

# Chapter 5

## Catalytic Mechanisms of Heme Peroxidases

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**Abstract** The hemoprotein peroxidases produce a reactive intermediate, Compound I, whose reactions are controlled by the protein environment. In conventional peroxidases with a histidine iron ligand, access to the Compound I ferryl species is restricted by the protein, favoring the transfer of single electrons from the substrate to an exposed heme edge. If the protein has a suitably placed oxidizable residue such as a tyrosine or tryptophan, it may be preferentially oxidized by the initial Compound I to give an alternative Compound I in which a ferryl species is paired with a protein rather than porphyrin radical cation. In conventional peroxidases, only small substrates have ready access to the ferryl oxygen and undergo a direct two-electron oxidation, although larger substrates can appear to undergo a two-electron oxidation through the stepwise removal of two electrons. In contrast, peroxidases with a thiolate iron ligand have more open distal active sites and are

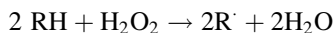
able to catalyze a range of two-electron oxidations in addition to one-electron peroxidative transformations.

## 5.1 Introduction

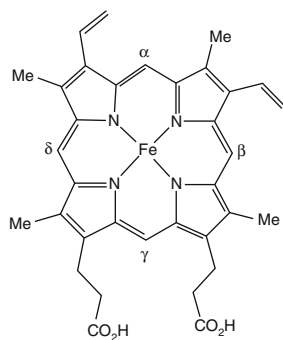
The hemoprotein peroxidases are ubiquitous proteins that catalyze the one-electron oxidation of their substrates employing a peroxide, usually  $\text{H}_2\text{O}_2$ , as the cosubstrate. Historically, the most studied and best characterized of the peroxidases are horseradish peroxidase (HRP) and cytochrome *c* peroxidase (CcP), although more recently enzymes such as lignin peroxidase (LiP) and ascorbate peroxidase have also been extensively investigated. In parallel with these studies of plant and fungal enzymes, much work has been done on the structure and mechanism of the mammalian peroxidases, particularly myeloperoxidase (MPO) and lactoperoxidase (LPO), for both of which crystal structures are now available. In all of these enzymes, the catalytic center is iron protoporphyrin IX (Fig. 5.1), although in the mammalian proteins this prosthetic group is autocatalytically modified. Despite the differences in the proteins, active sites, and even prosthetic groups, the catalytic mechanisms of all the peroxidases are sufficiently similar that they can be viewed, despite their differences, from a common perspective.

## 5.2 Peroxidase Catalytic Cycle

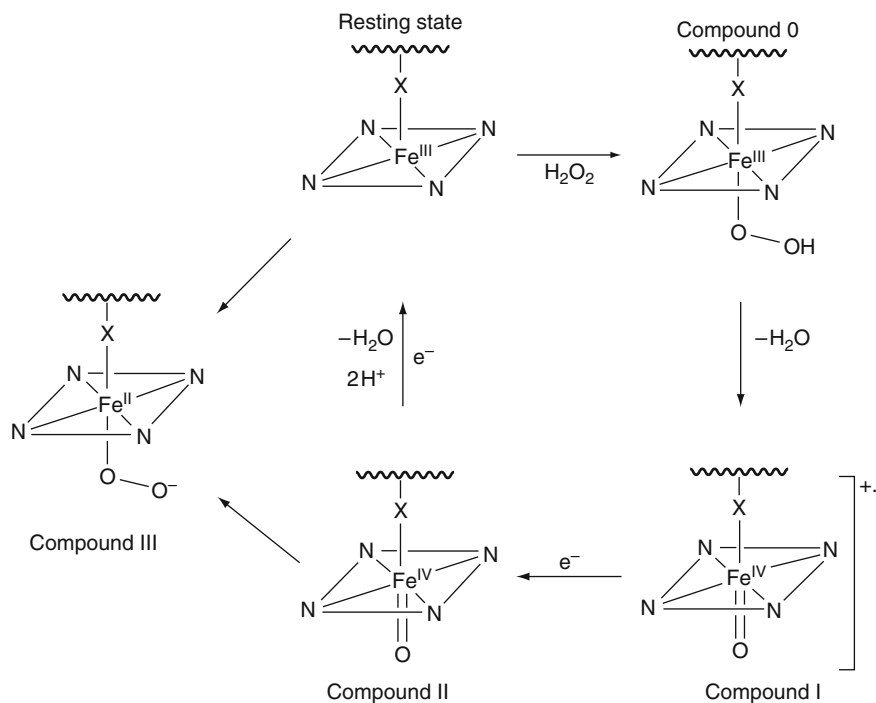
The common overall reaction of the peroxidases can be written as in the following equation, where RH is a suitable peroxidase substrate and  $\text{R}'$  is a free-radical product derived from it:



**Fig. 5.1** The structure of iron protoporphyrin IX, the prosthetic heme group of the peroxidases except where it is modified by the formation of covalent bonds to the methyl or vinyl groups. The  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -*meso*-carbon atoms, defined with respect to the pattern of the ring substituents, are labeled



This net transformation, however, encompasses a more complex mechanism that can be visualized in the generic catalytic cycle shown schematically in Fig. 5.2. Compounds I and II, the critical catalytic intermediates, are readily distinguished from the resting ferric state of the protein by their UV–visible absorption spectra (Table 5.1) [1–4]. Although the exact positions of the maxima show small variations, the spectroscopic properties of HRP are representative of those of all the peroxidases in which the heme iron atom is coordinated to a histidine nitrogen atom. The individual stages of the catalytic cycle are considered below.



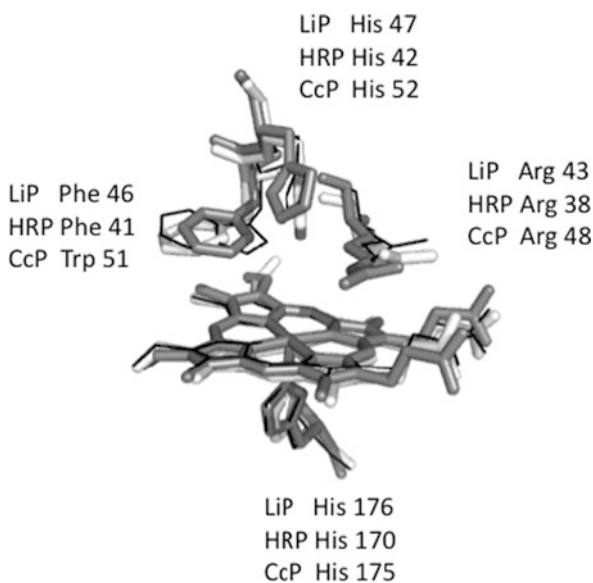
**Fig. 5.2** Generic peroxidase catalytic cycle. The *square* of four nitrogens around the iron atom is a representation of the prosthetic heme group of the peroxidase

**Table 5.1** Soret absorption maxima for resting state ferric HRP and the Compound I and Compound II catalytic intermediates [1–4]

HRP species	Soret (nm)	Molar absorptivity (mM <sup>-1</sup> cm <sup>-1</sup> )	Visible (nm)	Charge transfer (nm)
Ferric	403	102	498, 640	
Compound I	400	54	~525 (sh), 577	622, 651
Compound II	420	105	527, 555	
Compound III	417	108	544, 580	

### 5.2.1 Ferric Peroxidase

The prosthetic heme in the resting peroxidases is in the ferric state. In HRP and most peroxidases, the iron is five coordinate, high spin [5], with a histidine as the proximal iron ligand and a water molecule in the distal side that is not coordinated to the iron. In HRP, the oxygen of the water molecule is 3.20 Å from the iron [6] and in CcP at a distance of 2.33 Å [7]. In addition to the proximal histidine iron ligand, which in HRP is His170, in CcP His175, and in LiP His176 (Fig. 5.3), most peroxidases have a distal histidine not bound to the heme iron atom that is intimately involved in catalysis. In HRP, this residue is His42, in CcP His52, and in LiP His47. A second important catalytic residue is a distal arginine that through polar interactions facilitates heterolytic cleavage of the peroxide dioxygen bond. In HRP, this residue is Arg38, in CcP Arg48, and in LiP Arg43. Additional residues are thought to promote the formation of Compound I. In HRP, these include Asn70 on the distal side, which by hydrogen bonding to the N–H of His42 enhances its basicity, and Asp247 on the proximal side, which by accepting a hydrogen bond from the proximal histidine increases the negative electron density on the imidazole ring and thereby, through a “push” effect, facilitates O–O bond cleavage. The roles of these residues are supported by mutagenesis studies showing that loss of any of the indicated residues or interactions decreases the catalytic activities of the enzymes, with the distal catalytic histidine being the most important [9–13].



