HEME PEROXIDASES: STRUCTURE, FUNCTION, MECHANISM AND INVOLVEMENT IN ACTIVATION OF CARCINOGENS. A REVIEW

Marie STIBOROVÁ[a1,*], Markéta MIKŠANOVÁ[a], Václav MARTÍNEK[a] and Eva FREI[b]

[a] *Department of Biochemistry, Charles University, 128 40 Prague 2, Czech Republic; e-mail: [1] stiborov@prfdec.natur.cuni.cz*

[b] *Department of Molecular Toxicology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany; e-mail: e.frei@dkfz-heidelberg.de*

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Peroxidases are enzymes playing an important role in large and diverse numbers of physiological processes in organisms including human. We have attempted in this article to summarize and review the important structural and catalytic properties of principal classes of heme peroxidases as well as their biological functions. Major reactions catalyzed by these enzymes (a conventional peroxidase cycle, reactions using O_2 and halogenations) and their mechanism are reviewed, too. Moreover, the reaction mechanisms by which peroxidases are implicated in bioactivation of xenobiotic chemicals are presented. Numerous chemicals including protoxicants and procarcinogens are metabolized by equally numerous chemical reactions catalyzed by peroxidases. The unifying theme is the radical nature of the oxidations. The direct conventional peroxidase reaction forming reactive species is generally responsible for the activation of procarcinogenic substrates of peroxidases. The subsequent formation of a superoxide anion radical and peroxy radicals is necessary for activation of chemicals that are poor substrates for peroxidases. The significance of studies concerning the reactions catalyzed by peroxidases is underlined in the present review article. A review with 166 references.

Key words: Peroxidases; Enzyme structure and function; Reaction mechanisms; Carcinogens; Heme proteins; Porphyrins; Prostaglandins; Oxidations.

Abbreviations used: AA, arachidonic acid; AFB, aflatoxin B_1; AH_2, reducing substrate; $AH^{\bullet}$, corresponding radical; ANFT, 4-(5-nitro-2-furyl)thiazol-2-amine; anti-BPDE, *anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene-7,8-diol; BHA, butylated hydroxyanisole [*tert*-butyl(methoxy)phenols]; BHT, butylated hydroxytoluene [*tert*-butyl(methyl)phenols]; BP-7,8-diol, benzo[*a*]pyrene-7,8-diol; DES, diethylstilbestrol; FANFT, *N*-[4-(5-nitro-2-furyl)thiazol-2-yl] formamide; HOX, hypohalous acid; HRP, horseradish peroxidase; 2-NA, 2-naphthylamine; 6-OH-Sudan I, 1-(phenylazo)naphthalene-2,6-diol; PEI, poly(ethyleneimine); PAH, polycyclic aromatic hydrocarbon; PGH_2, prostaglandin H_2; PGG_2, prostaglandin G_2; $PGG_2^{\bullet}$, peroxy radical of prostaglandin G_2; PHS, prostaglandin endoperoxide H synthase; YH, substrate for halogenation; TLC, thin layer chromatography; X, halogen; Z,Z-DIES, β-dienestrol; S, spin.

1. INTRODUCTION

Peroxidases are enzymes, ubiquitous among the plant and animal kingdom, and exhibiting multimodal function in the organism. One of the most important functions of peroxidases, which might negatively influence human health, is their participation in activation of carcinogenic chemicals in extrahepatic tissues – namely, production of primary reactive radicals of reductive substrates followed by formation of cytotoxic active forms of oxygen and peroxy radicals, which are implicated in initiation and promotion phases of carcinogenesis. Detoxication of hydroperoxides, biosynthesis of essential substances such as prostaglandins and thromboxans, participation in antimicrobial protection in organisms and implication in detoxication reactions of several drugs, rank among peroxidase functions positively influencing human health. An interesting viewpoint concerning practical uses of peroxidases was established in the last two decades: they can be utilized alone or as organisms rich in peroxidase enzymes for removal of toxic pollutants from the environment[1–3].

Peroxidases are the subject of extensive investigation, which has a great significance for theoretical studies. Because primary products of their reactions are radicals, they serve as model enzymes to mimic a one-electron oxidation of substrates in organisms. They are also suitable models for kinetical studies characterizing mechanisms of two-substrate enzyme reactions. Peroxidases utilize peroxides (hydrogen peroxide and/or organic hydroperoxides) to catalyze the oxidation of a wide variety of organic and inorganic substrates. The two-substrate peroxidase reactions exhibit the irrevesible ping-pong mechanism. Most of peroxidases contain ferriprotoporphyrin IX as a prosthetic group in their active sites[4]. This prosthetic group exists in several oxidation states, and hence it is simpler to use the name heme, regardless of its oxidation state.

The irreversibility of peroxidase reactions is one of typical features for reactions catalyzed with these enzymes. Covalent compounds (not enzyme–

substrate complexes) are usual detectable intermediates in the peroxidase reactions. The consequence is that the rate of the reaction appears to have no upper limit[5]. The larger the concentrations of reactants the faster the reaction.

The present review is focused on explanation of multiple mechanisms of reactions catalyzed with heme peroxidases and on participation of peroxidases in activation of carcinogens. Other reviews on this subject were recently published[6–16] and should also be consulted for a more complete understanding of this enzyme system.

2. CLASSIFICATION AND CHARACTERIZATION OF PEROXIDASES

Several peroxidases have been isolated, sequenced and characterized. They have been classified essentially into two main superfamilies: (i) the plant peroxidase superfamily, and (ii) the animal peroxidase superfamily.

On the basis of sequence homologies, Welinder has proposed three classes of peroxidases which belong to the plant peroxidase superfamily (Class I, intracellular peroxidases of prokaryotic origin, Class II, intracellular fungal peroxidases and Class III, secretory plant peroxidases)[17,18].

Class I peroxidases do not contain any disulfide bridge, any carbohydrate, any calcium ions and any signal peptide for secretion. This class of peroxidases includes yeast cytochrome *c* peroxidase, plant ascorbate peroxidase and bacterial catalase-peroxidase[19]. In contrast to Class I enzymes, cysteine bridges are present in Class II and III, even if differently located; all the cysteine residues (around 8–10) form disulfide bridges which provide a high degree of rigidity to the protein[17,19]. These two classes contain also signal peptide sequence for their secretion[17] and two calcium ions required for formation of the structural environment of heme[11]. Finally, Class II and III peroxidases are glycosylated on the protein surface[17]. Tyrosine residues are absent in fungal peroxidases (Class II), except for one residue in one isoenzyme of lignin peroxidases[19]. This makes the reduction potential of the protein matrix for this class higher than in the others. However, despite the differences within the peroxidase families, the proteins have a similar folding pattern. Comparison of the amino acid sequences shows that pattern heme peroxidases from plants, fungi and bacteria are evolutionary related[11,17,18]. Lignin and horseradish peroxidases (HRP) are examples of Class II and III enzymes, respectively. Chloroperoxidase from *Caldariomyces fumago* is a fungal peroxidase, which is, however, very different in its structure and chemical properties from other peroxidases[15]. It does not fit into any of the three plant peroxidase classes.

