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Role of Active Site Residues in Peroxidase Catalysis : Studies on Horseradish Peroxidase

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Peroxidase catalyses oxidation of both aromatic and inorganic electron donors with H_2O_2 through the intermediate formation of compound I and compound II. Much information is now available on the mechanism of peroxidase-catalysed formation of enzyme intermediates as well as on the oxidation of electron donors using horseradish peroxidase (HRP) as a model enzyme. The recently available x-ray crystal structure of HRP has furnished valuable informations on the critical role of Arg-38, His-42, Phe-179 and heme propionates of the heme distal pocket in peroxidase catalysis. Site-directed mutagenesis studies have clearly established that histidine-42 and arginine-38 are actively involved in the heterolytic cleavage of H_2O_2 during compound I formation. NMR studies have indicated that inorganic donors such as iodide and thiocyanate bind at a site at equal distance from heme peripheral 1 and 8 CH_3 groups and the binding is controlled by protonation of an ionisable group of pK_a value around 4 presumably contributed by heme propionic acid. However, recent mutant and NMR studies have implicated the critical role of Phe-179 in aromatic donor binding which has been confirmed in the x-ray crystal structure also. Heme propionates also play an important role in donor oxidation by controlling the formation of compound I. Recent studies indicate that heme propionates maintain the proper orientation of the heme moiety with respect to its surrounding residues for catalytic formation of compound I. They appear to control inorganic donor oxidation by regulating the entry of the donors at the active site through the formation of salt-bridge with the nearby positively charged residue. HRP also shows oxidase activity through the intermediate formation of compound I, II and III by endogenous H_2O_2 through a peroxidase-oxidase oscillatory reaction. Under certain condition, the enzyme also shows reductive reaction with pseudocatalytic decomposition of H_2O_2 to O_2 . Except in the case of sulfoxidation of aryl thioethers by H_2O_2 in presence of molecular oxygen, HRP cannot show peroxygenase reaction by transferring the ferryl oxygen to the donor molecule due to steric hindrance caused by His-42 and Phe-41.

Key Words: Horeseradish peroxidase; Peroxidase intermediate formation; Aromatic donor oxidation, Inorganic ion oxidation, Heme propionates, Peroxidase catalysis, Peroxidase oxidase activity, Pseudocatalase with reductive activity, Peroxygenase activity

Introduction

Peroxidase, a widely distributed enzyme in both plant and animal kingdom (Saunders et al. 1964, Dunford & Stillman 1976), catalyses the oxidation of a large variety of substrates including aromatic electron donors and inorganic anions to exert a wide spectrum of biological functions. Thyroid peroxidase (Magnusson 1991), for example, catalyses the oxidation of iodide to form thyroid hormones. In salivary and lacrimal gland and their secretion (Banerjee & Dutta 1986, Mazumdar et al. 1996), the enzyme catalyses the oxidation of SCN- to form OSCN- to exert bactericidal action. Similar function has

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been attributed to lactoperoxidase (Pruitt & Tenovuo 1985) and eosinophil peroxidase (Slungaard & Mahoney 1991). Myeloperoxidase of neutrophil (Klebanoff & Clark 1978) oxidises chloride to hypochlorous acid which is highly bactericidal. Peroxidases are also present in the gastro-intestinal tract. While gastric peroxidase (De & Banerjee 1986, Das et al. 1995) may be involved in controlling acid secretion (Bandyopadhyay et al. 1992) and in preventing oxidative damage of the gastric mucosa (Das et al. 1997), intestinal peroxidase is mainly contributed by the invading eosinophil with bactericidal function (De et al. 1986). It is now almost established that peroxidase plays a very important role in host defense against oxidative damage of the cell by scavenging the intracellular H_2O_2. The following publications deal with the chemistry and biology of peroxidases (Saunders et al. 1964, Dunford & Stillman 1976, Klebanoff & Clark 1978, Greppin et al. 1986, Banerjee & Datta 1986, Banerjee 1988, Banerjee et al. 1990, Dunford 1991, Thomas et al. 1991, Magnusson 1991, Hurst 1991, Hendersen 1991, Ortiz de Montellano 1992).

For the last few decades, peroxidases from both plant and animal sources have been purified and characterized. The most extensively studied peroxidases in relation to structure-function mechanism are horseradish peroxidase from horseradish root, cytochrome c peroxidase from yeast, chloroperoxidase from *Calderomyces fumago*, lignin peroxidase from *Pharerochaete chrysosporium*, lactoperoxidase from milk and myeloperoxidase from neutrophil. The binding of electron donors at the active site and mechanism of oxidation of some of these peroxidases have also been studied (Smith 1995, Ortiz de Montellano 1992). However, several papers have appeared on horseradish peroxidase C (HRPC) which may be considered as the most extensively studied enzyme (Dunford 1991). It may act as a model peroxidase to understand the basic mechanism of peroxidation reaction catalysed by almost all peroxidases. In this review emphasis will be placed mainly on the role of active site residues of HRP in i) the formation of catalytic intermediates such as compound I and II with H_2O_2; ii) mechanism of oxidation of inorganic and aromatic electron donors at the active site and iii) the catalytic activities other than peroxidation e.g. oxidase, pseudocatalase and peroxygenase.