**Fig. 5.3** Location of critical catalytic residues relative to the heme group in the active site of LiP (*white sticks*), HRP (*gray sticks*), and CcP (*black line*). The figure is based on [8]

### 5.2.2 *Compound 0*

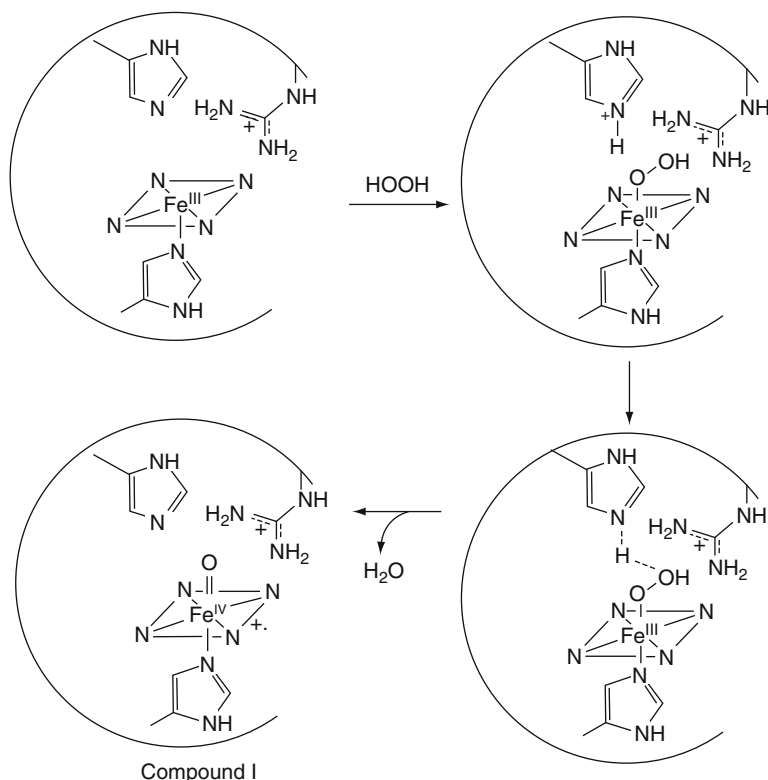
The first step of peroxidase catalysis involves binding of the peroxide, usually  $\text{H}_2\text{O}_2$ , to the heme iron atom to produce a ferric hydroperoxide intermediate  $[\text{Fe}(\text{III})\text{--OOH}]$ . Kinetic data identify an intermediate prior to Compound I whose formation can be saturated at higher peroxide concentrations. This elusive intermediate, labeled Compound 0, was first observed by Baek and Van Wart in the reaction of HRP with  $\text{H}_2\text{O}_2$  [14]. They reported that it had absorption maxima at 330 and 410 nm and assigned these spectral properties to the ferric hydroperoxide species  $[\text{Fe}(\text{III})\text{--OOH}]$ . They subsequently detected transient intermediates with similar spectra in the reactions of HRP with alkyl and acyl peroxides [15]. However, other studies questioned whether the species with a split Soret absorption detected by Baek and Van Wart was actually the ferric hydroperoxide [16–18]. Computational prediction of the spectrum expected for Compound 0 supported the structure proposed by Baek and Van Wart for their intermediate, whereas intermediates observed by others with a conventional, unsplit Soret band may be complexes of ferric HRP with undeprotonated  $\text{H}_2\text{O}_2$ , that is  $[\text{Fe}(\text{III})\text{--HOOH}]$  [19]. Furthermore, computational analysis of the peroxidase catalytic sequence suggests that the formation of Compound 0 is preceded by formation of an intermediate in which the undeprotonated peroxide is coordinated to the heme iron [20]. Indeed, formation of the  $[\text{Fe}(\text{III})\text{--HOOH}]$  complex may be required to make the peroxide sufficiently acidic to be deprotonated by the distal histidine residue in the peroxidase active site [21].

### 5.2.3 *Compound I: Porphyrin Radical*

The conversion of Compound 0 to Compound I requires protonation of the distal oxygen of the ferric hydroperoxide complex so that formation of the ferryl species can be linked to elimination of the distal oxygen as a molecule of water. It is thought that the distal histidine residue in the peroxidase active site is the base that deprotonates the peroxide in the formation of Compound 0. This proton is then delivered by the imidazole to the terminal oxygen of the ferric hydroperoxide complex, catalyzing the O–O bond cleavage that produces Compound I (Fig. 5.4). This general sequence, referred to as the Poulos–Kraut mechanism [22], was first postulated when the structure of CcP became available. The structure of CcP was the first of any peroxidase to be determined. Although more recent work has suggested refinements of this mechanism, its basic features have been confirmed by extensive work.

UV–visible [23], EPR [24, 25], Mössbauer [24], EXAFS [26], and resonance Raman [27] data indicate that Compound I of HRP consists of a ferryl  $[\text{Fe}(\text{IV})\text{=O}]$  species combined with a radical cation localized on the porphyrin framework [23, 24], although the porphyrin radical cation is difficult to observe directly by EPR [23, 25].

The crystal structure of Compound I has been determined after its generation by reaction of ferric HRP with peracetic acid [28]. The high reactivity of Compound I



**Fig. 5.4** Schematic representation of the Poulos–Kraut peroxidase mechanism in which the conserved distal histidine serves as an acid–base catalyst that transfers a proton from the  $\text{H}_2\text{O}_2$  to the terminal oxygen after formation of the  $[\text{Fe}(\text{III})\text{--OOH}]$  intermediate. The proximal histidine iron ligand and the catalytic histidine and arginine are shown. In HRP, these residues are His170, His 42, and Arg38, respectively

and its sensitivity to reduction by X-rays made it necessary to employ a multiple crystal strategy to obtain the structure. The iron–oxygen bond in the structure is 1.7 Å in length, a value that agrees reasonably well with the 1.64 Å obtained by extended X-ray fine structure (EXAFS) analysis [26]. In general, the iron–oxygen bond length in Compound I is in the range 1.67–1.76 Å [29], consistent with the presence of an unprotonated ferryl oxygen atom. The oxygen of the ferryl group in the HRP crystal structure forms hydrogen bonds with Arg38 and a water molecule that, in turn, is hydrogen bonded to His42 and Arg38 [26].

### 5.2.4 Compound I: Protein Radical

The Compound I species of many peroxidases, as illustrated for HRP, is characterized by an  $[\text{Fe}(\text{IV})\text{=O}]$  ferryl species coupled with a porphyrin radical cation.

However, in some peroxidases, the porphyrin radical cation, if formed at all, is transient and gives way to a Compound I intermediate in which the ferryl species is accompanied by a protein rather than porphyrin radical. The prototypical enzyme of this class is CcP in which Trp191, located adjacent to the proximal iron ligand His175 [30], is oxidized to the radical cation [31]. This intermediate in CcP was historically known as Compound ES to differentiate it from Compound I of enzymes like HRP, although in fact it is an alternative form of Compound I if this intermediate is defined as an enzyme species two oxidation equivalents higher than the resting ferric state. The finding that replacement in HRP of Phe172 by a tyrosine or Phe221 by a tryptophan gives mutant proteins in which the Compound I with a porphyrin radical decays to a Compound I with a protein radical indicates that a key determinant of this shift in the radical location is simply the presence of an appropriately located oxidizable residue [8, 32]. In the CcP Compound I structure, the Fe–O distance is 1.87 Å [7], consistent with the presence of a singly bonded, protonated ferryl oxygen [Fe(IV)–OH], such as that found in Compound II of all the peroxidases.

The different location of the radical in the two types of Compound I structure is relevant, as it causes differences in their spectroscopic properties and results in differential catalytic activities. These differences only apply to Compound I, as the spectroscopic and catalytic properties of Compound II, in which only the ferryl is retained (see below), are similar for all the peroxidases.

In some enzymes, the protein radical appears to participate in substrate oxidation. Evidence exists for the involvement of a surface tryptophan in the oxidation of veratryl alcohol by the ligninase from *Phanerochaete chrysosporium* [33]. Similarly, tryptophan radicals on the surface of the versatile peroxidases from *Pleurotus eryngii* and *Bjerkandera adjusta* [34–36], and a tyrosine in the LiP from *Trametes cervina* [33], are thought to be involved in substrate oxidation.