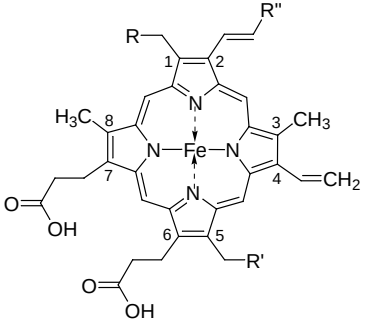
Correlations based on sequence data show that the animal peroxidases form a distinct peroxidase superfamily[20]. These closely related heme peroxidases have been detected in most mammalian exorcine glands and their secretions. Lactoperoxidase, thyroid peroxidase, myeloperoxidase and prostaglandin endoperoxide H synthase (prostaglandin H synthase, PHS) are examples of such enzymes. Two of them (myeloperoxidase and PHS) are dimers consisting of two identical subunits[15].

2.1. Structure of Heme Peroxidases

Plant and fungal peroxidases contain a heme *b* (Fig. 1) where, in the resting state, an iron ion in the oxidation state +3 is present. The iron is five-coordinated to the four pyrrole nitrogen atoms of heme and to the nitrogen of an axial histidine[21,22] (the so-called proximal histidine) with the exception of chloroperoxidase which contains a thiolate ligand[15]. Weak hydrophobic forces complete the binding of heme *b* to the protein.

Mammalian peroxidases (except prostaglandin H synthase with heme *b*) contain a heme *l*, where the positions 1 and 5 are hydroxymethylated[23,24] (Fig. 1). The heme *l* covalently binds to the protein matrix (to glutamic and aspartic acids) by two ester groups[25]. Amino acid sequences of these enzymes in the region of covalent linkage are highly conserved[25]. Myeloperoxidase contains a heme *m*, in which a methionine sulfonium linkage occurs between a vinyl side chain of the heme and a methionine residue[26,27] (Fig. 1).

For many years, the only peroxidase structure known at atomic resolution was that for cytochrome *c* peroxidase[28], which served as a structural



	R	R'	R"
Heme *b*	H	H	H
Heme *l*	HO	HO	H
Heme in LPO	^{275}Glu(O-)	^{125}Asp(O-)	H
Heme in MPO	^{242}Glu(O-)	^{94}Asp(O-)	^{243}Met(S^+-)

FIG. 1
The heme structure found in peroxidase enzymes. LPO, lactoperoxidase; MPO, myeloperoxidase

basis for analysis of properties and function of all other enzymes of this category. In the last decade, X-ray structures for several other peroxidases became available (for review, see ref.[15]), among them are: peanut peroxidase, barley peroxidase, myeloperoxidase, chloroperoxidase, HRP, PHS, lignin peroxidase[29,30], *Arthromyces ramosus* peroxidase[31], *Coprinus cinereus* peroxidase[32], manganese peroxidase[33], and pea ascorbate peroxidase[34]. Despite the low sequence homology among peroxidases (lower than 20%), the overal folding, the organization of the secondary structure, iron coordination and most of the residues in the active centre are conserved in the peroxidases sequenced up to now[35].

The peroxidase protein is divided in two different structural domains, enveloping the heme moiety, and originating from an early gene duplication event[34,35]. Typically, the structure is dominated by 10-11 α-helices, linked by loops and turns, while β-structure is a minor component. Several glycine and proline residues are conserved determining the correct backbone folding. Buried salt bridges are conserved in all peroxidases fixing the long loop connecting the two structural domains. The heme group of plant peroxidases is located between helices B and F (refs[36,37]) (Fig. 2).

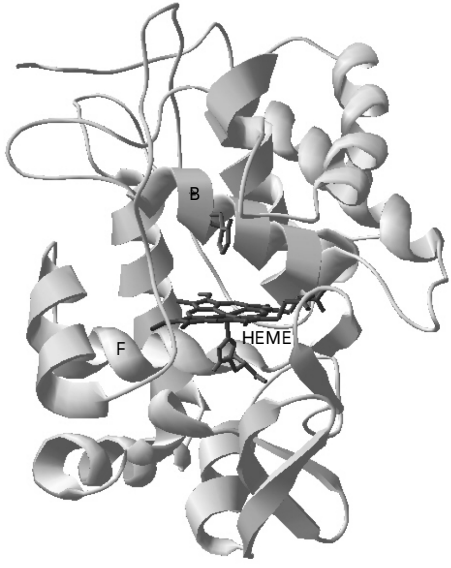


FIG. 2
Helical structure of yeast cytochrome *c* peroxidase. The heme is located between helices B and F (based on the Protein Data Bank file 1CCA)

Figures 3 and 4 show the heme pockets of pea ascorbate peroxidase[34,38] and cytochrome *c* peroxidase[39] with the most relevant residues, respectively. The iron ion is invariantly coordinated by the axial (proximal) histidine. This residue is characterized by an anionic character due to the presence of a strong hydrogen bond between its imidazole residue and a conserved[39,40] aspartate (Fig. 4). This property of axial ligand contributes to the low, negative reduction potential of the iron[11,39,40], which stabilizes high oxidation states of the active species of peroxidase. Globins, which are oxygen carriers, are characterized by the same coordination at the iron but there the iron has a positive reduction potential, thus stabilizing the reduced state. Indeed, in these heme proteins, the proximal histidine is weakly H-bound to a backbone carbonyl group. Reduction potentials of peroxidases, which range from –278 mV for HRP (ref.[41]) to –90 mV for manganese peroxidase[42], are modulated by the state of the axial ligand of the heme and consequently by the strength of the Fe–N bond in the porphyrine skeleton. The bond strength is in turn determined by the relative position and distance of the proximal histidine with respect to the iron[11,43,44].