Horseradish Peroxidase and its Active Site Architecture

Several isoenzymes of HRP have been isolated of which HRPC is the most extensively studied enzyme consisting of 308 amino acid residues with the ferric protoporophyin IX as heme prosthetic group and having 18% carbohydrate with two Ca^{2+} per molecule, the total molecular weight being 42100 (Dunford 1991). The complete primary sequence of HRPC alongwith other structural characteristics is now known (Welinder 1979). The amino terminal is blocked by a pyrrolidene-carbonyl residue and c-terminal peptide has been isolated with or without a terminal serine suggesting chemical lability. The heme iron (Fe^{3+}) is attached through fifth coordination position to the imidazole group of His-170 at the proximal side. The carbohydrate residues located at the surface of the molecule are attached to the asparagine residues at positions 13, 57, 158, 186, 198, 214, 250 and 268. Four disulfide bridges occur between positions 11-91, 44-49, 97-301 and 117-209. The glycosylation sites has now been identified in native HRPC from its x-ray crystal structure (Gajhede et al. 1997). All sites are N-glycosylation sites located at the loop regions which are exterior to the core. These sites are distributed over the entire surface of the molecule pointing outside thereby increasing solubility in water and rendering resistance to free radical induced cross-linking of the protein. A nonglycosylated recombinant HRPC* has been shown to regain its activity only in presence of heme and Ca^{2+} indicating that glycosylation is not essential for acivity (Smith et al. 1990). Of the two Ca^{2+} present per mole of enzyme, only

one Ca^{2+} maintains the protein structure at the heme environment favouring the enzyme activity (Shiro et al. 1986).

Peroxidases have been classified into three distinct types such as intracellular peroxidases of prokaryotic origin (class I), fungal (class II) and plant peroxidases (class III) of which HRPC is included in class III (Welinder 1992). The structural basis of the distinct differences of these peroxidases has been reported (Welinder et al. 1995). A wealth of information on the architecture of the active site of HRPC and its comparison with cytochrome P-450 system has been provided by heme alkylation using alkyl and aryl hydrazines (Ortiz de Montellano 1992). It is interesting to note that cytochrome P-450 transfers the ferryl oxygen to their substrate whereas peroxidases remove an electron from their donor without transferring the ferryl oxygen (formed by reaction with H_2O_2). Although both cytochrome P-450 and HRPC (henceforth termed as HRP) are inactivated by phenylhydrazine, the latter forms a stable phenyl-iron complex with P-450 but similar complex is not observed in case of HRP. Instead inactivation of HRP is associated with covalent binding to the protein alongwith incorporation of phenyl radical to the δ-meso carbon of the heme as well as formation of 8 hydroxymethyl heme derivative due to abstraction of a hydrogen from the 8-methyl group by the phenyl radical. Other hemoproteins such as myoglobin, hemoglobin etc also form phenyl-iron complexes (Ortiz de Montellano 1989). These results suggest that heme iron although accessible in other hemoproteins, is not accessible to the substrates in the case of HRP. Moreover, modification of δ-meso carbon and the nearby 8-CH_3 group indicates that substrates may interact at or near the δ-meso carbon which is not covered by the surrounding protein. A plausible model of the active site of HRP has been proposed by Ortiz de Montellano (1992) as shown in figure 1. In the cytochrome P-450 system, iron is easily accessible to the substrate

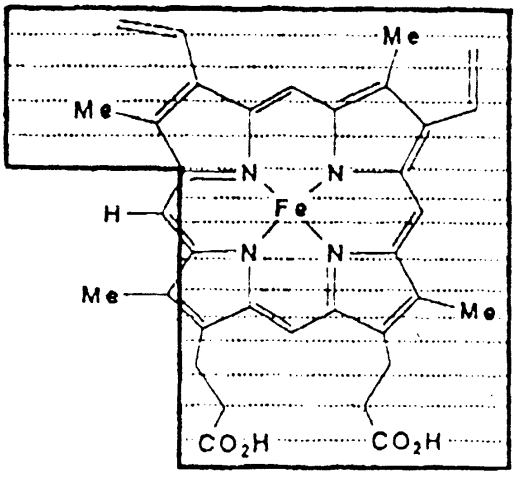

Figure 1 A plausible model of the active site architecture of HRP as proposed by Ortiz de Montellano (1992). The shaded area indicates that this part of the heme is covered by protein structure

resulting in the transfer of ferryl oxygen giving rise to monoxygenase activity while this activity is absent in HRP due to inaccessibility of the heme iron by the substrates because of the presence of some residues, especially the distal His-42 (Ortiz de Montellano 1992). The availability of recombinant expression system (Smith et al. 1990, Hartmann & Ortiz de Montellano 1992) has further helped identifying the residues governing the reactivity of the heme iron towards the substrates (Newmyer & Ortiz de Montellano 1995, 1996, Newmyer et al. 1996).

Although some structural similarity of HRPC has been evident with the peanut peroxidase (PNP) which is the first crystal structure of class III peroxidase available (Schuller et al. 1996), the full structural details of the active site of HRP are available recently when Gajhede et al. (1997) published the crystal structure of HRPC at 2.15 Å resolution. The crystal structure shows that distal heme pocket of HRPC does contain the conserved catalytic residues such as Arg-38, Phe-41, and His-42 (figure 2). The closest water molecule in the distal pocket is 3.2 Å away from the Fe^{III} position indicating that iron in heme is pentacoordinated. All glycosylation sites are distributed over the surface of the molecule perhaps to increase its solubility in