### 5.2.5 Compound II

One-electron reduction of Compound I produces Compound II, in which the iron is still present in the [Fe(IV)] state, but the porphyrin radical has been quenched. The crystal structure of HRP Compound II exhibits an iron–oxygen bond length of 1.8 Å [28]. In general, peroxidase Compound II structures have an iron–oxygen bond length of 1.86–1.92 Å, consistent with the presence of a single-bond between these atoms and therefore of a protonated oxygen [Fe(IV)–OH] [29]. The ferryl oxygen in HRP is hydrogen-bonded to Arg38 and to a water molecule that is hydrogen-bonded to His42 and Arg38.

### 5.2.6 Compound III

Compound III designates a complex in which a molecule of oxygen is bound end-on to the ferrous iron of the peroxidase. It is thus a structure that is close to those of the

oxygen complexes present in oxymyoglobin and oxyhemoglobin. The crystal structure of HRP Compound III shows the oxygen is bound in a bent conformation, with one oxygen atom bound to the iron at a distance of 1.8 Å and a Fe–O–O bond angle of 126° [28]. In HRP, the oxygen atom furthest away from the iron atom is hydrogen-bonded to His42, Arg38, and a water molecule.

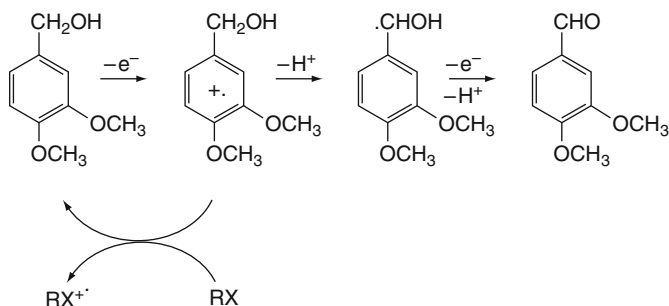
Compound III, in which the iron is in the ferrous state, is usually formed when there is a large excess of H<sub>2</sub>O<sub>2</sub>. It is likely that this intermediate is largely formed by combination of superoxide, generated by the oxidation of H<sub>2</sub>O<sub>2</sub>, with the ferric enzyme, although superoxide could also be generated by electron transfer from oxidized substrates to molecular oxygen. Compound III is not ordinarily a catalytically active intermediate, although it may play a role in the oxidation of isoniazid by the catalase–peroxidase KatG of *Mycobacterium tuberculosis* [37]. For a more detailed description of Compound III, refer to Chap. 11.

## 5.3 Self-Processing of Peroxidases

### 5.3.1 Protein Modifications

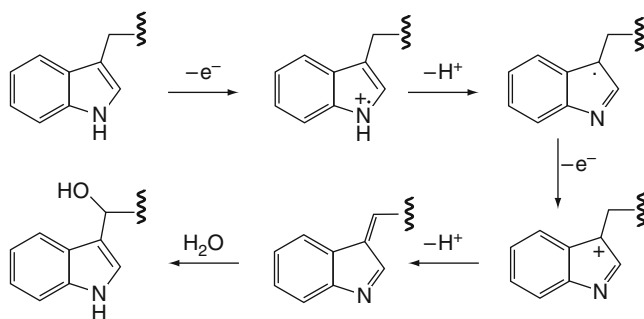
In view of the formation of a highly reactive Compound I ferryl species, and the fact that the porphyrin radical cation of this intermediate is reduced in enzymes such as CcP by a protein residue, it is not surprising that permanent covalent modifications are autocatalytically introduced into some protein frameworks. Two examples of autocatalytic protein modification, those of LiP and the catalase–peroxidases, are summarized here to illustrate the maturation of peroxidase protein structures that can have important functional consequences.

LiP catalyzes the degradation of lignin. The enzyme is commonly assayed by its ability to oxidize veratryl alcohol to a diffusible veratryl radical cation that can either oxidize other substrates or is further converted by a second electron removal to the aldehyde (Fig. 5.5). This reaction is thought to mimic the *in vivo* operation of



**Fig. 5.5** Oxidation of veratryl alcohol to a radical cation that can oxidize other substrates (RX) or can be further oxidized to give veratryl aldehyde

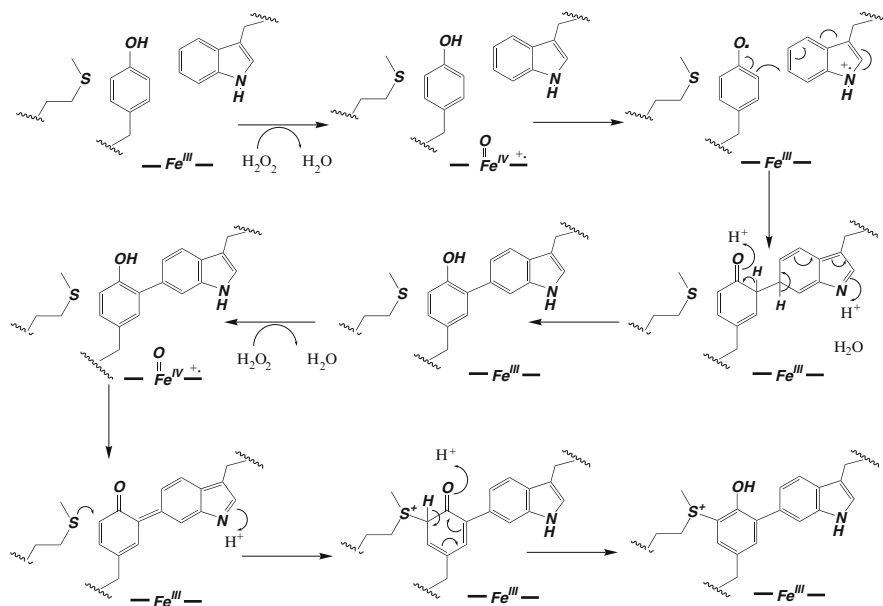




**Fig. 5.6** Reaction scheme showing a mechanism for intramolecular, autocatalytic oxidation of Trp71 in LiP to a hydroxylated tryptophan derivative

LiP, where a diffusible radical can facilitate degradation of the relatively impermeable lignin matrix. Interestingly, LiP undergoes a maturation process in which Trp171 is autocatalytically oxidized by insertion of a hydroxyl into the side-chain adjacent to the aromatic ring (Fig. 5.6) [38, 39]. Oxygen is not required for this hydroxylation reaction, suggesting that the hydroxyl group is introduced after formation of a conjugated system via two sequential one-electron oxidations by Michael addition of a water molecule, producing the modified Trp171 residue [38]. The finding that in the presence of veratryl alcohol more catalytic turnovers are required to introduce the modification indicates that the modification is not actually essential for oxidation of this substrate. However, the W171F and W171S mutants are unable to oxidize veratryl alcohol but still oxidize two other small substrates [39, 40]. These results suggest that the Trp171 locus is the binding site for oxidation of veratryl alcohol, but a second site exists for the oxidation of other substrates. It is not clear, however, whether the Trp171 modification is incidental or actually plays a role in the oxidation of veratryl alcohol.

The catalase–peroxidase KatG enzymes are unique in that they have high catalase activity but also a substantial peroxidase activity [41]. One of the unique, highly conserved features of this class of enzymes is the presence of a crosslinked Met–Tyr–Trp tripeptide. Expression of the *M. tuberculosis* KatG in *Escherichia coli* under conditions that prevent catalytic turnover yields an enzyme without the crosslinked peptide, but exposure to low levels of  $\text{H}_2\text{O}_2$  results in rapid, quantitative introduction of the crosslinks [42]. The tripeptide is clearly the result of an autocatalytic processing event. The crosslinked tripeptide is formed by a series of sequential peroxidative reactions terminated by addition of the Met sulfur to the resulting Michael acceptor (Fig. 5.7). Mutation of the residues involved in tripeptide formation results in a major loss of catalase activity, but conversely a small increase in peroxidase activity [43]. The role of the tripeptide thus appears to be related to the preservation of catalase activity, possibly by helping to reduce the catalase-inactive Compound II to the ferric state [43], but mostly, as the tripeptide radical, by accepting an electron from the Compound III [Fe(II)– $\text{O}_2$ ] intermediate,



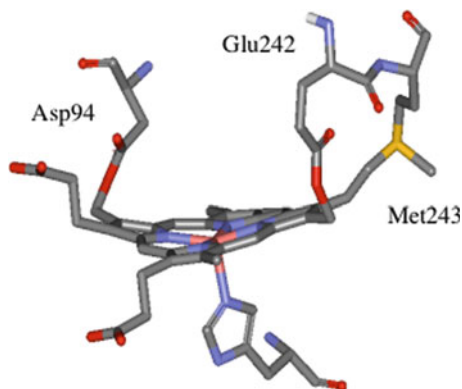
**Fig. 5.7** Mechanism proposed for autocatalytic formation of the Met–Tyr–Trp crosslinked tripeptide found in all KatG catalase–peroxidases based on experimental observations with the enzyme from *M. tuberculosis*. The iron between *two bars* represents the prosthetic heme group

generating a molecule of oxygen and returning the tripeptide radical to the neutral state and the iron to the ferric state [44, 45].