The distal cavity is the site of interaction with hydrogen peroxide and it is characterized by two invariant amino acids (the distal histidine and the distal arginine)[34,35,40] forming the hydrophilic pocket (Fig. 3). This is, again,

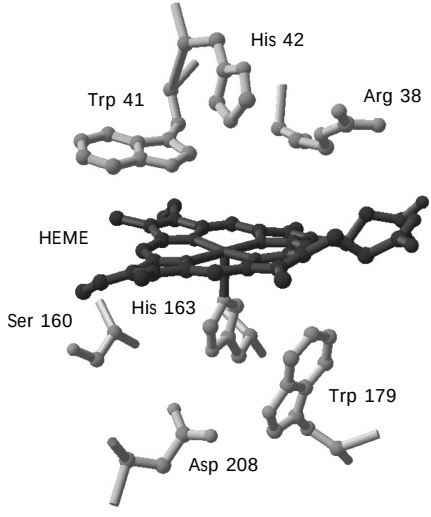


FIG. 3
Structural representation of the heme enviroment of recombinant pea cytosolic ascorbate peroxidase (based on the Protein Data Bank file 1APX)

a distinct property of peroxidases as compared to globins, which also have a histidine in the distal pocket, however, they are characterized by a largely hydrophobic cavity. The pentacoordination state of iron in peroxidases is determined by the presence of a strong histidine axial ligand, which pulls the iron out of the heme plane and would prevent a water molecule from binding on the other axial position[11]. Nevertheless, this iron state is also determined by the properties of the distal cavity[11]. The role of the distal histidine and arginine is well understood in terms of general acid-base mechanisms[45]. The distal histidine acts as a general acid-base catalyst and the distal arginine is a charge stabilizer[45]. The more basic group in peroxidases contributes a higher electron density to iron, which is believed to help to stabilize the iron(III) oxidation state.

All X-ray structures of peroxidases solved so far show in the heme active centre four well-ordered water molecules which are invariant in these structures[11]. Three of them are present in the distal cavity and, hydrogen-bonded to the hydrophilic residues, form an extensive network linking the distal histidine to arginine as well as to a hydrophilic moiety (a propionate side chain of heme). This hydrophilic group always forms the solvent exposed side of the heme. The other conserved water molecule is located on the proximal side, where it is invariantly bound by the hydrogen bond to

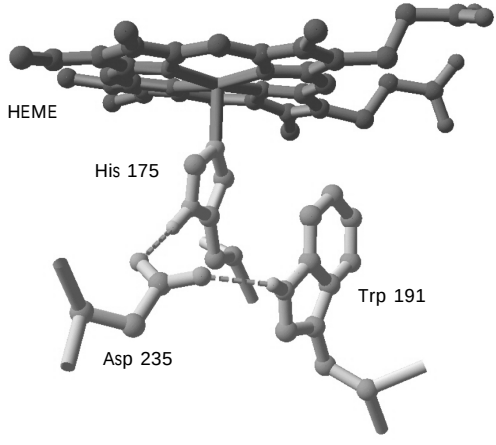


FIG. 4
View of the Asp-His-Fe triad on the proximal side of yeast cytochrome *c* peroxidase. The dashed lines indicate hydrogen bonds between Asp235 and both His175 and Trp191 radical centers

the proximal aspartate. These water molecules are quite rigid. Indeed, in molecular dynamics simulations they maintain the same position as in the X-ray structure along all the trajectory[11,46,47].

The specificity of peroxidases towards a large number of substrates depends on several factors. Among them is the suitable reduction potential of the active species (an iron ion and a center of a cation radical) with respect to those of the substrates. This property is mainly determined by covalent and electrostatic factors[11]. Structural properties of the protein are also relevant in defining the specificity towards different substrates. The protein structure can determine specific binding sites for substrates and mediators. This specificity is probably modulated by changes in the surface topography and by small number of amino acids substitutions, without any significant influence on the folding and arrangement of the secondary structure elements[40,48–50].

The structural and functional aspects of carbohydrates in peroxidases have not been explained as yet. The glycosylation site in peroxidases is usually located on the surface of the protein molecule. It might be assumed that the main purpose of glycosylations is to increase solubility of the enzyme in water and, perhaps, to increase resistance of the enzyme to radical-induced protein crosslinking[40]. This assumption is supported by the enhanced sensitivity of the recombinant enzyme (without glycosylation) to its inactivation with H_2O_2 (ref.[40]).

2.2. Oxidation States of Heme Peroxidases[15]

Five oxidation states of heme peroxidases are shown in Fig. 5.

Native peroxidase (ferric peroxidase, the resting state) contains a heme with five-coordinated iron(III). Positions 1–4 are occupied by four pyrrole nitrogen atoms of the porphyrin skeleton. Position 5 (the proximal side of the heme) is occupied by the imidazole side chain of the histidine residue. The sixth coordination position is free, thus determining a high spin (S = 5/2) state for the iron[21,22].

Compound I is formed from the native enzyme by reaction with peroxide. This results in reduction of peroxide to water and oxidation of the protein by two electrons. Compound I contains an oxyferryl (Fe^{IV}=O) center and an organic cation radical located either on the heme (as porphyrin π-cation radical) or on some amino acid residue, depending on the enzyme[11,15]. Compound I of the yeast cytochrome *c* peroxidase has its radical site on the tryptophan residue (on the proximal side of the heme)[51]. Some investigators still like to use the name compound ES as an indication of the unique

protein-radical found in the oxidized yeast enzyme[15]. Compound I of PHS exists both as porphyrin π-cation radical and as a protein radical (on the tyrosyl residue located on a proximal side of heme). Both radical forms are essential for PHS enzymatic activity[6,15]. Compound I oxidizes one substrate molecule to give a substrate radical and compound II.

Compound II also contains an oxyferryl (Fe^{IV}=O) group[11,15] (Fig. 5). This species is a one-electron oxidized form of the native enzyme and a one-electron reduced form of compound I. Two forms of compound II of cytochrome *c* peroxidase can exist (Fig. 5): the conventional ferryl (Fe^{IV}=O) form (as in the other peroxidases) and the radical form[51]. The radical form of compound II originates if the Fe^{IV}=O site of compound I is reduced first and the protein radical (on the tryptophan residue) remains without change[52]. If the ferryl (Fe^{IV}=O) group of compound II is reduced by one electrone, then the native enzyme (with Fe^{III}) and a molecule of water are formed.

