Haem iron-containing peroxidases

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Introduction

Haem-containing peroxidase enzymes are widely distributed throughout the plant and animal kingdoms and also have been isolated from bacteria, mould and micro-organisms [1]. Peroxidases serve the role of antioxidants, protecting cells, tissues and organs against the toxic effects of peroxides produced in vivo by oxidase activity [1]. They catalyse the oxidation of a variety of organic and inorganic compounds by hydrogen peroxide, organic hydroperoxides, peracids or inorganic oxides, such as periodate or chlorite. The general peroxide-dependent reaction catalysed by a peroxidase is shown in eqn. (1):

\[
\text{ROOH} + 2\text{AH}_2 \xrightarrow{\text{Peroxidase}} 2\text{AH}^- + \text{ROH} + \text{H}_2\text{O}
\]  

(1)

So far, 13 haem-containing peroxidase crystal structures have been reported, including Arthromyces ramosus peroxidase [2], ascorbate peroxidase [2], peanut peroxidase [2], Coprinus cinereus peroxidase [3], cytochrome c peroxidase (CCP) [3], lignin peroxidase [3], myeloperoxidase [3], prostaglandin H2 synthase [3], Caldariomyces fumago chloroperoxidase (CPO) [4], manganese peroxidase [5], di-haem CCP from Pseudomonas aeruginosa [6] and, most recently, horseradish peroxidase (HRP) isozyme C [2] and barley-grain peroxidase [5]. All of these peroxidases contain the haem (iron protoporphyrin IX) prosthetic group with a histidine proximal ligand, with

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the exception of the cysteinate-ligated CPO. The overall folding patterns of
the histidine-ligated peroxidases are quite similar despite relatively little
similarity of their primary sequences [7].

**Peroxidase reaction sequence**

HRP has been used as the paradigm for the biochemical study of peroxidases.
It is the most extensively studied member of the plant peroxidase superfamily
[1]. The peroxidase mechanism to be discussed below has been derived
primarily from studies of HRP.

The peroxidase mechanism comprises four steps, as shown in Figure 1. The native ferric state (structure 1, see Figure 1) reacts with hydrogen peroxide
on the distal side of the haem to generate a transient hydroperoxide adduct,
compound 0 (2), which breaks down rapidly (so fast that it cannot normally be
observed) to give an oxoferryl (sometimes called oxyferryl) porphyrin π-
cation radical, compound I (3). Peroxidases can also utilize organic hydroper-
oxides, peracids and other oxygen-atom donors in place of hydrogen peroxide
to generate compound I. Next, compound I is reduced by an electron-rich
substrate (AH2) to generate compound II (4) and a cation radical of the sub-

![Diagram of the peroxidase cycle]

**Figure 1. Peroxidase cycle**
The porphyrin ring of the haem is represented by a parallelogram with the four pyrrole nitrogens at the corners.
strate, which, depending on its acidity, will release H\(^+\). Alternatively, AH\(_2\) can contribute a hydrogen atom (H\(^{•}\)) to produce AH\(^•\) and Fe(IV)-OH; the latter can lose a proton to become Fe(IV)=O. This will be discussed later. A second AH\(_2\) can then react with compound II to regenerate the ferric resting state (I). In this reaction, the substrate contributes a H\(^•\) (or an electron plus a proton), and the resulting Fe(III)-OH is protonated and releases H\(_2\)O. The two radical-product molecules disproportionate non-enzymically to form a two-electron oxidized-product molecule (A) and a regenerated substrate molecule (AH\(_2\)), see eqn. (2). The rate of substrate oxidation by compound I of HRP is usually 10–100-fold faster than that by compound II [1]. This may be due to reorganization energy differences between the two processes. The reduction of compound I to II merely requires the delivery of an electron to the porphyrin \(\pi\)-cation radical. However, the reduction of compound II to the ferric state requires the delivery of an electron and two protons (or a hydrogen atom plus a proton), so that H\(_2\)O can be released. In this process, the haem undergoes a reorganization to 5-co-ordinate iron.

\[
AH^- + AH^• \rightarrow AH_2 + A
\]  

(2)

The peroxidase catalytic reaction cycle, illustrated with structures of the haem prosthetic group in Figure 1, involves two high-valency oxoferryl intermediates. Compound I (3) is two oxidation equivalents above the native ferric state of the haem, and compound II (4) is one oxidation equivalent above native ferric haem (1). The ferric-hydroperoxide complex (2), designated compound 0, is thought to be formed after mixing HRP and hydrogen peroxide en route to the generation of compound I.