### 5.3.2 Heme-Protein Crosslinking

Although the mammalian peroxidases catalyze conventional peroxidation reactions, their physiological functions generally involve oxidation of a halide ( $X^-$ ) to the halogenating and microbicidal product HOX [46]. This difference in physiological function may help explain why the prosthetic heme group in LPO [47], MPO [48], eosinophil peroxidase [49], and probably thyroid peroxidase is covalently bound to the protein. The crystal structures of LPO and MPO clearly show that covalent ester bonds are formed between the 1- and 5-methyl groups and the carboxyl group of nearby Asp or Glu residues [47, 48]. In MPO, these bonds involve the carboxyl groups of Asp94 and Glu242 (Fig. 5.8). However, a recent study has suggested that the ester bond with Glu242 in the protein isolated from a native source may only be present in a subset of the protein molecules [49]. Unusually, in MPO there is an additional bond between the heme 2-vinyl group and the sulfur of a methionine residue [48]. The two ester bonds to the heme appear

**Fig. 5.8** The active site of MPO showing the covalent bonds between the 1- and 5-methyls of the heme and the carboxyl groups of Glu242 and Asp94, respectively. The covalent bond between the 2-vinyl of the heme and the sulfur atom of Met243 is also shown



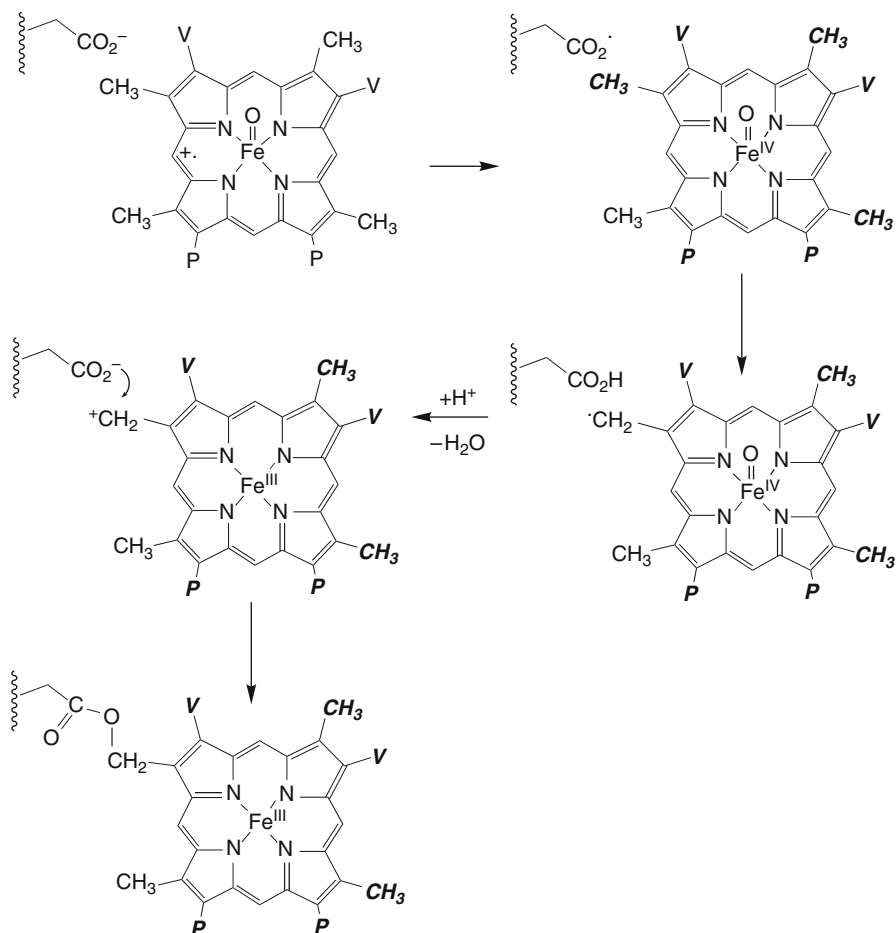
to be present in eosinophil peroxidase, although they have not been as precisely established [50].

Heterologous studies clearly demonstrate that covalent heme binding is the result of an autocatalytic processing event. Expression of LPO in a baculovirus system yields a protein in which only a fraction of the protein has a covalently bound heme [51]. Incubation of this expressed protein with  $H_2O_2$ , however, results in a high level of covalently bound heme [51, 52]. The mechanism in Fig. 5.9 has been proposed to explain the covalent binding sequence, which in these proteins must occur twice to form the two ester bonds present in the native protein. Interestingly, introduction by site-specific mutagenesis into HRP of a carboxyl group near one of the heme methyl groups yields a protein, the F41E mutant, which on incubation with  $H_2O_2$  quantitatively forms a covalent bond to the heme [53], presumably by the mechanism shown in Fig. 5.9.

The third covalent bond present in MPO is also thought to form by an autocatalytic process, although it has not been clearly demonstrated to do so. However, incubation of an ascorbate peroxidase mutant into which a methionine has been introduced by mutagenesis has been shown to result in covalent attachment of the Met sulfur atom to a heme vinyl group [54]. Although the link is not identical to that in MPO, this finding provides strong circumstantial evidence that the bond in MPO is also formed by an autocatalytic mechanism.

### 5.3.3 Heme Modification by Metabolites

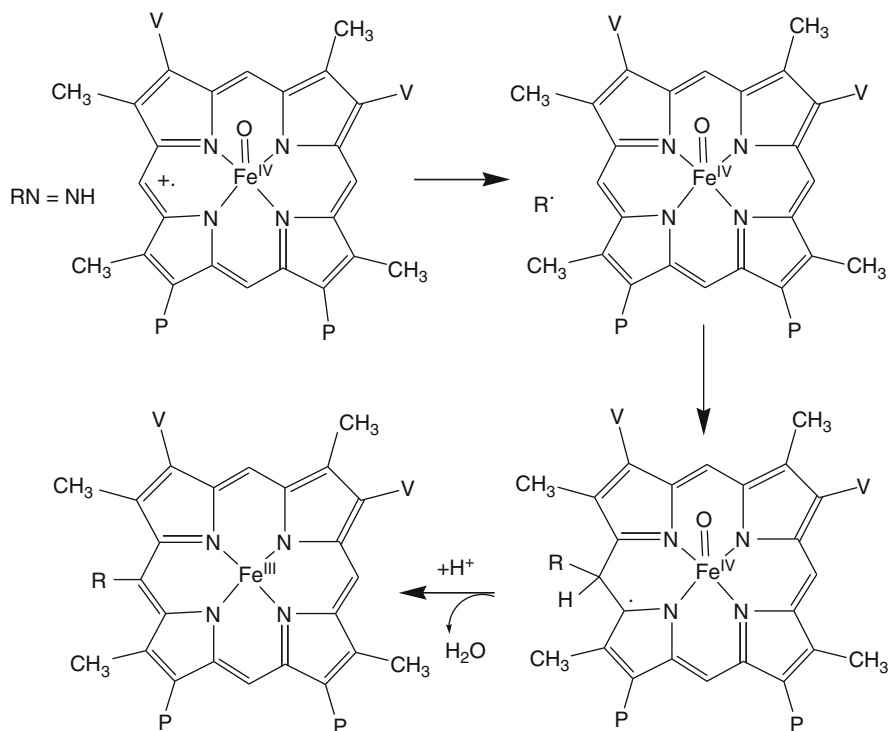
The free radical products generated by peroxidases are highly reactive species that can, in some instances, covalently modify the heme group of the enzyme (Fig. 5.10). In the case of HRP, the substrates for which this type of reaction has been observed include aryl and alkyl hydrazines [55, 56], the azide anion [57], nitromethane [58], cyclopropanone hydrate [59], and alkyl acids [60]. Free radicals



**Fig. 5.9** Mechanism proposed for formation of a covalent bond between a heme methyl and a protein carboxyl group. A similar sequence must occur again to form the second heme–protein ester link in the mammalian peroxidases. In the heme structure, V stands for  $-\text{CH}=\text{CH}_2$  and P for  $-\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$

of relatively high energies readily add at the  $\delta$ -*meso* carbon of the heme group, as shown in Fig. 5.10, whereas radicals of low reactivity (e.g., nitrite radical) can only add efficiently to the heme vinyl groups (Fig. 5.11) [61]. The C–H bond dissociation energy for the radical of around  $90 \text{ kcal mol}^{-1}$  appears to define the limit below which addition to the *meso*-position does not occur, although addition to the vinyl groups can, in principle, occur with all the radicals (Table 5.2).