Ferrous peroxidase (reduced form of native peroxidase) is another oxidation state of peroxidases. The reduction potential of the iron(III)/iron(II) pair for peroxidases is low[53,54]. A strong reducing agent (such as sodium dithionite) is, therefore, required to obtain the ferrous enzyme and the reaction has to be accomplished anaerobically[55]. A unique preparation of fer-

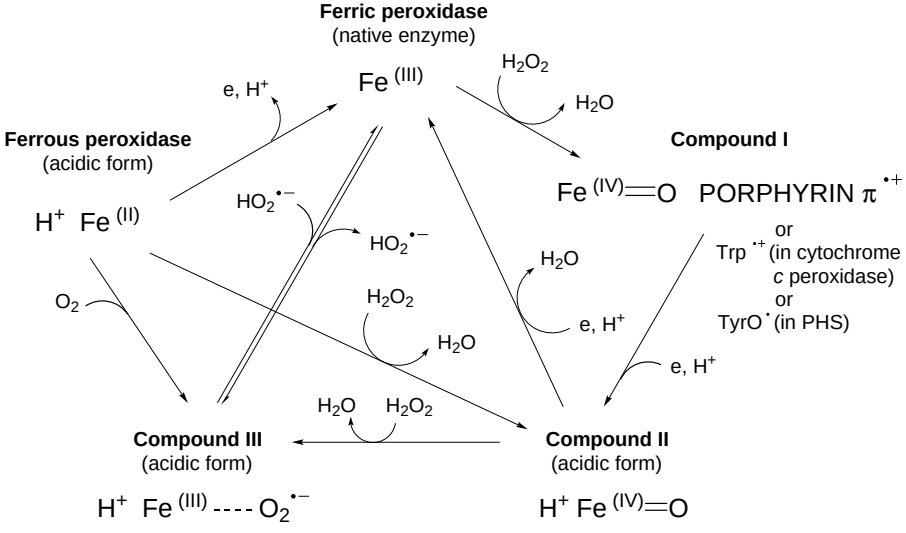


FIG. 5
Five principal oxidation states of peroxidases and their conversions

rous peroxidase was obtained from the reaction of NADH with peroxidase[55]. This marked the discovery of the famous peroxidase–oxidase oscillatory reaction[56].

Compound III (oxyperoxidase) is the peroxidase form spectrally similar to oxymyoglobin and oxyhemoglobin. However, unlike the oxyglobins, oxyperoxidase is unstable[57]. Three routes of its formation are known; reaction of the ferrous enzyme with O_2, that of the ferric enzyme with the superoxide anion radical, $O_2^{\bullet -}$ (or its protonated form, hydroperoxy radical $HO_2^{\bullet}$), and reaction of peroxidase with excess of H_2O_2. The oxyperoxidase formation from native peroxidase and a superoxide anion radical (and the reversibility of the reaction) occur in several reactions, in which peroxidases act like oxidases[58].

2.3. Reactions Catalyzed by Heme Peroxidases

2.3.1. Conventional Peroxidase Reaction (Cycle)

The establishment of the mechanism of peroxidase reaction had a long and tortuous path, complicated by the dominance of concepts about enzyme–substrate complexes. The most common mechanism that appears to be universally accepted today is the following[59]:

native peroxidase (Porph Fe^{III}) + H_2O_2 → compound I ($^{\bullet +}$Porph Fe^{IV}=O) + H_2O
compound I ($^{\bullet +}$Porph Fe^{IV}=O) + AH_2 → compound II (Porph Fe^{IV}=O) + $AH^{\bullet}$
compound II (Porph Fe^{IV}=O) + AH_2 → native peroxidase (Porph Fe^{III}) + $AH^{\bullet}$ + H_2O

The symbol AH_2 stands for a reducing substrate, and $AH^{\bullet}$ for the corresponding radical. The resting ferric enzyme reacts rapidly with peroxide to form compound I, an oxy-ferryl species, in which one electron has been withdrawn from the heme group to form a porphyrin π-cation radical (see above). This intermediate is reduced in two sequential one-electron steps through compound II (into which the porphyrin cation radical has been reduced).

The feature that is typical of classic heme peroxidases is their ability to efficiently cleave peroxide bonds heterolytically[60]. This reaction takes place on the distal side of the heme. The distal histidine and arginine residues play the key role in the mechanism for the transfer of an oxygen atom from a peroxide to iron(III) of native peroxidase[20]. The distal histidine is a proton acceptor from peroxide and the distal arginine is a charge stabilizer[20,61] (Fig. 6).

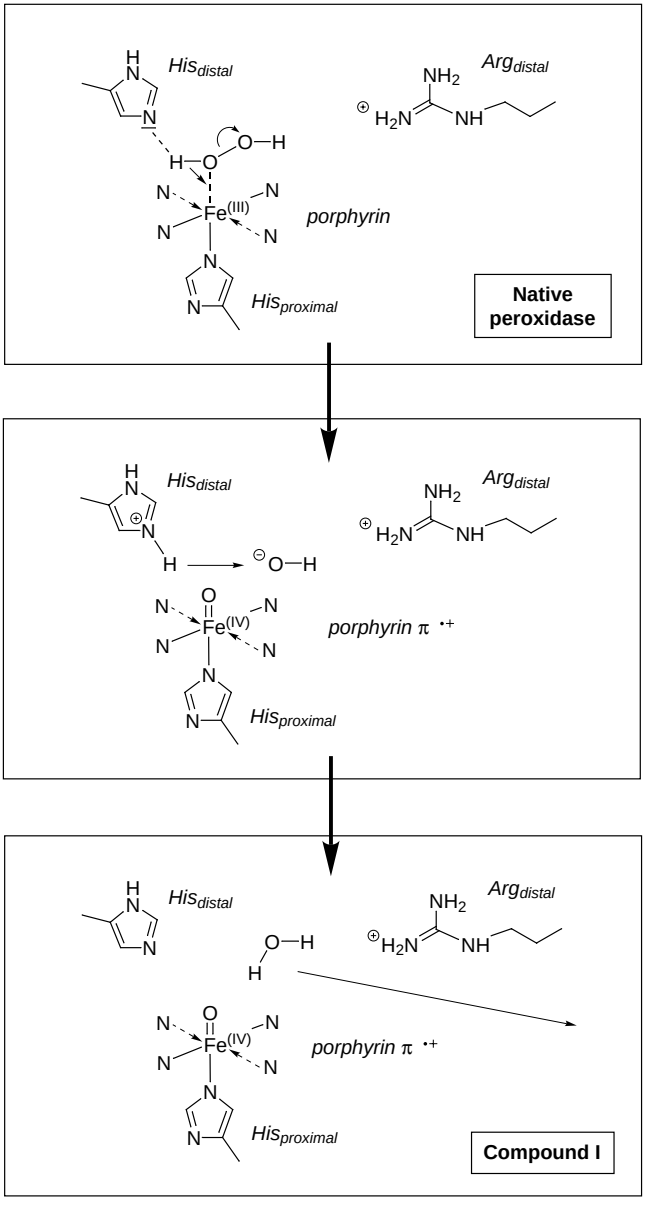


FIG. 6
The key amino acid residues implicated in peroxidase acid-base catalysis. Two residues in the distal heme pocket (Arg, His) are implicated in acid-base catalysis and cleavage of the peroxidase O–O bond in compound I formation