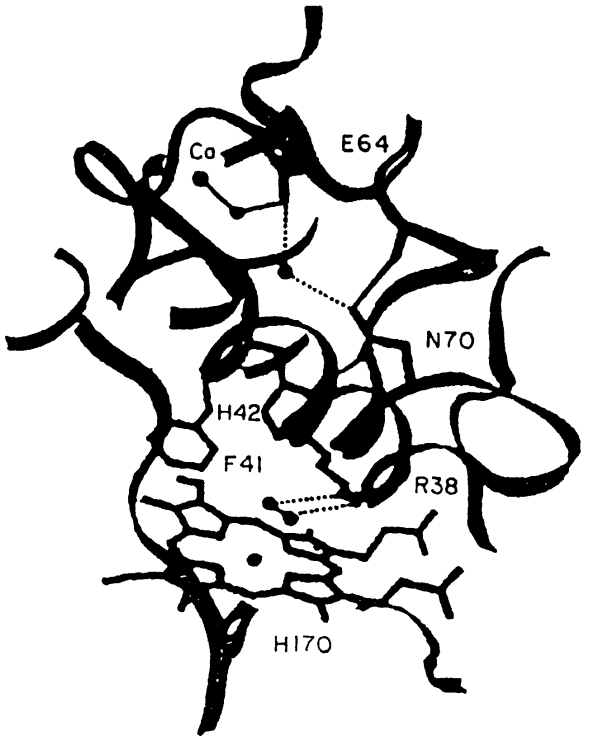

Figure 2 X-ray crystal structure of HRPC as deduced by Gajhede et al. (1997).

water and protection from H_2O_2 and radical-induced damage. The Ca^{2+} site is structurally coupled to the active site through Asp-43. As Asp-43 is close to distal histidine, Ca^{2+} depletion may affect the catalytic activity by distrubing the position of His-42. The heme iron is covalently bonded to the His-170 in proximal side. The $N\delta_1$ of the proximal heme ligand His-170 is hydrogen bonded to Asp-247 which may increase the basicity of His-170 proximal ligand relative to the globins. This may stabilise the high oxidation state of the catalytic intermediates with H_2O_2 by a charge relay system maintaining the heme iron in five-coordinated state. The hydrogen bonding network surrounding the heme which plays an important role in catalytic activity, has also been evident in the crystal structure (Gajhede et al. 1997). Direct hydrogen bond exists between the protein and the heme propionates through Glu-176, Ser-73, Ser-35 and Arg-31. The distal pocket is connected to the proximal side by a hydrogen bonding network through Arg-38 which is hydrogen bonded through water to the heme propionate. A substrate access channel has also been evident from the crystal structure. It has a peripheral hydrophobic layer contributed by several Phe residues and the heme 8-CH_3. The inner channel and lining of the heme cavity has an overall positively charged character due to presence of Arg-38. Phe-68, Phe-142, and Phe-179 form a hydrophobic region near the exposed heme edge which may be involved in aromatic donor binding. This site may take part in the preelectron transfer complex formed between the substrate and the catalytic intermediates of HRP.

Electron Donors

HRP catalyses oxidation of a variety of electron donors (second substrate) by H_2O_2 (primary substrate) through intermediate formation of compound I and compound II. The electron donors include inorganic anions such as I^-, SCN^-, NO_2^-, SO_3^- etc. HRP can also catalyse the oxidation of various aromatic donor molecules such as phenols and aromatic amines. HRP also catalyses oxidation of sulphydryl compounds, NADH, NADPH, indoleacetic acid, epinephrine etc by its oxidase activity. The mechanism of oxidation of some of these electron donors has also been reviewed (Dunford & Stillman 1976, Dunford 1991). The only known peroxygenase reaction catalysed by HRP is the sulphoxidation of aryl thioethers where ferryl oxygen is transferred to the substrate, the mechanism of which has also been reported (Ortiz de Montellano et al. 1995).

Mechanism of Catalytic Intermediate Formation

The formation of catalytic intermediates of HRP with H_2O_2 to form compound I and compound II was studied in great detail by several workers of whom Theorell, George, Chance and Keilin need special mention (Saunders et al. 1964). HRP in normal ferric state reacts with H_2O_2 to form a transient green complex, compound I, which

undergoes a one-electron reduction by an electron donor to form relatively stable compound II (Chance 1949, 1952). The latter further undergoes a one-electron reduction by a second electron donor to regenerate ferriperoxidase to start a new cycle as follows :

$$Fe^{3+}\text{-HRP} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$
$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + AH \quad (2)$$
$$\text{Compound II} + AH_2 \rightarrow Fe^{3+}\text{-HRP} + AH \quad (3)$$
$$AH + AH \rightarrow A + AH_2 \quad (4)$$

where AH_2 represents the aromatic electron donor and $AH^{\bullet}$ is the corresponding one-electron oxidation product (free radical). The radicals may dimerise to generate stable oxidation product A. The electronic structure of the primary green compound I is two-oxidizing equivalents above the resting ferric state (Dunford & Stillman 1976, Dolphin et al. 1971). Magnetic susceptibility data and Mossbauer data (Theorell & Ehrenberg 1951, Schultz et al. 1979) suggest low spin iron (iv) cf S = 1 for both HRP compound I and II, indicating that the second oxidizing equivalent of compound I resides in a free radical of porphyrin. Dolphin et al. (1971) proposed an iron (iv) porphyrin π cation radical for HRP compound I which was later established by extended x-ray absorption fine structure spectroscopy (Penner-Hahn et al. 1986). The heme structure of compound II has been investigated by Mössbauer spectroscopy, electron nuclear double resonance, extended x-ray absorption fine structure spectroscopy and resonance Raman studies and has been found to contain a ferryl heme with an oxene ligand ($Fe^{iv} = O$) (Penner-Hahn et al. 1986).