CPO purified from the mould \textit{C. fumago} stands out among the peroxidases due to its versatile reactivities. Along with the typical peroxidase activity, CPO has a unique ability among peroxidases to catalyse the chloride ion-dependent chlorination of certain organic substrates [8]. CPO utilizes intermediates similar to those observed with HRP to catalyse three distinctly different peroxide- or peracid-dependent reactions (Figure 2): (i) halogenation of organic compounds with Cl\(^•\), Br\(^•\) or I\(^•\) as the halogen source (5\(\rightarrow\)6\(\rightarrow\)7\(\rightarrow\)9\(\rightarrow\)5; see Figure 2); (ii) a typical peroxidase activity (5\(\rightarrow\)6\(\rightarrow\)7\(\rightarrow\)8\(\rightarrow\)5); and (iii) a catalase (disproportionation of hydrogen peroxide) activity (5\(\rightarrow\)6\(\rightarrow\)7\(\rightarrow\)5). The halogenation reaction is thought to proceed through compound X (9), a ferric hypochlorite species.

Whereas the proximal ligand of most other peroxidases is the nitrogen of a histidine residue, CPO is ligated by the anionic sulphur of a deprotonated cysteine (cysteinate) residue [8]. This unique property of CPO has qualified it as a particularly useful protein model for reactions of cytochrome P450, another cysteinate-ligated haem-containing enzyme (see Chapter 5 in this volume). The enzymes of the cytochrome P450 superfamily consist of mono-oxygenases that activate molecular oxygen to catalyse an extensive variety of oxygen-transfer reactions ([8] and Chapter 5 in this volume).
**Compound I**

**Physical properties**

This compound has a characteristic green colour with an oxidation state two equivalents above the ferric enzyme [1,3]. Establishing the distribution of electrons around the haem, the iron and the protein in compound I has been a challenge. Three resonance forms could contribute to its structure (structures 3, 3a and 3b, see Figure 3). Figure 3 also displays an alternative structure, 3c, for the compound I of CCP (originally called compound ES, CCP-ES) that will be discussed below.

**HRP**

Various lines of evidence have led to the conclusion that structure 3, a green oxoferryl porphyrin π-cation radical, is the predominant structure of HRP compound I (HRP-I) [9,10]. The UV-visible absorption spectrum of HRP-I features a Soret band at 400 nm that is only about half the intensity of the native HRP Soret peak at 402 nm (Figure 4a). The loss of absorbance intensity is attributed to the loss of aromaticity in the porphyrin on forming the
In Figure 4, the UV-visible absorption spectra of other HRP intermediates (i.e. HRP-II and HRP-III) and CCP-ES are also displayed. In the visible region, peaks present at 498 and 640 nm in the spectrum of native ferric HRP disappear upon formation of compound I, leaving a broad series of weakly absorbing peaks. The lack of distinct bands in the visible region is generally cited as evidence for the presence of a porphyrin radical [8].

Magnetic susceptibility measurements have shown that HRP-I is paramagnetic with three unpaired electrons ($S = \frac{3}{2}$), suggesting a formal iron oxidation state of $V$ for HRP-I [1]. However, Mössbauer spectroscopy [11] showed that the oxidation state of the haem iron in HRP-I is Fe(IV), implying that the fifth oxidation equivalent is not on the iron. Extended X-ray absorption fine structure spectroscopy (EXAFS) of the haem iron site in HRP-I demonstrated that the Fe–O bond length is 1.64 Å [10,12]. This short bond for HRP-I is consistent with an oxoferryl complex [Fe(IV)=O]. Moreover, electron-nuclear double resonance spectroscopy (ENDOR) confirmed that HRP-I contains an oxo ligand [13,14].