The existence of a two-electron oxidized form of the native enzyme (compound I) is consistent with a two-electron reduction of peroxide[62]. Compound I is reduced in two sequential one-electron steps through compound II. Both compound I and II usually react with the same reducing substrate (AH_2). Therefore, once the reaction between compound I and AH_2 starts, the formation of compound II and its reaction with reducing substrate also begins. Reducing substrates such as phenols and aromatic amines are hydrogen atom donors to the enzyme, not simply one-electron donors[63]. Hydrogen atom donation results in formation of a radical (usually O-, N- or C-centered). The radicals can undergo a variety of reactions depending on circumstances.

2.3.2. Halogenations

Halous ions behave differently from the reduction substrates (AH_2) of a conventional peroxidase reaction. Their oxidation proceeds by a two-electron mechanism to produce an ion in a +1 oxidation state[64]. The halogen (X) in a free hypohalous acid (HOX) or in peroxidase-bound hypohalous acid (FeOX) has the formal +1 oxidation state. Experimental data are consistent with a mechanism, in which the substrate for halogenation (YH) binds to the halogenating intermediate (FeOX) of the peroxidase, and the halogen atom is transferred directly from FeOX to YH (ref.[65]). If a poorly oriented or nonbinding substrate is present, then free hypohalous acid is released. The reactions proceed as follows:

native peroxidase (Porph Fe^{III}) + H_2O_2 → compound I ($^{\bullet+}$Porph Fe^{IV}=O) + H_2O

H^+-compound I ($^{\bullet+}$Porph H Fe^{IV}=O) + X^- → enzyme-bound HOX (Porph H Fe^{III}–O–X)

either

enzyme-bound HOX (H Fe^{III}–O–X) + YH → native peroxidase (Fe^{III}) + YX + H_2O

or

enzyme-bound HOX (H Fe^{III}–O–X) → native peroxidase (Fe^{III}) + HOX

Myeloperoxidase[66], chloroperoxidase[67], lactoperoxidase, lignin peroxidase, HRP and thyroid peroxidase are able to oxidize halides[64,68].

2.3.3. Reaction of Peroxidase Using O_2

The carbon-centered radicals generated by peroxidases are, in some cases, scavenged by O_2 in a nonenzymatic process and an organic hydroperoxide is generated. Hence, oxygen is incorporated into some oxidation products[15,69]. One example of such reactions is the oxidation of indole-3-acetic acid. The ability of HRP to degrade indole-3-acetic acid in the absence of hydrogen peroxide was reported in 1955 (ref.[70]). One of the difficult facts to establish was the mechanism of reaction initiation. Hydrogen peroxide is not required and hence at pH 7.4, the obvious choice appears to be trace autoxidation of indoleacetate to form a hydroperoxide that is sustained in a branching chain reaction[71]. The branched-chain reaction is shown in Fig. 7. Each peroxidase cycle produces two cation radicals of indoleacetate by reduction of compound I and II of HRP. These cation radicals are capable of producing another hydroperoxide {[(indol-3-yl)methyl]hydroperoxide}, *via* decarboxylation to form the skatole radical. The carbon-centered radical is scavenged by molecular oxygen to form the hydroperoxy radical, which is followed by hydrogen atom abstraction. The hydroperoxide molecule can initiate another peroxidase cycle[71].

An additional example showing the reaction using O_2 by peroxidase is the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2) catalyzed by PHS (refs[72–74]). Besides the peroxidase activity, this enzyme catalyzes a unique reaction, the addition of two moles of oxygen to one mole of AA and forms a cyclic endoperoxide hydroperoxide, prostaglandin G_2 (PGG_2)[6]. This reaction commonly called the cyclooxygenase activity of PHS (Fig. 8) is dependent on the tyrosyl residue present on the proximal side of the heme. The endoperoxide hydroperoxide is subsequently reduced to the corresponding alcohol, PGH_2. The PHS peroxidase reaction takes place on the distal side of the enzyme molecule.

A major question of the conversion of AA to PGH_2 is the efficiency of PGG_2 diffusion from one side of the enzyme to the other. One possible mechanism suggested by Dunford and co-workers[15,75,76] is shown in Fig. 9. Compound I formed in the first step of the peroxidase cycle contains its additional oxidizing equivalent on porphyrin ($^{\bullet+}$Porph Fe^{IV}=O···TyrOH). Then, the electron is transferred from tyrosyl residue (on the proximal side of heme) to the porphyrin π-cation radical[77] (Porph Fe^{IV}=O···$^{\bullet+}$TyrOH). This electron transfer is essential for the cyclooxygenase activity of PHS (refs[78,79]). The radical structure abstracts a hydrogen atom from arachidonic acid (AA) and forms the carbon-centered radical (AA$^{\bullet}$) and enzymatic product (Porph Fe^{IV}=O···TyrOH). The AA$^{\bullet}$ is subsequently scavenged by oxygen

to form PGG_2 hydroperoxide radical ($PGG_2^{\bullet}$) on the distal side of heme. One small molecule reductant (AH_2) is consumed in converting this $PGG_2^{\bullet}$ radical into PGG_2, and the molecule of PGG_2 can then initiate a new peroxidase cycle. The Porph Fe^{IV}=O···TyrOH species is converted into a normal compound II (refs[75,76], Fig. 9).