The formation of compound I occurs by heterolytic cleavage of the O-O bond of H_2O_2 through an electron "push pull" mechanism (Poulos 1987, Dawson 1988, Dunford 1991) originally proposed in case of cytochrome c peroxidase (Poulos & Kraut 1980). The positive distal histidine imidazole (His-42) provides the pull and a partially or fully deprotonated proximal histidine (His-170) provides the push. Similar to cytochrome c peroxidase (Poulos & Kraut 1980), the following steps (figure 3) have been proposed for HRP-compound I formation (Dunford 1991, Ortiz de Montellano 1992). The un-ionized H_2O_2 is converted into a much better nucleophil upon transfer of its proton to a distal

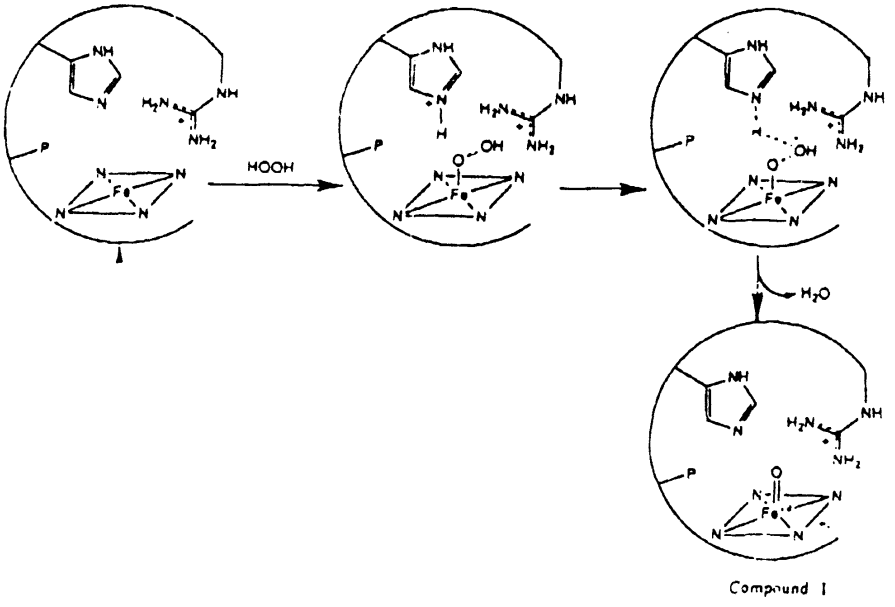

Figure 3. Plausible mechanism of compound I formation of HRP in presence of H_2O_2 (rtiz de Montellano, 1992).

basic group, His-42. Formation of iron-peroxide bond is facilitated by the positive charge on the proximal His-170. Electron flow which occurs from the distal site of the heme to the iron in the first part of the reaction is reversed in the later stages. Negative charge on His-170 and positive charge on His-42 and Arg-38 facilitate the heterolytic cleavage of the O-O bond of H_2O_2 leading to the formation of ferryl group (Fe = O) and H_2O as a leaving molecule.

The one-electron reduction of compound I to compound II by aromatic electron donor (Oertling & Babcock 1988) is associated with a transfer of a proton from the donor to the imidazole nitrogen of distal His-42, which is ultimately released as a second molecule of H_2O when compound II is further reduced to ferric state by another donor molecule as shown in figure 4 (Oertling & Babcock 1988). It is now believed that the aromatic donor is hydrogen bonded to the distal His-42 (Oertling & Babcock 1988) when it binds at a hydrophobic site at the heme distal pocket. When an electron is transferred from the aromatic donor (HA) to

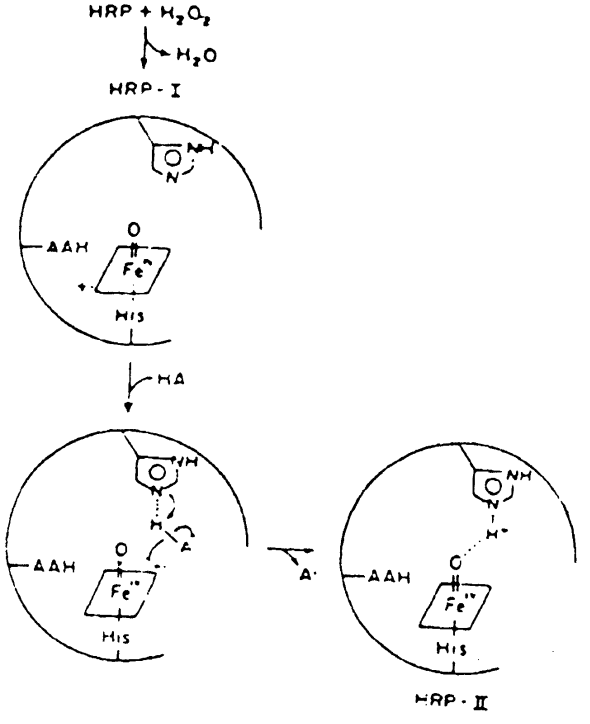

Figure 4 Mechanism of one-electron reduction of compound I by aromatic electron donor (Oertling & Babcock 1988)

the porphyrin π cation radical of compound I, a proton concurrently passes to the distal histidine along the hydrogen bond, resulting in the homolytic cleavage of H-O bond of the donor to form a free radical ($\cdot A$) (Shiga & Imizumi 1975, Bhattacharyya et al. 1993). The inhibitor studies using substituted hydrazines (Ortiz de Montellano 1987, Ator & Ortiz de Montellano 1987) indicated that while electron transfer occurs in the region of δ-meso carbon and 8-methyl group of the heme, concomitant proton transfer takes place from the donor to the distal His-42.