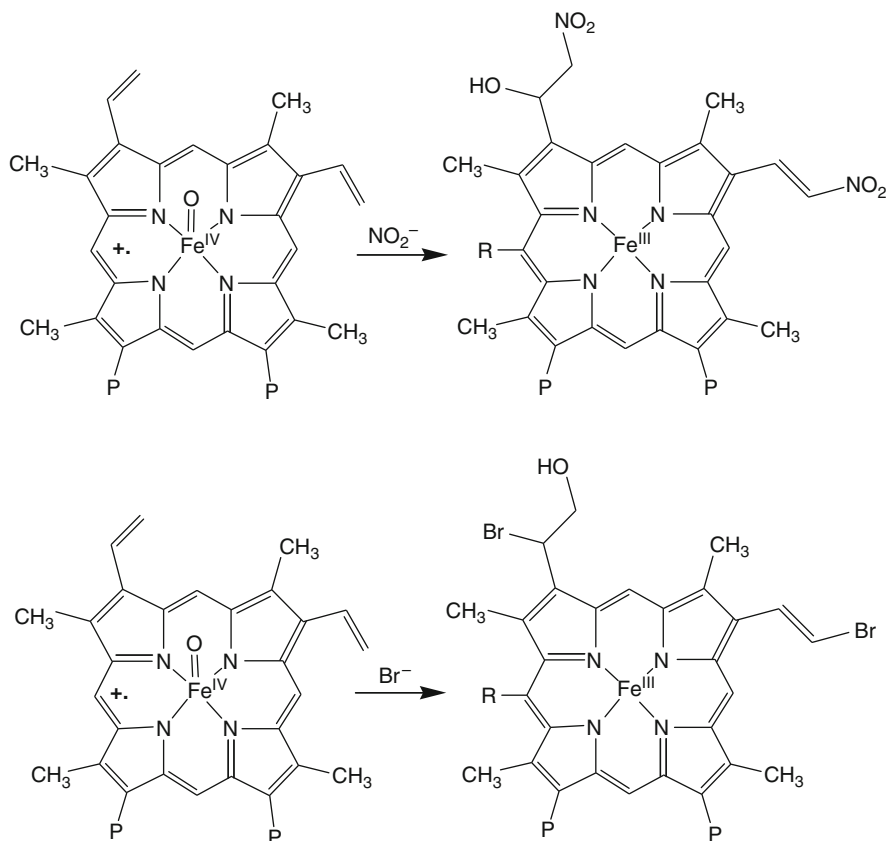
Prosthetic heme modification also occurs in some instances with the two-electron oxidation products formed by peroxidases when they oxidize halide and pseudohalide ions. Thus, the oxidation of bromide by HRP results in the addition of HOBr to one or both of the heme vinyl groups (Fig. 5.11) [62]. Similar reactions are



**Fig. 5.10** Addition to the  $\delta$ -meso position of the prosthetic heme of the carbon radical generated when HRP oxidizes a diazene ( $\text{RN}=\text{NH}$ ). The diazene is itself generated by peroxidatic oxidation of the precursor hydrazine ( $\text{RNHNH}_2$ )

observed with the HOSCN produced by HRP from thiocyanate ion [63]. Even the oxidation of chloride ion, which is mediated very poorly by HRP, results in addition of HOCl to the vinyl groups [62]. The electrophilic metabolites may also react with nucleophilic groups of the protein [64].

Heme modification by the products of peroxidase catalysis has been observed with peroxidases other than HRP, but it does not occur with all peroxidases. Some peroxidases are resistant to these types of reactions. In particular, the mammalian peroxidases are resistant to heme modification by both the free radical and electrophilic metabolites [63]. This resistance is due, at least in part, to the covalent bonds that link the heme to the mature protein. A similar resistance to modification by the HOBr produced by HRP is observed when the reaction is carried out with the F41E mutant in which a covalent bond to the heme has been introduced [65]. However, resistance to radical products can occur even without the presence of covalent links between the heme and the protein. Thus, LiP has a heme that is resistant to modification by phenylhydrazine or azide, although the protein is apparently inactivated by modifications of the protein [66].



**Fig. 5.11** Modification of the vinyl groups of the prosthetic heme of HRP upon catalytic oxidation of nitrite to  $\text{NO}_2^-$  or  $\text{Br}^-$  to HOBr. For each reaction, two different modifications of the vinyl groups are shown. These modifications can occur in different combinations. For example in the  $\text{Br}^-$  reaction, both of the vinyls could be present as bromohydrin [ $-\text{CHBrCH}_2\text{OH}$ ] adducts

**Table 5.2** Relationship between the energy of a radical and its ability to add to the *meso*-position of the heme group in HRP [61]

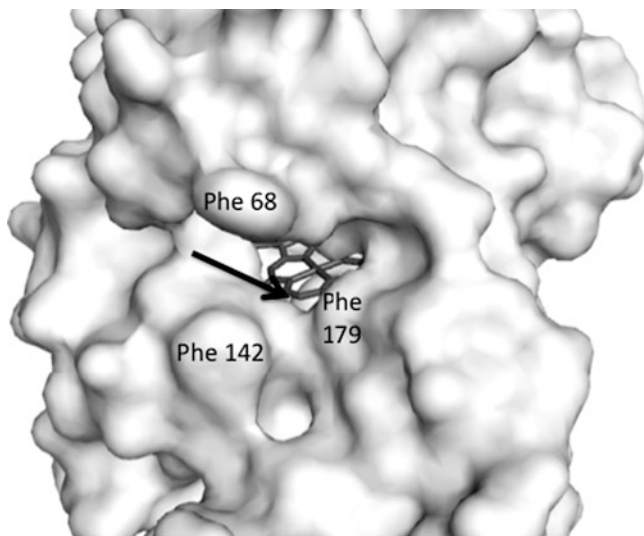
C-H bond	DH <sub>298</sub> (kcal mol <sup>-1</sup> )	Site of addition to heme of HRP <sup>a</sup>
HCN	126	$\delta$ - <i>meso</i>
C <sub>6</sub> H <sub>5</sub> -H	113	$\delta$ - <i>meso</i>
CH <sub>3</sub> CO <sub>2</sub> H	112	$\delta$ - <i>meso</i>
CH <sub>3</sub> -H	105	$\delta$ - <i>meso</i> , vinyl
HCl	103	$\delta$ - <i>meso</i> , vinyl
CH <sub>3</sub> CH <sub>2</sub> -H	101	$\delta$ - <i>meso</i>
HNCS	96	$\delta$ - <i>meso</i>
HN <sub>3</sub>	92	$\delta$ - <i>meso</i>
CH <sub>3</sub> OO-H	88	vinyl
HBr	88	vinyl
HNO <sub>2</sub>	79	vinyl

<sup>a</sup>In some instances, the F41M mutant of HRP was used to decrease the steric barriers to the active site

## 5.4 Substrate Oxidations

### 5.4.1 Oxidative Properties

Peroxidases catalyze the one-electron oxidation of sufficiently oxidizable substrates but generally do not catalyze oxygen transfer reactions in which the ferryl oxygen is transferred to the substrate. This is a key distinction between the peroxidases and monooxygenases despite the fact that both enzymes employ analogous Compound I catalytic species. Computational comparison of Compound I in the peroxidases and cytochrome P450 enzymes suggests that the peroxidase Compound I should actually be competent in oxygen transfer reactions [67]. However, steric constraints imposed on the peroxidase active site play a major role in limiting peroxygenase reactions in favor of simple one-electron oxidations. The crystal structure of HRP shows that the heme group is embedded in a protein crevice with only the  $\delta$ -*meso*-edge of the heme exposed to the medium (Fig. 5.12) [6]. The heme iron atom is protected, among others, by the catalytic histidine residue (His42) and an adjacent phenylalanine (Phe41). A consequence of this active site topology is that substrates are oxidized by electron transfer to the exposed heme edge without interacting directly with the ferryl oxygen, a reaction trajectory that precludes transfer of the ferryl oxygen to the substrate. This heme edge reactivity was first unmasked by studies showing that the inactivation of HRP and other peroxidases by alkyl and aryl hydrazines resulted from addition of the resulting carbon radicals exclusively to the  $\delta$ -*meso* carbon atom of the prosthetic heme group (Fig. 5.10)



**Fig. 5.12** The HRP substrate access channel. The aromatic residues flanking the access channel are *highlighted*. The *arrow*, located in the access channel, points to the  $\delta$ -*meso*-carbon atom

[55, 56]. The bottom line is that for substrates larger than one or two atoms in size, steric restrictions imposed on the peroxidase active site prevent oxygen transfer reactions and confine substrate–enzyme interactions to the heme edge.