In the reactions of peroxidase using O_2, no direct participation of the enzyme in the reduction of O_2 was proved. The so-called oxidase reaction of peroxidase is usually understood to describe the reaction in the system

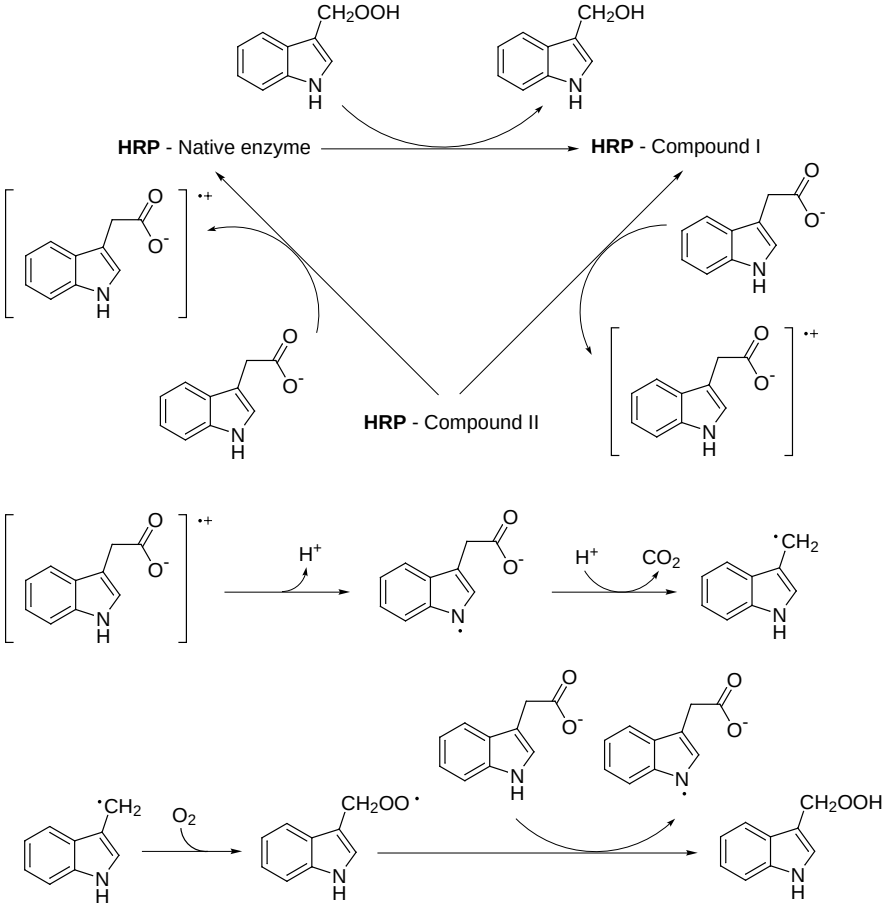


FIG. 7

Branching chain reaction of indole-3-acetate with horseradish peroxidase (HRP). Not shown are the inactivation steps or the very slow spontaneous conversion of indole-3-acetate to the corresponding cation radical

peroxidase–O_2–NADH (ref.[80]). However, it is known at the present time that no direct electron transfer occurs from peroxidase to O_2. The oxidase terminology is, therefore, incorrect but deep-rooted in the literature. The mechanism of the reaction is as follows: NADH is unstable in acid solution and is capable of generating a trace of H_2O_2 to start the peroxidase reaction forming compound I. Both compound I and II oxidize NADH to the radical. This liberated radical reduces O_2 to superoxide[81], which is subsequently protonated to produce H_2O_2 by the reaction with NADH. Hydrogen peroxide can thereafter act in a clasic peroxidase reaction.

2.3.4. Unique Reactions Catalyzed by Peroxidases

Extracts of the marine acorn worm (*Balanoglossus biminiensis*) were found to oxidize luciferin[82]. During this reaction strong light emission is produced[83]. The enzyme called luciferase was found to function as a peroxidase (requir-

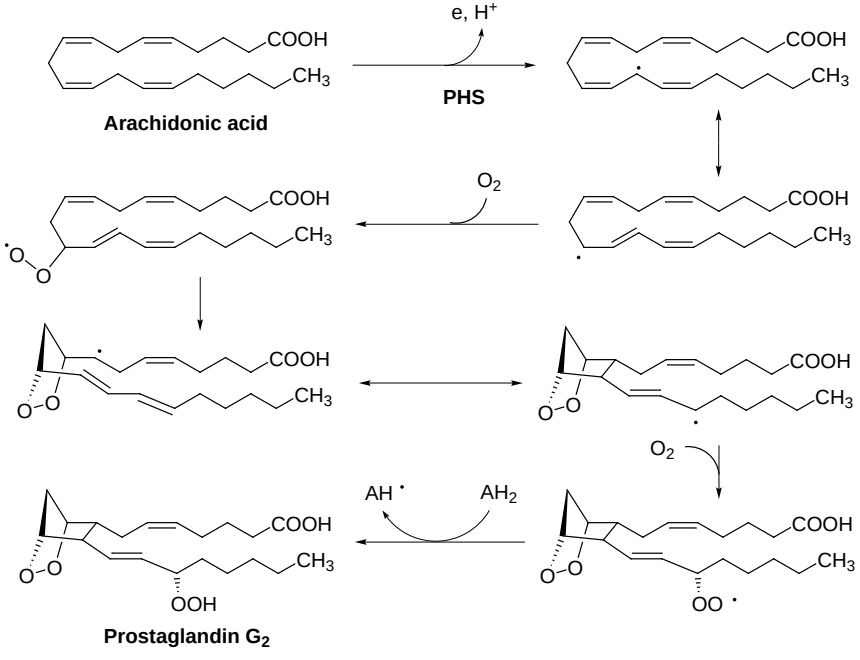


FIG. 8
Reaction scheme for conversion of arachidonic acid into prostaglandin G_2. PHS, prostaglandin H synthase; AH_2, a reducing substrate of PHS; $AH^{\bullet}$, the corresponding free radical

ing hydrogen peroxide) and HRP could be substituted effectively[84]. Pyrogallol[85], linear aldehydes[86] and 9-methylcarbazole[87] are examples of compounds that also produce light-emitting species when reacted with HRP. The reaction of luminol[88] and isobutyraldehyde[89] with HRP has been studied in more detail to elucidate the mechanism.