Mechanism of Inorganic Donor Oxidation

The mechanism of oxidation of inorganic electron donors such as iodide and thiocyanate has been extensively studied. Of many inorganic compounds, iodide is the most attractive substrate because it plays an important role in thyroid hormone biosynthesis catalyzed by thyroid peroxidase (Morrison & Schonbaum 1976). Oxidation of iodide occurs in the manner of a single two-electron transfer directly to compound I in contrast to the two one-electron transfers for aromatic donor molecules (Roman & Dunford 1972, Morrison & Schonbaum 1976) according to the following reactions :

$$Fe^{3+}\text{-HRP} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (5)$$
$$\text{Compound I} + I^- \rightarrow Fe^{3+}\text{-HRP} + I^+ \quad (6)$$

The reaction between compound I and iodide takes place through the intermediate formation of enzyme-hypoiodous complex, [EOI] (Morrison & Schonbaum 1976) as follows :

$$E + H_2O_2 \xrightarrow{I^-} EO \rightarrow [EOI] \rightarrow E + IO^- \quad (7)$$
$$IO^- + I^- + H^+ \rightarrow I_2 + OH^- \quad (8)$$
$$I_2 + I^- \Leftrightarrow I_3^- \quad (9)$$

where E represents Fe^{3+}-HRP and EO is compound I. As pK_a of hypoiodous acid is 11, the reactions may be written as

$$[EOI] \xrightarrow{H^+} E + HOI \xrightarrow{I^-} I_2 + \hat{U} \Leftrightarrow OH^- I_3^- \quad (10)$$

These reactions form the basis for the assay of peroxidase activity by I_3^- formation at 353 nm using iodide as electron donor.

The interaction of iodide at the heme distal pocket of HRP has been extensively studied by different workers. Kinetic studies (Bjorkstein 1970, Pommier et al. 1973) suggest that iodide may form a complex near the heme moiety of HRP. Ugarova et al. (1981) reported that iodide does not quench the porphyrin fluorescence at neutral *p*H whereas the quenching is markedly enhanced with lowering of *p*H. This finding suggests that iodide interacts with the heme distal pocket at acidic *p*H. Sakurada et al. (1985), using I^{127}-NMR techniques, reported a strong interaction of I^- with HRP in acid *p*H. It seems likely that the line broadening at acidic *p*H is associated with binding of iodide to HRP. Using transferred nuclear overhauser technique (TRNOE), Sakurada et al. (1987) proposed that iodide binds within 10 Å (most likely within 6 Å) from the heme peripheral 1- and 8-methyl protons. They also suggested that interaction of iodide with HRP depends on the protonation of an ionizable group with the pK_a value of 4-4.3. Similar pK_a value (4.6) was also obtained by others (Roman & Dunford 1972, Ugarova et al. 1981) using stop-flow technique for the reaction between compound I and iodide. The finding that iodide binds to HRP at acidic *p*H generated the idea that protonation of an ionizable group of pK_a value around 4 breaks up the salt-bridge of heme propionic acid with some nearby amino acid residue of the heme pocket facilitating the entry of iodide to the heme crevice which is a aprerequisite for the electron transport from iodide to the heme ferryl group (Sakurada et al. 1987). Moreover, Sakurada et al. (1987) observed from the kinetic studies with catalytically active enzyme that Km of iodide is 4 mM which is far below the K_d value (100 mM) obtained in the binding of iodide to the native enzyme. This suggests that the affinity of iodide to compound I is higher than the native enzyme. Two plausible explanations for this difference were suggested : (a) the formation of Fe (iv) = O and subsequent hydrogen bonding with N-H of imidazole of distal histidine (Hashimoto et al. 1986) may cause some conformational change at the distal side and (b) the π cation radical of compound I may enhance the electrostatic attraction between iodide and the heme. However, from a model on HRP active site based on heme alkylation studies (Ortiz de Montellano 1987) and kinetic and binding data (Sakurada et al. 1987, Harris et al. 1993), it is plausible that iodide binds near the δ-meso carbon in close proximity to the aromatic donor binding site. That both inorganic and aromatic donors bind near the δ-meso heme edge with aromatic site close to 8-CH_3 and inorganic site between 1- and 8-CH_3 groups has been evident from NMR nuclear overhauser interaction with 1- and 8-CH_3 groups (Sakurada et al. 1987, Modi et al 1989, La Mar et al. 1992) and sensitivity to the δ-meso ethyl substitution studies (Harris et al. 1992). Chemical modification studies with diethylpyrocarbonate indicate that distal His-42 is also involved in iodide oxidation (Bhattacharjee et al. 1992). However, it is not clear yet as to which residue is involved in iodide binding in native HRP. Recently iodide binding site has been studied in Arthromyces remosus peroxidase by x-ray crystallography, ^1H and ^{127}I NMR and kinetic studies (Fukuyama et al. 1997). X-ray analysis of the peroxidase crystal soaked in KI solution showed that iodide binds about 10 Å away from the heme peripheral methyl groups at the entrance of the substrate access channel to the distal side of the heme in between two peptide segments, Phe^{90}-Pro^{91}-Ala^{92} and Ser^{151}-Leu^{152}-Ile^{153} and at a distance of 12.8 Å from the heme iron. Binding of iodide is controlled by protonation of an amino acid residue with a pK_a value of 5.3 presumably contributed by the distal histidine. The authors suggest that the distal histidine may take part in electron transfer from bound iodide to the heme ferryl group, as we suggested earlier (Bhattacharyya et al. 1993).