Work on both protein and model compounds supports the conclusion that one of the oxidation equivalents resides on the haem as a π-cation radical. Resonance Raman [15] and NMR [16] data, as well as iterative extended Hückel calculations [17], have all substantiated that a π-cation radical actually resides
Figure 4. Spectra of HRP species in various states

(A) UV-visible absorption spectra of HRP species and CCP-ES at neutral pH, and (B) UV-visible absorption spectra of HRP species at neutral pH. Spectra were recorded at 4°C at pH 7.0 in 100 mM potassium phosphate buffer.
on the haem. The Mössbauer and EPR data for HRP-I [18] show that the \( S = 1 \) spin of the Fe(IV)=O group is weakly coupled to a spin \( S' = 1/2 \) radical on the haem. EPR studies of Zn-substituted HRP further demonstrate that the porphyrin can be oxidized to a \( \pi \)-cation radical with hexachloro-iridate [9]. In contrast to the iron, the Zn cannot be oxidized, so that the oxidation equivalent has to reside on the ring. This Zn-porphyrin radical has a visible spectrum, which is very similar to normal compound I that contains iron, including the same lower-intensity optical Soret band; thus the loss of aromaticity of the porphyrin ring upon oxidation is associated with the lower-intensity Soret band.

**CCP**

In most peroxidases, the haem group in compound I is in the +5 oxidation state (ferryl iron coupled to the porphyrin radical). However, with CCP, rapid intramolecular electron transfer from a nearby amino acid residue (Trp-191) to the nascent porphyrin \( \pi \)-cation radical generates a stable compound, ES (3c, Figure 3, and see Figure 7 later). This complex, which is two oxidizing equivalents above the ferric state, consists of a protein-based tryptophan cation radical and an oxoferryl haem centre equivalent to HRP-II [19]. As expected, the UV-visible absorption (Figure 4a) and magnetic CD spectra of CCP-ES are different from those of HRP-I, but are similar to those of HRP-II [20]. EXAFS measurements [21] show that the Fe–O bond length in CCP-ES is \( 1.67 \pm 0.04 \) Å, which is characteristic of the oxoferryl complex, Fe(IV)=O, in HRP-I [10,12]. In addition, resonance Raman spectroscopy verified the presence of an Fe(IV)=O bond in CCP-ES [22]. The crystal structure of this intermediate confirmed the presence of an oxoferryl complex \([Fe(IV)=O]\) [19].

Identification of the site of the amino acid radical in compound ES was more elusive. Unlike HRP-I, which is green, CCP-ES is red and exhibits an EPR signal typical of a free radical, suggesting that a free radical resides on an amino acid [23]. Initially, several amino acids were considered to harbour the free radical, although further investigations placed the free radical on Trp-191 in the active site [3]. EPR of CCP mutants, with Trp-51 and Trp-191 separately replaced by Phe, showed that Trp-191, rather than Trp-51, was the site of the tryptophan radical [3]. ENDOR studies on mutants containing perdeuterated Trp residues showed that the second oxidizing equivalent resided on Trp-191 in the wild-type CCP-ES [24]. Furthermore, when the Trp-191→Phe mutant was mixed with hydrogen peroxide in a stopped-flow spectrophotometer, a porphyrin \( \pi \)-cation radical was observed with a half life of \( \approx 14 \) ms [3]. Recent ENDOR data have confirmed that CCP-ES consists of an oxoferryl haem coupled by weak exchange to the Trp-191 \( \pi \)-cation radical [25].

**CPO**

Compound I of CPO (CPO-I) exhibits a Soret band at 368 nm and a distinguishing peak at 690 nm. Like other peroxidases, CPO can form compound I upon reaction with peracids and organic hydroperoxides [26].
The weak intensity of the Soret band and the lack of distinct bands in the visible region of the spectrum of CPO-I indicate the formation of a porphyrin π-cation radical [8]. The EPR and Mössbauer spectroscopy of CPO-I are indicative of a porphyrin π-cation radical with a stronger coupling to the haem ferryl iron than is the case with HRP-I [8]. The properties are consistent with the model of an exchange-coupled spin $S=1$ Fe(IV) ion and a spin $S'=1/2$ porphyrin radical [8], like that of HRP.

The nature of the amino acid residues and the environment that is present in the pocket containing the haem, on both the proximal and distal sides, is likely to be of great influence on the structure and reactivity of intermediates, including compound I. The proximal ligand appears to be less important than other factors. In CCP, for example, the proximal histidine ligand was mutated to glutamine with no decrease in the rate of reaction with hydrogen peroxide; spectroscopically, both the ferric and compound-I states of this mutant closely resemble those of the wild-type enzyme, presumably due to ligation of the oxygen of the glutamine side chain to haem iron. However, the stability of compound I was drastically reduced [27].