Chloroperoxidase is a versatile heme-containing enzyme which exhibits peroxidase, catalase and cytochrome P450-like reactions in addition to catalyzing halogenation reactions (for example chlorination)[90]. The enzyme is a heme peroxidase-cytochrome P450 functional hybrid[91]. The proximal side of the heme in chloroperoxidase resembles cytochrome P450 because a

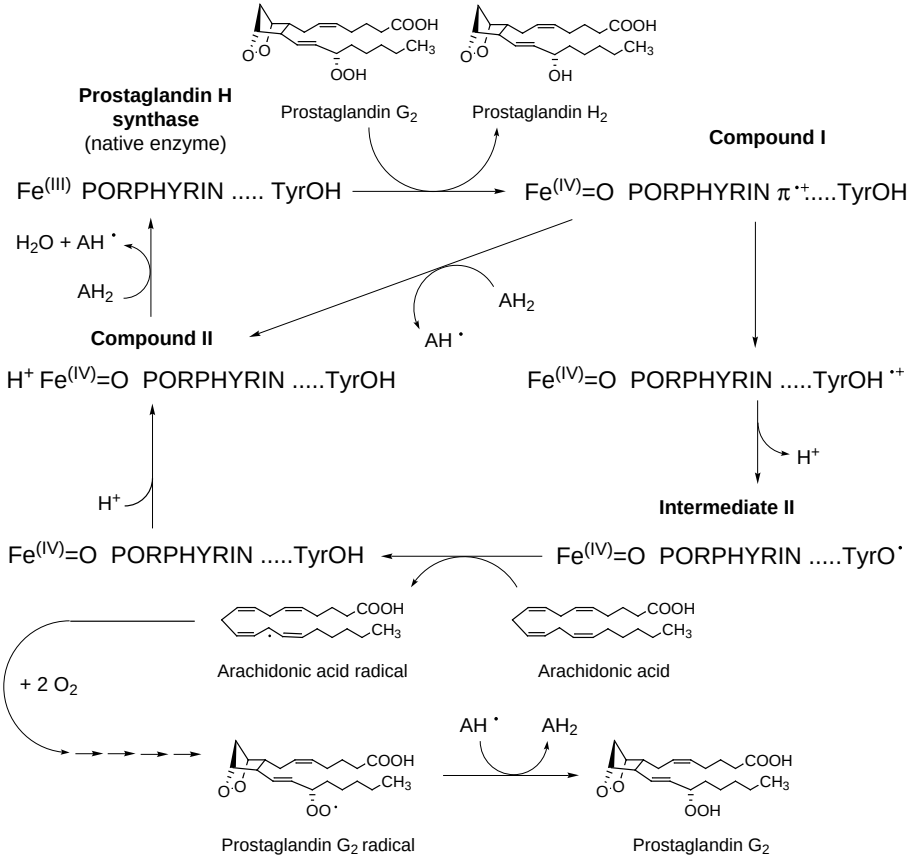


FIG. 9

Schematic representation of the tightly coupled mechanism of the action of prostaglandin H synthase (PHS). AH_2, a reducing substrate of PHS; $AH^{\bullet}$, the corresponding radical

cysteine residue serves as an axial heme ligand, whereas the distal side of heme is peroxidase-like in polar residues from the peroxide-binding site[91].

3. MULTIMODAL FUNCTION OF PEROXIDASES

Multiple functions of peroxidases have been determined[68,72,92–96]. Only some of them (the most important) are discussed in the present review. The primary function of plant peroxidases is believed to be defence mechanism against diseases, insect predators and in wound healing. The degradation of indoleacetate[92] (the substrate promoting plant growth) and the lignin biosynthesis (required for growth) are both catalyzed by peroxidases. Hence, either a delicate balance or the compartmentalization within the cell is required for the processes[93]. Peroxidase activity was also found to be an undissociable factor from auxine and polyamine metabolism in the induction of rooting and flowering[94].

Lignin degradation is accomplished aerobically by a narrow array of microbes (or fungi) which have their own hydrogen-peroxide-generating system[95]. Hence, the microbes can penetrate the plant cell wall and spread into the plant or utilize lignin as a source of carbon.

Mammalian secretory fluids maintain antimicrobial activity in addition to a nonspecific defence mechanism for the protection of mucosal surfaces against invading bacteria, parasites and viruses. Of the agents providing antimicrobial protection in these fluids, the products of peroxidase-driven oxidation of halous and pseudohalous ions catalyzed by peroxidases seem to be the most important ones[96]. The PHS peroxidase is essential for the first step of the arachidonic acid cascade (see above)[72]. The thyroid peroxidase is important for the synthesis of thyroxine (by iodination of tyrosine)[68].

Plant and animal peroxidases are also generally important for catalyzing the removal of H_2O_2, which is cytotoxic and can be formed during the oxidation chain. However, the peroxidase activity is a twin-edged sword. It protects against hydrogen peroxide but it is also responsible for formation of radicals from reduction substrates[63], which reduce the oxidized form of native peroxidase[62] (compound I and II) to the native enzyme closing one cycle of the peroxidase reaction.

An important peroxidase function having significant biological implications is their participation in activation of carcinogenic chemicals in mammals. Although the cytochrome P450 family enzymes has been recognized as the most common enzyme system for converting chemicals to reactive electrophiles, which are carcinogenic, the peroxidative oxidation of various

chemicals also results in carcinogen activation. Peroxidase enzyme systems are involved in bioactivation of chemicals in extrahepatic tissues, where the monooxygenase system has low activity. This peroxidase function is discussed in more detail later on the review. Mammalian peroxidases such as PHS, myeloperoxidase, eosinophil peroxidase[6–8,97], and probably even other peroxidases, play a role in processes of carcinogenesis[6–8,98–101].

3.1. Mechanisms of Carcinogen Oxidation

Peroxidase-dependent bioactivation of carcinogenic chemicals occurs by several mechanisms (Fig. 10). First, the peroxidase directly oxidizes the carcinogen by a conventional peroxidase reaction to form a reactive species modifying macromolecules (DNA, RNA, proteins) in organisms. A large number of procarcinogenic compounds can serve as reducing cosubstrates for peroxidases in a conventional reaction[79,102]. Therefore, such direct oxidation of these substances is a potentially very important mechanism of peroxidase-catalyzed bioactivation of procarcinogens.

The second mechanism of this process is based on the formation of compound III, which is able to oxidize/oxygenate several procarcinogens. Hydroquinones and quinones are oxidized using this route[103,104].

The third mechanism of procarcinogen bioactivation is linked with the secondary reactions of the radicals formed in conventional peroxidase reactions. These radicals are a source of activated oxygen, which can oxidize xenobiotics. Carcinogenic *N*-nitrosamines (*i.e.* *N*-methyl-*N*-nitrosoaniline) seem to be oxidized by this mechanism in a peroxidase-dependent reaction[105].

Peroxy radicals derived from primary radicals and O_2 are utilized in the fourth possible mechanism. Xenobiotic chemicals are oxidized by a hydroperoxide-dependent mechanism. An example of such peroxidase activation mechanism is found with the nonsteroidal anti-inflammatory drug phenylbutazone. This substance is oxidized with peroxidase (*i.e.* PHS) to a carbon-centered radical. The carbon-centered radical traps molecular oxygen to form a peroxy radical[106]. This peroxy radical can bioactivate other compounds. Additional information on peroxy radical-dependent bioactivations is available in other review articles[6,7,107,108].