CcP, in contrast to HRP, has two sites for interaction with substrates. Cytochrome *c*, its physiological substrate, binds primarily at a protein surface site facing the carboxyl groups of the heme from which it delivers an electron to the Compound I or II intermediate [68]. The oxidation of guaiacol by CcP, however, is apparently mediated at the  $\delta$ -*meso* edge of the heme. Autocatalytic inactivation of the enzyme by phenylhydrazine or azide, which results in covalent binding of the phenyl or azide group, respectively, to the  $\delta$ -*meso* carbon of the heme inhibits oxidation of the small substrates to a much greater extent than it does cytochrome *c* oxidation [69]. Furthermore, reconstitution of the protein with  $\delta$ -*meso*-substituted hemes suppresses oxidation of guaiacol but has only modest effects on cytochrome *c* oxidation. A channel is present in the CcP crystal structure that leads from the outside of the protein into the active site crevice and terminates near the  $\delta$ -*meso* edge of the heme [30]. Two sites thus exist for substrate oxidation in CcP, one at the protein surface for the normal protein substrate and a second one for small substrates such as guaiacol within the heme cavity.

As already mentioned, in some enzymes radicals generated on surface tryptophan and tyrosine radicals by electron transfer to the ferryl species are involved in abstraction of electrons from substrates [33–36, 40]. Mutation of Trp171 on the surface of *P. chrysosporium* LiP to a phenylalanine or serine completely suppresses the veratryl alcohol oxidizing activity of the enzyme [40]. A similar depression in the oxidation of veratryl alcohol occurs on mutation of Trp164 in the versatile peroxidase from *P. eryngii* [34, 35].

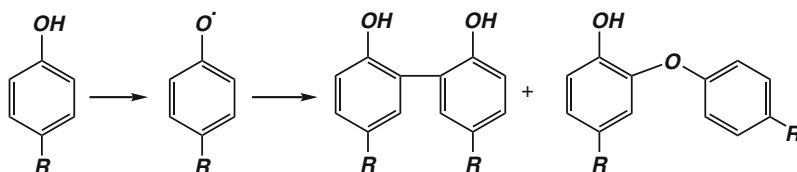
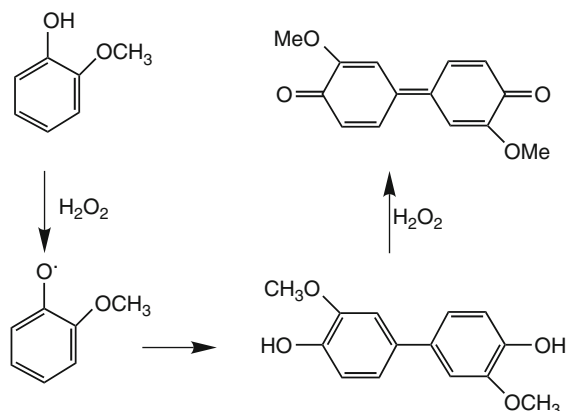
## 5.4.2 One-Electron Oxidations

### 5.4.2.1 Phenols

The classic reaction catalyzed by peroxidases is the one-electron oxidation of phenols. The guaiacol assay for peroxidase activity, an assay that derives from the earliest observation of peroxidase activity [70], involves the one-electron oxidation of guaiacol to a free radical that undergoes subsequent radical–radical combination to give a colored dimeric product (Fig. 5.13) [71]. The ability of substituted phenols to act as substrates and reduce HRP Compound I is controlled by the electronic nature of the substituent [72]. At pH 7.0, the linear Hammett correlation observed between the substituent  $\sigma$  value and the rate of Compound I reduction adheres to the equation  $\log k_X/k_H = -6.92\sigma$ , where  $k_X$  is the rate of the reaction of the substituted phenol and  $k_H$  is the rate of the reaction of the unsubstituted phenol. The ability of substituted phenols to reduce Compound II also correlates with the substituent  $\sigma$ -values according to the equation  $k_X/k_H = -4.6\sigma$  [73]. As might be expected, correlations have also been shown to exist between the



**Fig. 5.13** The oxidation of guaiacol to colored dimeric products underlies the use of guaiacol in a classical peroxidase activity assay. Additional products can be formed



**Fig. 5.14** A common peroxidase reaction is the oxidation of phenols to phenolic radicals that condense to give dimeric or oligomeric products. As shown, two orientations are possible for the condensation of the phenolic radicals if the *para*-position is blocked by a group R

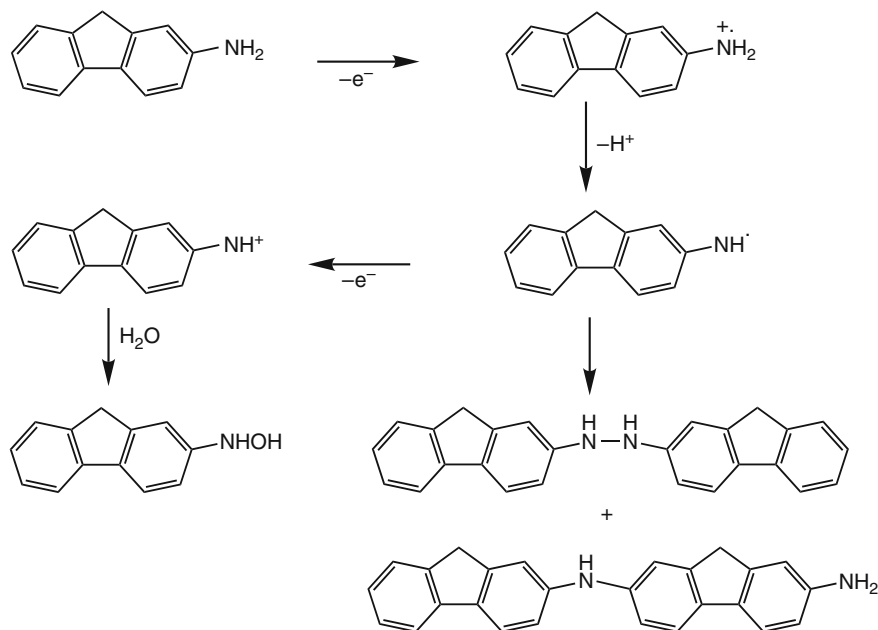
calculated redox potentials of the phenols, which are themselves related to the electronic properties of the substituents, and the rates of their oxidation by HRP [74, 75]. These relationships establish that the ability to reduce both Compound I and Compound II increases as the electron donating strength of the substituent increases, with Compound I reduction being more responsive to the electron density in the phenol ring. As the difference in the redox potentials of Compounds I and II at pH 7.0 is small [76], something else must account for the difference in the sensitivity of the rates to the electronic nature of the substituents. One possibility suggested by the evidence that electrons are transferred to the heme edge is that electron transfer is more facile to the heme edge of Compound I, in which the porphyrin is a radical cation, than of Compound II, in which it is an electroneutral porphyrin.

The final products of phenol oxidation are generated by secondary reactions of the radicals produced by the peroxidase. One very common pathway involves dimerization or oligomerization of the radicals, as illustrated in Fig. 5.14 for the oxidation of a *para*-substituted phenol such as tyrosine [R = CH<sub>2</sub>CH(NH<sub>2</sub>)CO<sub>2</sub>H]. The dimerization can occur between two ring carbon atoms or by addition of the oxygen of one phenoxy radical to a ring carbon of the other.