Urano et al. have suggested that the oxygen-radical resonance form of compound I (3a, Figure 3) is more likely to be able to abstract a hydrogen atom from the substrate than is a porphyrin oxoferryl π-cation radical (3, Figure 3) [28]. In fact, CPO-I and HRP-I are even able to abstract a hydrogen atom from organic oxidants, such as alkyl hydroperoxides and peracids, as will be discussed later in more detail. Consequently, it is reasonable to ask which of the resonance structures of compound I displayed in Figure 3 are more energetically favoured and what are the determining factors?

Champion [29] proposed that the presence of a cysteiny1 sulphur on the proximal side, as found in cytochrome P450 and CPO-I, favours the oxygen-radical-type species (7a, Figure 5) over the oxoferryl π-cation-radical form (7, Figure 5), and Urano et al. [28] used synthetic models to obtain experimental evidence for this proposal. Loew et al. [30] presented calculations for cytochrome P450 indicating that an oxo-radical species would be within 0.7 kcal/mol of the ground state. They speculated that this oxo-radical species is apparently stabilized as the iron–oxygen bond is lengthened.

From his study with cytochrome P450, Champion argued that the sulphur→iron π-electron donation appears to have mechanistic consequences for the O–O cleavage [29]. In thiolate-ligated enzymes, compound I has a neutral overall charge due to the negative charge on the proximal sulphur, thus favouring the oxo-radical configuration over the higher energy π-cation radical on the haem (Figure 5, top). However, in histidine-ligated enzymes, the presence of nitrogen with a neutral charge results in an overall charge of +1 for compound I (Figure 5, bottom). The formation of a π-cation radical is required to diminish the unfavourable effect of this charge by delocalizing it over the extensive π-bonding system of the haem. Thus, the π-cation radical form (3, Figures 3 and 5) is favoured over an oxo-radical type (3a, Figures 3
and 5). Moreover, the absence of the sulphur allows the Fe(dπ) orbitals to couple more effectively with the oxygen, contributing to a shorter Fe–O bond [29]. The π-cation radical is more likely to be reduced by electron transfer, whereas the oxo-radical configuration is more likely to abstract a hydrogen atom. Nevertheless, Mössbauer and EPR data suggest that CPO, which has a proximal cysteine ligand, favours the π-cation radical configuration [8]. Champion suggests that in contrast to cytochrome P450, where the distal side contains primarily non-polar residues, CPO contains polar residues to help stabilize the π-cation radical.

**Reactivity**

Compound I is an intermediate that has been proposed to be directly involved in the enzymic mechanism of many haem-containing enzymes. In cytochrome P450, for example, it is thought to be the reactive intermediate responsible for abstracting a hydrogen atom from the organic substrate [8]. CPO-I, formed by reaction of the ferric enzyme with an alkyl hydroperoxide, has been shown by
EPR spectroscopy to abstract a hydrogen atom from a second alkyl hydroperoxide (ROOH) molecule to form compound II and a peroxyl radical (ROO’’) [31]. Spin traps were used to detect the peroxyl radicals and the molecules formed from the breakdown of those radicals. Compound II then abstracts a hydrogen atom from a third molecule of alkyl hydroperoxide to generate the native ferric enzyme. Figure 6 illustrates the reaction sequence proposed by Chamulitrat et al. [31]. Similar conclusions for the reaction of CPO with excess peracid were obtained directly using stopped-flow spectrophotometry [26].

A spectroscopic and kinetics study produced results with HRP [32] that are also consistent with the reaction sequence described by Chamulitrat et al. for CPO [31]. These experiments showed that a peracid (metachloroperbenzoic acid) will react with HRP-I to generate compound II and an organic radical. The latter either diffuses away or, approximately 30% of the time, inactivates the enzyme by alkylating the haem.

The results obtained for the reactions of CPO and HRP with alkyl hydroperoxides or peracids demonstrate both the oxidizing potency of

Figure 6. Scheme for the reaction of CPO with alkyl hydroperoxide
compound I and its ability to abstract a hydrogen atom (or an electron and a proton), not only from organic substrates, but also from either alkyl hydroperoxides or peracids [26]. In keeping with this, the oxidation potentials for compound I/compound II and for compound II/Fe(III) of HRP have each been estimated to be approximately +1 V (versus the normal hydrogen electrode) [33].

