thanabal et al. [55] also tentatively assigned Tyr-185 to the aromatic donor binding site. However, Veitch and Williams [56] have considered the role of several conserved phenylalanine residues in the haem pocket, of which Phe-172 is a likely candidate. This diversity of opinion regarding the role of tyrosine or phenylalanine in aromatic donor binding will be resolved in future by X-ray crystallographic studies. Recently Smith et al. [24] reported that mutation of Phe-41 (which is conserved in several peroxidases [50,54]) with valine in their recombinant HRP-C* leads to increased *p*-aminobenzoic acid oxidation but decreased ABTS oxidation, suggesting that Phe-41 modulates the reactivity of different reducing substrates by perturbing the neighbourhood of the aromatic donor binding site. However, the mutation experiments reported so far [24,25,57] are conflicting, possibly owing to alterations of the haem-linked hydrogen bonding network in the haem distal pocket [54,58]. Recently, Banci et al. [52] proposed the location of a *p*-cresol binding site between the 8-CH₃ and Phe-68 moieties and suggested that hydrophobic interaction between the substrate and the protein residues might pull the donor away from other possible primary sites.

We speculate that if the primary binding site consists of a hydrophobic pocket created by Arg-183 and Tyr-185 near the 8-CH₃ group at the substrate access channel [14,51], aromatic donors will be primarily oxidized by electron transfer from this site to the haem ferryl group through the nearby δ -mesocarbon [44] owing to the electron attracting effect of compound I, before proceeding to the more hydrophobic site. The aromatic donor is hydrogen-bonded to the distal His-42 [3,59] when it binds at the hydrophobic site [14]. When an electron is transferred from the

donor to compound I, a proton passes to the distal histidine along the hydrogen bond, resulting in homolytic cleavage of the H–O bond of the donor to form a free radical and compound II [59]. We have shown that His-42 controls the oxidation of aromatic donor by regulating the electron transfer mechanism through concomitant proton flow along the hydrogen bond without affecting the donor binding [27]. We now conclude that the aromatic donor binds at the active site consisting of an arginine and a tyrosine residue, of which the former plays an obligatory role, presumably through hydrogen bonding, and the latter plays a facilitatory role through hydrogen bonding and/or hydrophobic interaction.

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