### 5.4.2.2 Aromatic Amines

Peroxidases also catalyze the one-electron oxidation of aromatic amines to the corresponding radical cations. Studies of the oxidation of substituted anilines by HRP Compounds I and II have established that these reactions are also linearly correlated with the electron donating nature of the substituents [72, 73]. The Hammett equation for the Compound I oxidation of anilines was  $k_X/k_H = -7.00\sigma\sigma$ , and for the corresponding reaction of Compound II (obtained by a different group) was  $k_X/k_H = -5.75\sigma$  [77]. A comparison of the absolute rates of the oxidation of substituted anilines and phenols indicates that the phenols are generally oxidized more rapidly by 1–2 orders of magnitude [72, 77].

The oxidation of aminofluorene by HRP provides a suitable example [78]. The amine group is oxidized to the radical cation that, upon deprotonation, yields the aminofluorenyl radical. This radical can dimerize by nitrogen–nitrogen coupling or by unsymmetrical combination, yielding the products in Fig. 5.15. The nitrogen radical can also be further oxidized to a nitrogen cation that combines with water to give the hydroxylamine derivative. In reality, these products can be further oxidized by HRP, so that higher oxidation products are obtained.



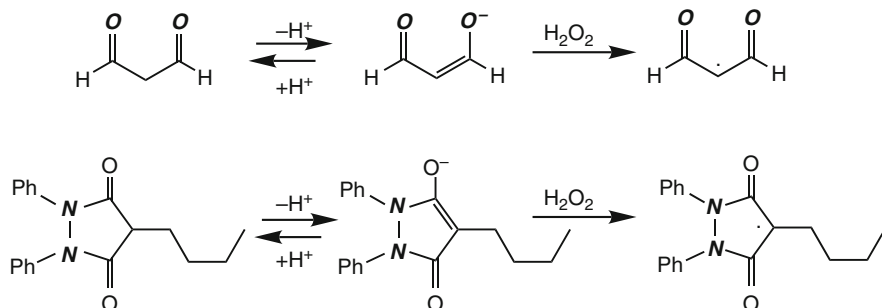
**Fig. 5.15** Initial products formed in the oxidation of aminofluorene by HRP. Further oxidative products can occur under the continued catalytic action of the peroxidase

### 5.4.2.3 Carbon Oxidation

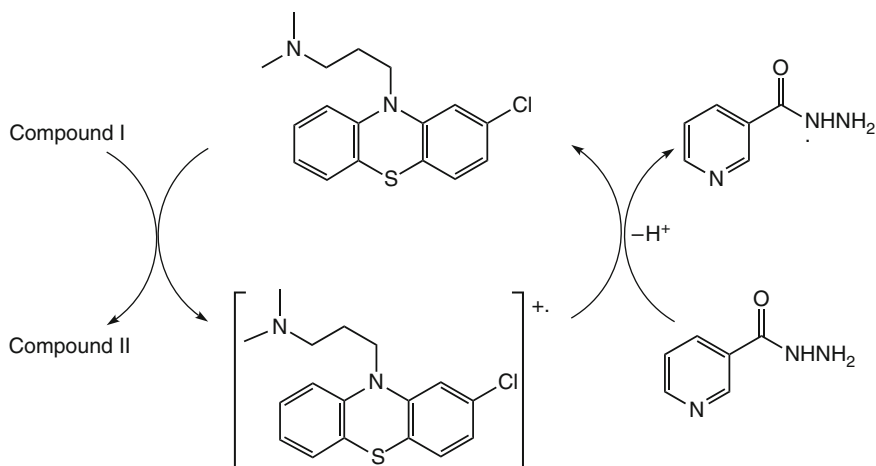
Carbon atoms can be oxidized when they are anionic or bear substantial negative charge due to their incorporation into an appropriate functional group. For example, HRP oxidizes malondialdehyde to a free radical product (Fig. 5.16; [79]). The carbon between the two carbonyl groups in this molecule has a pKa of 4.65 and is therefore present at pH 7 largely in a deprotonated state with the negative charge delocalized over the conjugated system. Phenylbutazone, with a pKa of 4.5 for the carbon between the two carbonyl groups, is also highly ionized in solution and is oxidized by HRP to the corresponding free radical [80].

### 5.4.2.4 Electron Transfer Relay

Efficient, diffusible peroxidase substrates can play a role in the oxidation of secondary, less efficient, peroxidase substrates. As already mentioned, the veratryl radical produced by LiP can serve to oxidize lignin at sites inaccessible to the enzyme (Fig. 5.5). The same mechanism operates with manganese peroxidases, which oxidize Mn(II) to Mn(III) [81]. The higher valent manganese then diffuses into lignin to mediate the oxidation of relatively inaccessible residues. This mechanism is not limited to lignin degrading enzymes, however. For example, a similar relay has been shown to improve the oxidation of isoniazid by HRP in the presence of chlorpromazine [82]. Chlorpromazine, an efficient substrate for HRP, is converted to a radical cation that, in turn, oxidizes isoniazid (Fig. 5.17). Isoniazid itself is a substrate for HRP, but it is an inefficient substrate and also irreversibly inactivates the enzyme. Using the chlorpromazine radical cation as a mediator results in a rate of isoniazid oxidation several orders of magnitude faster than in its absence. Furthermore, the chlorpromazine electron relay completely protects the peroxidase enzyme from irreversible inactivation.



**Fig. 5.16** HRP-catalyzed oxidation of malondialdehyde and phenylbutazone, two carbons with an acidic carbon atom



**Fig. 5.17** Role of chlorpromazine as an electron relay in the oxidation of isoniazid by HRP. Although isoniazid is directly oxidized by HRP, the reaction is much faster when chlorpromazine, a better substrate, is used as a relay in the oxidation of isoniazid

## 5.4.3 Two-Electron Oxidations

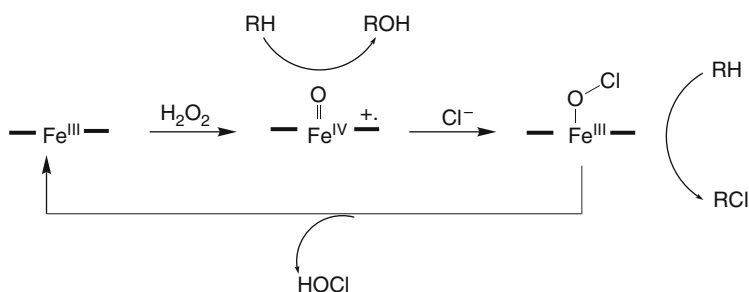
### 5.4.3.1 Halogenation

Direct, two-electron oxidations are rare for most peroxidase enzymes. The one broad exception is the oxidation of halide and pseudohalide ions, specifically  $\Gamma^-$ ,  $\text{Br}^-$ ,  $\text{Cl}^-$ , and  $\text{NCS}^-$ . Fluoride ion, in contrast, is not known to be oxidized by these enzymes. The oxidation of  $\Gamma^-$  and  $\text{NCS}^-$  is common for the peroxidases, whereas that of  $\text{Br}^-$  is widespread but is usually less effective, and that of  $\text{Cl}^-$ , among the conventional peroxidases, is only important in the case of MPO [46, 83]. The halogenation activities of the mammalian peroxidases are compared in Table 5.3. As the table shows, chloride ion is oxidized by MPO, particularly at pH 5, but it is a very poor substrate for EPO and LPO.  $\text{Br}^-$ ,  $\Gamma^-$ , and  $\text{SCN}^-$  are readily oxidized by all three enzymes, but most efficiently by EPO at pH 5 [84–86].

The plant and fungal peroxidases with a histidine iron ligand also do not detectably oxidize chloride ion, although the demonstration that oxidation of chloride ion at acidic pH by HRP and *Arthromyces ramosus* peroxidase results in addition of HOCl to the prosthetic heme vinyl groups illustrates that a low level of activity actually exists [62, 87]. However,  $\text{Cl}^-$  ions are readily oxidized by the special class of peroxidases exemplified by the haloperoxidases from *Caldariomyces fumago* and *Agrocybe aegerita* [88]. These chloroperoxidases are distinguished by the fact their proximal iron ligand is not a histidine but rather a cytochrome P450-like thiolate ion [89]. Furthermore, the distal catalytic histidine is replaced in chloroperoxidase by a glutamic acid (Glu183), which plays a catalytic role similar to that of the histidine in HRP [90, 91].