**Mechanism of formation**

HRP-I is readily formed when either hydrogen peroxide or any of a number of alternative oxygen-atom donors, such as organic hydroperoxides and peracids, is mixed with ferric HRP. The proposed pathway for the formation of HRP-I from an alkyl hydroperoxide is illustrated in Figure 7. Initially, the distal histidine residue acts as a general base to form a ferric alkyl hydroperoxide, $2a$. Evidence for formation of such a species will be discussed below. The positively charged distal arginine is thought to stabilize the developing negative charge on the $\beta$-oxygen during heterolytic O–O bond scission ($2b$). The transiently protonated histidine acts as a general acid to protonate the leaving alkoxide and generate compound I ($3$), and ROH is released from the active site. As described above, the distal histidine and

![Figure 7. Formation of HRP-I](image)
arginine residues play significant roles in the formation of HRP-I. The exact role of the proximal histidine ligand is still being examined [27].

**Compound II**

**Physical properties**

HRP-II has a characteristic red colour with a Soret absorption band at approximately 420 nm (Figure 4a). Magnetic-susceptibility measurements of compound II are consistent with the presence of two unpaired electrons [1] and Mössbauer spectra confirm that HRP-II indeed contains Fe(IV) [1]. The initial EXAFS study of HRP-II by Penner-Hahn et al. reported that the Fe–O bond was very short, approximately 1.6 Å [10,12]. EXAFS results reported by Chance et al. suggested that the length of the Fe–O bond was pH dependent, short at alkaline pH, and ≈1.93 Å at neutral pH [34]. Possible structures for HRP-II at neutral and alkaline pHs based on these EXAFS results are shown in Figure 8 (4a and 4, respectively). Sitter et al. also observed pH-dependent properties in the resonance Raman spectra of HRP-II [35]. However, the magnitude of the shift in the Fe–O vibrational frequency as a function of pH was too small to indicate complete conversion from an Fe=O double-bonded unit to an Fe–OH single-bonded ligand. Thus it was proposed that, above pH 8.7, the oxygen of the oxoferryl species was hydrogen-bonded to an imidazolium hydrogen atom from the distal histidine residue in the active site. A non-hydrogen-bonded oxoferryl structure, Fe(IV)O, was proposed as the predominant structure below pH 8.7 [35]. Further investigations will be necessary to better establish the structure of HRP-II as a function of pH.

**Mechanism of formation**

Compound I is reduced to compound II upon interaction with a substrate in the active site. One suggestion is that a hydrogen atom is transferred from the substrate to the oxoferryl oxygen/porphyrin π-cation-radical unit. The electron from the hydrogen atom would then reduce the porphyrin π-cation radical and the remaining proton would protonate the distal histidine [3,36],

![Figure 8. Structures of HRP-II](image)

The formal oxidation state of the iron is IV. The total charge for structure 4 is 0, and for 4a is +1.
leaving the oxoferryl complex intact. Ortiz de Montellano and co-workers have suggested an alternative, namely that the electron transfer (or hydrogen-atom abstraction) could take place at the edge of the haem rather than at the oxoferryl [Fe(IV)=O] centre [37], and the resulting cation radical of the substrate would disproportionate.

**Compound III**

HRP-III has a characteristic dark red colour and is equivalent to the oxyferrous complex of HRP, Fe(II)–O₂ or Fe(III)–O₂⁻ [1]. This complex is also at the same oxidation level and is very similar to oxymyoglobin or oxyhaemoglobin (see Chapter 6 in this volume). The main features in the UV-visible absorption spectrum are the Soret band at 416 nm and two peaks at 546 and 583 nm [1] (Figure 4b). Compound III is not part of the HRP catalytic cycle (and therefore is not shown in the catalytic schemes in this Chapter), but forms upon addition of a large excess of hydrogen peroxide to the native ferric enzyme [1]. It is known as compound III because it is the third complex of HRP to be observed, after compounds I and II, following the addition of hydrogen peroxide. Titration with dimethyl-p-phenylenediamine has shown that compound III is three oxidation equivalents above ferric HRP [1], so that it has the formal oxidation state of 6.

**Mechanism of formation**

HRP-III can be made by three methods. The first involves adding excess hydrogen peroxide to ferric HRP. This reaction proceeds via the generation of compound I and then compound II, which then converts relatively slowly to compound III [1]. Also, the reaction of HRP-II with excess hydrogen peroxide directly yields compound III [38]. A second method involves generation of oxyferrous HRP by addition of molecular oxygen (O₂) to ferrous HRP [1]. Finally, it is reported that compound III can be formed when superoxide anion (O₂⁻) is added to ferric HRP [1].