Besides iodide, thiocyanate, a pseudohalide is known to be oxidized by HRP (Modi et al. 1989). Although lactoperoxidase (LPO)-catalyzed SCN^- oxidation has been extensively studied and occurs through a direct two-electron transfer to form stable $OSCN^-$ (Aune & Thomas 1977, Thomas 1981, Modi et al. 1991), literature is very scanty on the mechanism of HRP-catalyzed SCN^- oxidation. By ^1H and ^{15}N-NMR studies, SCN^- has been shown to bind to HRP away from the distal histidine, near 1- and 8-CH_3 heme groups with a K_d value of 158 ± 19 mM (Modi et al. 1989). The oxidation product of SCN^- by HRP in presence of H_2O_2 is $(SCN)_2$, which is detected by NMR study (Modi et al. 1991). Recently we have shown that catalytic turnover of HRP-catalyzed SCN^- oxidation is 100-fold lower than that of LPO (Adak et al. 1997). The mechanism has been extensively studied. Unlike LPO, HRP catalyses one-electron oxidation of SCN^- to sulphur-centered thiocyanate radical ($^{\cdot}SCN$) which by dimerisation forms $(SCN)_2$. The latter is immediately hydrolysed to produce CN^- which reversibly inactivates HRP leading to slow turn over of SCN^- oxidation. The plausible mechanism of SCN^- oxidation by HRP is shown below :

$$Fe^{3+}\text{-HRP} + H_2O_2 \rightarrow \text{Compound 1} + H_2O \quad (11)$$

$$\text{Compound I} + SCN^- \rightarrow \text{Compound II} + {}^{\cdot}SCN \quad (12)$$

$$\text{Compound II} + SCN^- \rightarrow Fe^{3+}\text{-HRP} + {}^{\cdot}SCN \quad (13)$$

$$2\,{}^{\cdot}SCN \rightarrow (SCN)_2 \quad (14)$$

$$3(SCN)_2 + 4\,H_2O \rightarrow 5SCN^- + SO_4^{=} + 8H^+ + CN^- \quad (15)$$

$$Fe^{3+}\text{-HRP} + CN^- \longleftrightarrow Fe^{3+}\text{- HRP - } CN^- \quad (16)$$

^1H and ^{15}N NMR studies indicate that SCN^- binding is facilitated by protonation of an acid group with pK_a 4.0 (Modi et al. 1989). Similar pK_a value was also observed in case of binding of iodide to HRP (Sakurada et al. 1987). A salt bridge between heme propionate (pK_a 4.0) and a distal amino acid presumably arginine residue controls the entry and binding of SCN^- at the active site (Modi et al. 1994). Binding studies by optical difference spectroscopy indicate that both iodide and guaiacol compete with SCN^- for binding suggesting that binding sites for inorganic anions and aromatic donor are close to each other (Harris et al. 1993, Adak et al. 1997).

Mechanism of Aromatic Donor Oxidation

Unlike inorganic electron donors, extensive work has been done on the mechanism of aromatic donor oxidation with special emphasis on the residues involved in aromatic donor binding in HRP. The oxidation of aromatic donors by HRP occurs with two one-electron transfer reactions through the intermediate formation of compound I and compound II as shown in reactions 1-4. Extensive investigations have been carried out earlier by spectral, NMR, chemical modification and mutation studies to find out the location of the residues involved in aromatic donor binding. A number of workers reported on the basis of optical difference spectra that the enzyme in native form interacts with the aromatic donor molecule to form 1:1 complex (Critchow & Dunford 1972, Schejter et al. 1976, Paul & Ohlsson 1978, Schonbaum 1993) through a combination of hydrophobic interaction and polyfunctional hydrogen bonding. Binding was proposed to occur at the heme edge near 8-CH_3 group, about 8-11 A° away from the heme iron (Sakurada et al. 1986, Thanabal et al. 1988). On the basis of the computer modelling studies and transferred nuclear overhauser effect (TRNOE), aromatic donors were proposed to bind to a hydrophobic pocket composed of 8-CH_3, Tyr-185 and Arg-183 (Sakurada et al. 1986). From the proposed active site model based on heme alkylation studies, aromatic donors were suggested to interact near the δ-meso carbon at the exposed heme edge close to 8-CH_3 group (Ortiz de Montellano 1987, 1992, Harris et al. 1993). NMR studies further indicate that benzhydroxamic acid, an aromatic donor, binds to the heme distal site near 8-CH_3 and two unidentified Phe A and Phe B residues (Veitch 1995, Veitch & Williams 1995) through

interaction with distal His-42 and Arg-38 (Rodriguez-Lopez et al. 1996). Site-directed mutagenesis of Arg-38 with lysine in recombinant HRP-C* has shown a loss of binding of benzhydroxamic acid (Smith et al. 1993) indicating the role of Arg-38 in the process. We also identified the role of an active site arginine residue in aromatic donor binding by chemical modification studies (Adak et al. 1996). Banci et al (1994) have proposed from their molecular dynamics studies that p-cresol (aromatic donor) may bind at a site between the 8-CH_3 and Phe-68 moieties. Although the involvement of Arg-38 has been evident from mutant studies (Rodriguez-Lopez 1996, Howes et al. 1997), recent site-directed mutagenesis and ^1H NMR studies have identified the critical role of Phe-179 in close proximity with the 8-CH_3 group for complex formation with aromatic donor molecules (Veitch et al. 1997). The recently published x-ray crystal structure of HRPC clearly indicates that Phe-68, Phe-142 and Phe-179 form a distinct hydrophobic patch near the exposed heme edge and Phe-179 has been indicated to play a critical role in aromatic donor binding (Gajhede et al. 1997). It is reasonable to suggest that this hydrophobic site is the site for the formation of pre-electron transfer complex between aromatic donor and catalytic intermediates of HRP. The pathway of electron transfer to the heme ferryl group is not clear yet. However, distal His-42 may control aromatic donor oxidation by regulating electron transport from bound donor to the ferryl heme presumbaly by catalysing the proton transfer process (Bhattacharyya et al. 1993).