**Table 5.3** Apparent second order rate constants for the oxidation of halides and thiocyanate at pH 7 and 4.5 by Compound I of MPO, EPO, and LPO

	MPO [84] $\times 10^4$ ( $M^{-1} s^{-1}$ )		EPO [85] $\times 10^4$ ( $M^{-1} s^{-1}$ )		LPO [86] $\times 10^4$ ( $M^{-1} s^{-1}$ )
	pH 7	pH 5	pH 7	pH 5	pH 7
$Cl^-$	2.5	390	0.31	2.6	—
$Br^-$	110	3,000	1,900	11,000	4.1
$I^-$	720	6,300	9,300	>11,000	12,000
$SCN^-$	960	7,600	10,000	>11,000	20,000

**Fig. 5.18** Schematic representation of the mechanism of haloperoxidases. In the presence of  $Cl^-$ , HOCl is formed that (a) diffuses from the active site and oxidizes substrates in the medium, although in some cases, (b) oxidation may occur within the active site. In the absence of  $Cl^-$ , thiol-ligated haloperoxidases can (c) catalyze oxygen transfer to their substrates in a cytochrome P450-like reaction

The oxidation of halides by peroxidases results in the formation of the corresponding hypohalides: i.e., HOI, HOBr, HOCl, and HOSCN. Secondary reactions of these products with the halide ions can lead to additional oxidizing species. Thus, HOSCN can produce NCS–SCN by reaction with another NCS<sup>-</sup> ion [92], and HOCl can be similarly converted to BrCl by bromide ions [93]. The substrate halogenation reactions are the result of reaction with these diffusible metabolites, although it has been argued that substrate halogenation may occur within the active site of the enzyme, either by reaction with the hypohalide before it diffuses out or with a precursor of the hypohalide. Formation of the hypohalide is thought to involve addition of the halide ion ( $X^-$ ) to the oxygen of Compound I (Fig. 5.18) [84], yielding a transient Fe–OX intermediate that, at least in principle, can itself mediate halogenation reactions before it dissociates from the iron atom to give the hypohalide HOX. Furthermore, it has recently been reported that chlorination by a flavin-dependent halogenase involves not only HOCl but also a chlorinated active site lysine residue that can mediate further substrate halogenation [94]. This is reminiscent of a species called Compound X formed in the reaction of HRP with chlorite, in which a chlorinated protein residue was proposed to be involved in substrate halogenation [95]. Analogous protein-mediated halogenations may occur

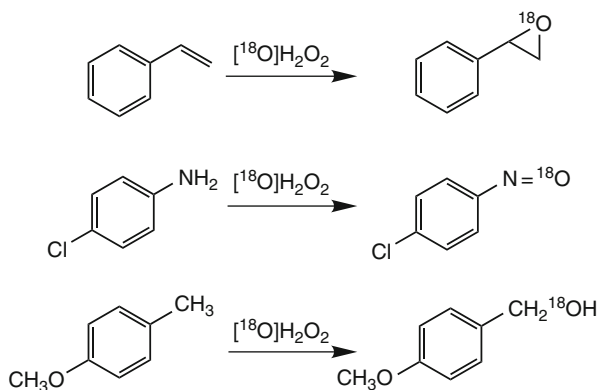
with the hemoprotein haloperoxidases, but there is no evidence that this is a significant reaction pathway.

### 5.4.3.2 Oxygen Transfer

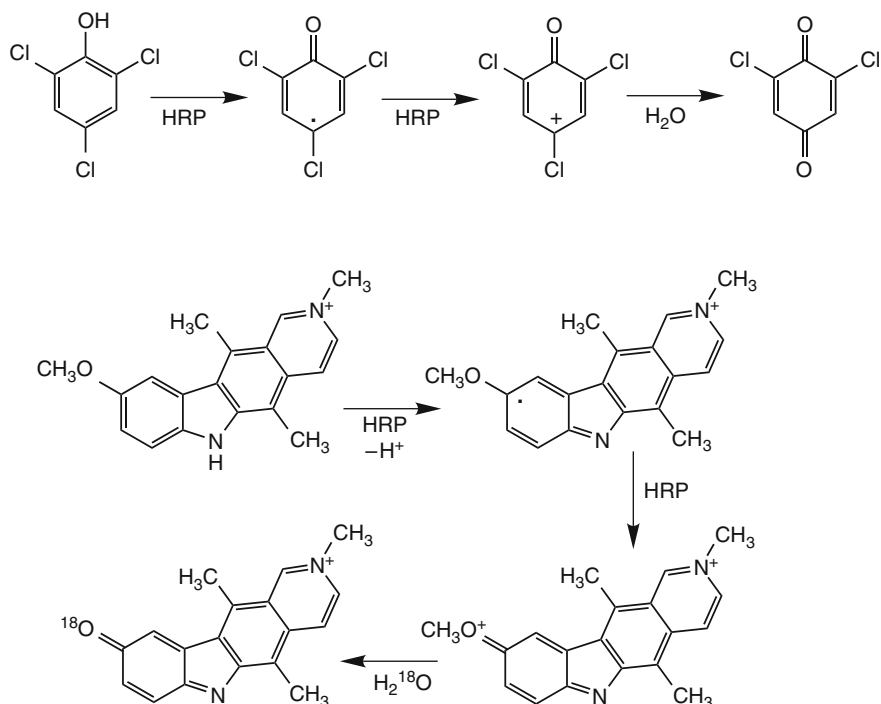
As already indicated, direct oxygen transfer from the peroxidase Compound I intermediate to the substrate does not occur for peroxidases except when the substrate is a small halide or pseudohalide ion. This generalization does not apply, however, to the thiolate-coordinated haloperoxidases. These enzymes not only have a thiolate proximal iron ligand but also a more open distal active site pocket that enables the binding and oxidation of small organic molecules [90, 91]. The oxygen transfer reactions catalyzed by the *Caldariomyces fumago* chloroperoxidase have been extensively investigated and are sufficiently robust to be of biotechnological interest [96]. These reactions include epoxidation of olefins, sulfoxidation of thiol groups, and oxidation of activated (e.g., allylic, propargylic, benzylic) hydrocarbon bonds. In several of these reactions, including the epoxidation of styrene [97], nitrogen oxidation of *p*-chloronitroaniline [98], and hydroxylation of 4-methylanisole [99], it has been specifically demonstrated by  $^{18}\text{O}$ -labeling studies that the oxygen incorporated into the substrate derives quantitatively from the peroxide cosubstrate (Fig. 5.19), as required by direct oxygen transfer from Compound I (Fig. 5.18). Analogous reactions are mediated by the thiolate-ligated haloperoxidase from *A. aegerita* [88].

### 5.4.3.3 Sequential One-Electron Oxidations

Many reactions are known in which oxidation of a substrate by a peroxidase produces a two-electron oxidized product. In general, these reactions can be



**Fig. 5.19** Three oxygen transfer reactions catalyzed by the chloroperoxidase from *Caldariomyces fumago* in which the oxygen transfer was firmly established by  $^{18}\text{O}$  isotopic labeling studies



**Fig. 5.20** Two examples of reactions in which the final metabolites are obtained by two one-electron oxidations catalyzed by the peroxidase

rationalized by formation of a free radical metabolite followed by a second oxidation of the free radical to the final observed product. The two oxidations can be mediated by sequential electron transfer to Compound I and then Compound II of the same peroxidase or by the action of two different peroxidases. The oxidation of veratryl alcohol to veratryl aldehyde (Fig. 5.5) is a reaction of this type. Two further examples of such reactions are the HRP-catalyzed dechlorination of 2,4,6-trichlorophenol and the oxidation of 9-methoxyellipticine to a quinone–imine (Fig. 5.20) [100, 101]. In the latter reaction, the oxygen of the iminoquinone derives from water, as expected from the indicated mechanism.

## 5.5 Conclusions

The peroxidases produce a reactive intermediate, Compound I, whose reactions are controlled by the protein environment. In conventional peroxidases with a histidine iron ligand, access to the Compound I ferryl species is restricted by the protein, favoring the transfer of single electrons from the substrate to an exposed heme edge. If the protein has a suitably placed oxidizable residue such as a tyrosine or

tryptophan, it may be preferentially oxidized by the initial Compound I intermediate to an alternative Compound I in which the ferryl species is paired with a protein rather than porphyrin radical cation. In most conventional peroxidases, only small substrates have ready access to the ferryl oxygen and are subject to a two-electron oxidation, although larger substrates can undergo an apparent two-electron oxidation by the stepwise peroxidative removal of two electrons. In contrast, peroxidases with a thiolate iron ligand have more open distal active sites and are able to catalyze a range of two-electron oxidations in addition to one-electron peroxidative transformations.

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