**Compound 0**

The process leading to the formation of compound I in the HRP catalytic cycle is not well understood. Preceding the formation of compound I, HRP interacts with hydrogen peroxide to generate compound 0 (2, Figure 1). The many questions that remain concerning the nature of this compound must be addressed to more fully understand the mechanism of compound-I formation.

Baek and Van Wart [39] observed that compound 0 was a transient intermediate en route to formation of compound I. At cryogenic temperature, they used rapid-scan spectroscopy to follow the reaction of ferric HRP with hydrogen peroxide or organic hydroperoxides. At ambient temperature, the half-life of the ferric peroxide species (compound 0) has been estimated to range from 0.05 to 0.1 ms, depending on the peroxidase examined. Kinetics and thermo-
dynamics of the formation of compound 0 have also been investigated. Unfortunately, it has not been possible to generate high-enough concentrations of compound 0 for examination with other spectroscopic methods such as Mössbauer spectroscopy, EPR, EXAFS or resonance Raman spectroscopy.

The best evidence for the involvement of compound 0 in formation of compound I comes from kinetic studies. The rate of formation of HRP-I reaches a limiting value at high concentrations of hydrogen peroxide or other alkyl hydroperoxides [39]. This indicates that a precursor to compound I, namely compound 0 (2, Figure 1), is formed reversibly en route to the generation of compound I (3, Figure 1). Similar results were obtained using a water-soluble haem model for peroxidases [40]. In the studies with both HRP and the water-soluble peroxidase model, a transient spectrum was observed that had the surprising features of a “hyperporphyrin” or “split Soret” UV-visible absorption spectrum, with two intense Soret transitions at 400 and 330 nm [40,41]. Such spectra have been observed for both the cytochrome P450 enzymes and CPO [8]. This was the first time such spectra had been observed for a histidine-ligated peroxidase. Thorneley and co-workers carried out rapid-scan stopped-flow studies of compound-I formation in an HRP mutant that had Arg-38 on the distal side replaced by leucine [41]. They observed changes in the Soret absorption bands around 400 nm, but the additional peak at 330 nm was missing.

Harris and Loew [42] have calculated the electronic structure and spectra of resting-state HRP and of two possible forms for compound 0. They concluded that the split Soret UV-visible absorption peaks of compound 0, observed with both wild-type HRP and the water-soluble peroxidase model, represent the ferric anion peroxide complexes [Fe(III)–OOH], whereas the single Soret absorption peak observed with the Arg-38→Leu-mutant HRP was due to the ferric hydrogen peroxide HRP complex, Fe(III)–HOOH [41]. These calculations affirm the conclusions from the above experiments [39–41] that Arg-38 has mechanistic importance in the formation of compound I; these authors suggested that the absence of Arg-38 interferes with proton transfer from hydrogen peroxide to the imidazole of the distal histidine, hindering the O–O heterolytic cleavage and, thus, slowing the rate of compound-I formation [42].

**Differences in the catalytic activities of P450 and peroxidases**

The reactive-oxygen catalytic species of the cytochrome P450 enzymes and the peroxidases seem to be similar. Both involve oxidation of the ferric resting state to an oxoferryl intermediate and either a porphyrin radical or a protein-based radical. Based on these similarities, the question must be asked, why are the catalytic outcomes of these two systems so very different? Mono-oxygenases transfer directly the ferryl-bound oxygen to substrate, whereas the
peroxidases abstract an electron from their substrates. Unlike the cytochrome P450 enzymes, most peroxidases do not accept electrons from associated electron-transfer proteins and cannot activate dioxygen via the generation of a metal-peroxide species. Cytochrome P450 enzymes can utilize peroxides (peroxigenase rather than oxygenase activity) to support catalysis (no P450 reductase, NADPH or O₂ is required; see Chapter 5 in this volume) while retaining the differences in catalytic mechanism from peroxidases mentioned above.