Residues Involved in Catalytic Intermediate Formation with H_2O_2

Site-directed mutagenesis studies have shown that His-42 when replaced with alanine, decreases the rate of reaction with H_2O_2 to form compound 1 by a factor of 10^5 relative to the wild type HRP indicating that distal His-42 is involved in compound 1 formation (Newmyer & Ortiz de Montellano 1995). Recently Rodriguez-Lopez et al. (1996) have shown that the apparent compound I formation rate in Arg-38-Leu mutant is 10^3 order less than the wild type HRPC indicating the involvement of Arg-38 also in the process. Replacement of conserved Phe-41 adjacent to His-42 with valine revealed an eight fold decrease in compound 1 formation indicating that Phe-41 also controls heme reactivity with H_2O_2 (Smith et al. 1992). Mutation of His-42 and Arg-38 further indicates that these two residues play an important role in the reaction of ferrous HRP with dioxygen to form oxyperoxidase (Rodriguez-Lopez et al. 1997). Thus His-42 and Arg-38 play an important role in the reactivity of ferric HRP with H_2O_2 and ferrous HRP with oxygen to form the catalytically active compound I and oxyperoxidase respectively. His-42 is also involved in the autoreduction of compound I to compound II by electron flow from endogenous source (Bhattacharyya et al. 1993).

The Role of Heme Propionic Acid in Catalysis

A substantial amount of work has been done on the role of heme propionates on peroxidase catalysis. After cleavage of HRP to apoperoxidase and heme, the enzyme can be successfully reconstituted into active form when heme is allowed to bind with the apoenzyme(Gjessing & Sumner 1942). Although the enzyme reconstituted with proto, meso, deuteroheme or protoheme monomethyl ester can catalyse compound I formation, the enzyme reconstituted with protoheme dimethyl ester where both propionic acids of heme have been esterified cannot do so (Tamura et al. 1972). Enzyme containing protoheme monomethyl ester is 20% active while dimethyl ester is inactive. The studies indicate that the side chains at 2 and 4 positions of porphyrin ring are not essential whereas the carboxyl groups at 6 and 7 positions are obligatory (Tamura et al. 1972). The role of 6 and 7 heme substituents in apoperoxidase binding and enzyme activity, is more critical and was studied by either blocking the heme propionate carboxyl groups using protoheme diamide and dialcohol hemin or

increasing the chain length using dibutyric acid hemin (DiNello & Dolphin 1981). The low activity, atypical spectra and slow binding to apoenzyme by diamide and dialcohol substituted enzyme indicate that free carboxylates are essential for rapid generation of active enzyme. On the other hand, although carboxylates are present in dibutyric acid derivatives, very slow binding and low catalytic activity suggest that chain length is also a determining factor in the binding of carboxylates in the narrow pocket of the apoperoxidases (DiNello & Dolphin 1981). Recently we have further investigated the role of heme propionates on catalytic intermediate formation and binding of electron donors after reconstitution of ferric protoporphyrin IX dimethyl ester (PPDME) into apoperoxidase (Adak & Banerjee 1998). The reconstituted enzyme neither oxidises guaiacol nor iodide specially due to block of compound I/II formation. Loss of heme CD spectrum indicates a loss of asymmetry of the heme-protein interaction due to change of heme orientation because of the loss of interaction with the surrounding residues via the heme propionates. Binding studies indicate no significant change in the K_d value of guaiacol while the K_d for SCN^- binding is twenty fold decreased indicating increased affinity of the propionate esterified enzyme to the inorganic donor. This indicates that heme propionates normally restrict the entry of inorganic substrates to the active site presumably due to salt bridge formation with nearby positively charged group as proposed earlier (Ugarova et al. 1981, Sakurada et al. 1987, Modi et al. 1989, 1994).

Catalytic Activity of HRP Other than Peroxidation

Oxidase Activity

HRP is known to have a number of catalytic activities other than its usual peroxidation reaction. Swedin and Theorell (1940) first reported the oxidase activity of HRP which catalyses the oxidation of dihydroxyfumaric acid by molecular oxygen. It is now known that various substrates including indoleacetic acid, thiols, NADH, NADPH etc are oxidised by HRP without added H_2O_2 and the mechanism of oxidation has been extensively studied (Yamazaki & Piette 1963, Yamazaki & Yokota 1967, Olsen & Davis 1976, De Sandro et al. 1991, Saikumar et al. 1994). It is now generally accepted that oxidase activity is mediated through intermediate formation of compound I, compound II and compound III through a peroxidase-oxidase oscillatory reaction (Yamazaki & Yokata 1967, Metodiewa et al. 1992, Scheeline et al. 1997, Hauser & Olsen 1998). Recently we have shown that peroxidase-oxidase reaction is also involved in the oxidation of epinephrine which occurs aerobically through intermediate formation of $O_2^{\cdot}$ and H_2O_2 and via the generation of compound I, II and III as intermediates (Adak et al. 1998). Binding studies indicate that for oxidase activity, epinephrine binds near the heme iron close to anion and aromatic donor binding sites (Adak et al. 1998).