The reaction of phenylhydrazine with haem proteins such as P450 and myoglobin, in which the haem iron is readily accessible to benzene-ring-bearing substrates or ligands, results in the formation of a phenyl–iron complex [37]. This is not the case for HRP, which undergoes addition of the phenyl group to the porphyrin δ-meso position [37]. Substitution at the haem δ-meso hydrogen with ethyl- or benzene-ring-bearing ligands has been observed to inhibit peroxidase activity in both HRP and CCP [43]. However, replacement of an active-site phenylalanine residue in HRP with smaller amino acids (by site-directed mutagenesis) allows increased peroxygenase activity (sulphoxidation of phenyl alkyl thioethers increases by up to 18-fold) and even styrene epoxidation to occur, presumably by altering the protein structure around the haem to increase accessibility to the oxoferryl moiety [44]. Analysis of available peroxidase crystal structures clearly shows the only accessible haem site to be the peripheral δ-meso position [2,5,45]. Thus one difference is in the accessibility of the distal haem site to organic molecules. The peroxidases are hydrophilic and restrict access to the distal side of the haem. This encourages electron-transfer reactions with the porphyrin and interactions with H₂O and hydrogen peroxide. The distal sides of P450 enzymes are more open and hydrophobic, encouraging oxygenations of organic substrates.

The reaction of peracids (e.g. metachloroperbenzoic acid) and alkyl hydroperoxides with P450s and peroxidases gives significantly different results, although compound I forms with both. Whereas the haem in cytochrome P450 is totally destroyed in seconds, the haem in HRP is only occasionally modified [32], and the haem in CPO is not damaged to any significant extent [26]. The results of such studies support the notion that a major reason the peroxygenase activity of typical peroxidases is suppressed is because of the steric and polar constraints of the protein, which limits access of organic substrates to the ferryl oxygen [37]. Perhaps this is the principle reason why cytochrome P450 enzymes and peroxidases behave so differently.

**Perspectives**

This Chapter has focused on the different peroxidase states, including their structures, how they can be formed, and some of the reactions they can undergo. Using various spectroscopic techniques and site-directed
mutagenesis, many discoveries have been made to elucidate the sequence of reactions and the reactive intermediates involved.

In the case of HRP and other histidine-ligated peroxidases, the final piece of the puzzle seems to be the crystal structure. The crystal structures of the numerous enzymes and some of their derivatives have confirmed many of the suggestions put forth for the structures of these peroxidases, while refuting others. These crystal structures also clarified their catalytic nature by identifying the individual amino acid residues involved in the reaction sequence. Such knowledge is invaluable for protein engineering that aims to construct various types of enzyme to service many specific tasks.

On the other hand, in the case of CPO, more work is still needed. CPO is unique among peroxidases. Whereas other peroxidases have a histidine proximal haem-iron ligand, CPO employs cysteinate. Such proximal ligation has earned CPO special recognition because it is similar to cytochrome P450. This resemblance implies similar chemical properties and similar reactive intermediates, and has been the basis for CPO being used as a reasonable protein model for the study of P450. Determination of the properties of the high-valency intermediates in the CPO reaction cycle has proven to be more elusive than with other peroxidases such as HRP. Stopped-flow spectroscopic techniques (including rapid-scan methods) will be required to obtain the appropriate kinetics data for delineating intermediates in the reactions. Rapid freeze-quench methodologies can then be used to capture the elusive CPO-intermediate species for further characterization by magnetic-resonance techniques. This will enable determination of the redox and co-ordination states of the intermediates.

The potential for using haem-containing peroxidases and other haem-containing enzymes in bioremediation processes in the environment is enormous. For that specific reason, better understanding of the structure–function relationships of peroxidase enzymes is necessary for constructing specifically engineered peroxidases to accomplish many of these difficult and otherwise unfeasible tasks.

**Summary**

- Peroxidases are enzymes that utilize hydrogen peroxide to oxidize substrates.
- A histidine residue on the proximal side of the haem iron ligates most peroxidases.
- The various oxidation states and ligand complexes have been spectroscopically characterized.
- HRP-I is two oxidation states above ferric HRP. It contains an oxoferryl (=oxyferryl) iron with a π-radical cation that resides on the haem. HRP-II is one oxidation state above ferric HRP and contains an oxoferryl iron. HRP-III is equivalent to the oxyferrous state.
Only compounds I and II are part of the peroxidase reaction cycle.

CCP-ES contains an oxoferryl iron but the radical cation resides on the Trp-191 residue and not on the haem.

CPO is the only known peroxidase that is ligated by a cysteine residue rather than a histidine residue, on the proximal side of the haem iron. CPO is a more versatile enzyme, catalysing numerous types of reaction: peroxidase, catalase and halogenation reactions.

The various CPO species are less stable than other peroxidase species and more elusive, thus needing further characterization.

The roles of the amino acid residues on the proximal and distal sides of the haem need more investigation to further decipher their specific roles.

Haem proteins, especially peroxidases, are structure–function-specific.

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