Pseudocatalase Activity with Reductive Reaction

In normal peroxidative cycle, peroxidase decomposes H_2O_2 to H_2O in presence of an electron donor while in some cases peroxidase can produce oxygen from H_2O_2 like catalase through oxidation of the electron donor, which is called its pseudocatalase activity and is associated with subsequent reduction of the oxidised donor (reductive reaction). Magnusson et al. (1984a) reported the pseudocatalase activity of thyroid peroxidase in presence of low concentration of iodide. They proposed the following reactions for a possible mechanism of iodide-dependent pseudocatalase activity of thyroid peroxidase and lactoperoxidase (Magnusson et al, 1984b).

$$E + H_2O_2 \rightarrow EO \xrightarrow{I^-} [EOI]^- \Leftrightarrow E + IO^- \quad (17)$$

$$IO^- + H_2O_2 \rightarrow O_2 + I^- + H_2O \quad (18)$$

As reaction 18 indicates the reduction of IO^- to I^- by H_2O_2, this reaction may be termed as reductive activity of the peroxidase with pseudocatalatic decomposition of H_2O_2 to O_2. Further studies on pseudocatalase activity has been reported in EDTA, ABTS [2-2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] and chloropromazine oxidation by HRP or lignin peroxidase (Banerjee et al. 1986, Shah & Aust 1993, Barr & Aust 1993). The mechanism of reductive and pseudocatalase activity of HRP using H_2O_2, EDTA and iodine has been extensively studied in our laboratory (Banerjee et al. 1986, Banerjee 1989, Bhattacharyya et al. 1993, 1994, Adak et al. 1995) where EDTA (N-N) is oxidised to EDTA dication radical (N^+-N^+) and iodine is reduced to iodide. It is now clear that EDTA binds close to the iodide binding site (Bhattacharyya et al. 1993) and iodine binds with the heme propionate (Adak et al. 1995, Adak & Banerjee 1998) and the peroxidative and reductive reactions take place concurrently at the active site through intermolecular electron transfer via the formation of an active enzyme-EDTA - I^+ ternary complex (Adak et al. 1995). The EDTA dication radical oxidises H_2O_2 to evolve O_2 which is the basis for the pseudocatalase activity as shown in the following reactions (Adak et al. 1995) :

Fe^{3+} -HRP + $H_2O_2 \rightarrow$ Compound I + H_2O (19)

Compound I + I^+ + (N-N) $\rightarrow$ Compound I-I^+-(N-N) (20)

Compound I-I^+- (N-N) $\rightarrow$ Compound II-I+(N^+ -N^+) (21)

Compound II-I+(N-N) $\rightarrow$ Compound II-I+(N-N) (22)

Compound II-I-(N-N) $\rightarrow$ Fe^{3+}-HRP + I^-+(N^+-N^+) (23)

2 (N^+ - N^+) + $2H_2O_2 \leftarrow$ 2 (N - N) + $2O_2$ + $4H^+$ (24)

Peroxygenase Activity

HRP catalyses the sulphoxidation of aryl thioethers by H_2O_2 in presence of molecular O_2 (Kobayashi et al. 1987) by incorporation of ferryl oxygen into the sulphur-centered cation radical intermediate. Normally electron donors do not directly interact with the ferryl oxygen due to steric barriers caused by His-42 and Phe-41 (Ortiz de Montellano 1987, Swandon et al. 1991, Newmyer et al. 1995). The peroxygenase activity of HRP could be expressed by mutation of the His-42 and Phe-41 offering lower barrier to the heme ferryl group (Newmyer et al. 1995, Ortiz de Montellano et al. 1995). Replacement of His-42 with alanine creates a cavity in the active site favouring monooxygenase reaction.

Conclusion

The active site topology of HRPC and the role of active site residues in catalysis have been illuminated by the heme alkylation and chemical modification studies, site- directed mutagenesis and NMR studies and by recent x-ray crystal structure studies. The heme periphery of HRPC is almost covered by protein structure except a small region near δ-meso carbon and 8-CH_3 group where most of the electron donors are supposed to interact for the delivery of electron to the heme ferryl group. The proximal His-170 binds the heme with the apoprotein and keeps its stable configuration with the help of several hydrogen bonding network with the distal residues. The residues in the distal heme pocket play a very important role in peroxidase catalysis. His-42 and Arg-38 are involved in heterolytic cleavage of O-O bond of H_2O_2 to form catalyticallly active compound I. These residues specially His-42 also restricts the electron donor to come in direct contact with the heme ferryl group and prevent monooxygenase activity. Heme propionates also play a critical role in compound I formation by keeping the right orientation of the heme with respect to the distal residues specially with His-42 and Arg-38 through hydrogen bonding network. It also controls the entry of inorganic electron donors to the heme pocket through salt bridge formation with the nearby positively charged residue such as Arg-38. The exact residue involved in the binding of inorganic donors in native HRP has not been identified yet. Probably they interact near the δ-meso heme edge at a site at equal distance from 1- and 8-

methyl groups. However, Phe-179 is now considered to play a critical role in aromatic donor interaction at the substrate access channel of HRPC. X-ray crystal structure of HRPC with the bound donors will in future exclusively identify the exact binding site of the donor molecules for catalysis. Secondly, the pathway of electron transfer from bound donor to the heme ferryl group is still in the dark except that His-42 may take part in the process. Thirdly, as native HRP is catalytically inactive and binding studies of electron donors with catalytically active intermediates are extremely difficult, the question remains unanswered whether the binding sites identified in the native enzyme will be the same as in compound I/II states where the oxidation-reduction potential between the electron donors and the catalytic intermediates is probably the main driving force for oxidation. Future studies should be directed to answer these unsolved questions on peroxidase catalysis.

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