In the case of PHS, another unique mechanism of carcinogen activation was described[6]. This mechanism is, unlike a peroxidase-mediated reaction of PHS-dependent bioactivation, indirectly linked to the cyclooxygenase activity of the enzyme (Fig. 9). In the cyclooxygenase-catalyzed conversion of

arachidonic acid to PGG_2, peroxy radicals are formed as reaction intermediates (see above). Instead of their implication in initiation of a PHS peroxidase catalytic cycle, these peroxy radicals directly react with carcinogens. This mechanism of PHS-dependent oxidation is important in the epoxidation of several carcinogenic bay-region polycyclic aromatic hydrocarbon (PAH) diols.

Peroxidase-dependent bioactivation of procarcinogens might also proceed indirectly. The highly reactive radicals generated by peroxidase can react with a second compound resulting in its bioactivation under appropriate cir-

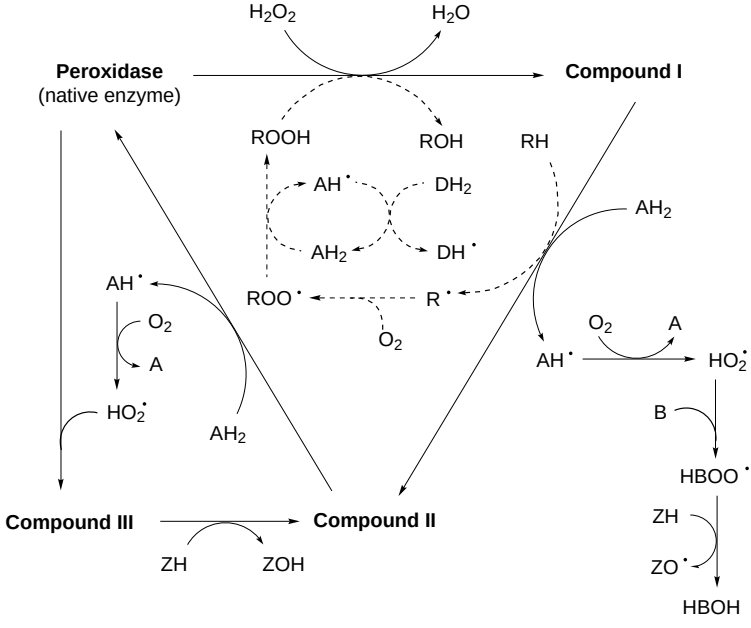


FIG. 10

Principal reaction mechanisms of bioactivation of xenobiotic chemicals by peroxidase. The reactions denoted with dotted arrows indicate the reactions which are alternative to those assigned with full arrows. AH_2, a reducing substrate of peroxidase; $AH^{\bullet}$, its corresponding radical; A, an oxidized form of AH_2; DH_2, a reducing compound (which is not the substrate of peroxidase); $DH^{\bullet}$, its corresponding free radical; ZH, a substrate for hydroxylation (oxidation/oxygenation) reactions; ZOH, $ZO^{\bullet}$, the oxidized forms of ZH; RH, a reducing substrate, which forms its corresponding hydroperoxide radical; $R^{\bullet}$, carbon-centered radical of RH; $ROO^{\bullet}$, the peroxy radical of RH; ROOH, the hydroperoxide of RH; ROH, a reduced form of the corresponding hydroperoxide; B, a reducing compound reacting directly with peroxy radical; $HBOO^{\bullet}$, a hydroperoxide radical of B; HBOH, a reduced form of the corresponding hydroperoxide ($HBOO^{\bullet}$)

cumstances (Fig. 10). The studies of Thompson and co-workers[109] with phenolic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), provide an interesting example of this mechanism. The mechanism is discussed in more detail later on the review.

3.2. Biological Implications of Carcinogen Activation

It was demonstrated that many carcinogenic chemicals metabolized to their ultimate forms by peroxidase oxidation induce carcinogenesis in experimental animals and humans. Tumors are frequently observed in tissues that have relatively high activity of peroxidases (*i.e.* urinary bladder with PHS, bone marrow with myeloperoxidase).

Aromatic amines are a class of chemicals, of which several induce extrahepatic neoplasia. For example, benzidine and its congeners induce bladder cancer in humans and dogs[110–113]. Benzidine is an excellent substrate for several peroxidases including urinary bladder PHS. It undergoes a one-electron oxidation by peroxidases to cation radicals that can undergo a second one-electron oxidation to diimine[114–116]. This oxidation appears to result in the highest degree of covalent binding to DNA but the studies are difficult to fully interpret, because polymer formation can interfere with the assay. Nevertheless, two deoxyguanosine adducts formed in DNA by benzidine activated with peroxidases *in vitro* were found in DNA of dogs and human exposed to this carcinogen *in vivo* (refs[6,101]).

Kadlubar and co-workers[117] characterized the peroxidase-catalyzed formation of three unique DNA adducts formed from another bladder carcinogen, 2-naphthylamine (2-NA). These adducts accounted for ≈60% of the total DNA binding obtained by incubation of [^{3}H]2-NA with PHS *in vitro* and for 20% of the [^{3}H]2-NA bound to dog urothelial DNA *in vivo*. The remaining adducts were identical to those previously reported as products of the reaction of *N*-(2-naphthyl)hydroxylamine with DNA (refs[6,117]).

We have also attempted to characterize the peroxidase-catalyzed formation of DNA adducts from a carcinogenic aromatic amine (the urinary bladder carcinogen 2-methoxyaniline[118–122]). The studies[123] indicate covalent DNA binding of this carcinogen ([^{14}C]2-methoxyaniline) in the oxidation of the carcinogen with peroxidases (HRP, lactoperoxidase and PHS). Reactive metabolites of this carcinogen such as primary formed radicals, as well as the secondary formed products (quinone imine and/or diimine) indirectly determined as products of oxidation of 2-methoxyaniline with HRP (ref.[121]), were suggested to be the ultimate forms of the carcinogen. We directly determined the formation of such products (unpublished results) and

prepared two of them (quinone imine and diimine) to test their reactivities with nucleophiles. The ^{32}P-postlabelling analysis[124] of adducts revealed that diimine generates adducts with deoxyguanosine 3′-monophosphate having the same chromatographic properties (TLC on PEI-cellulose) with those formed by the carcinogen activated with lactoperoxidase and HRP (unpublished results). These data indicate that peroxidase-mediated activation of 2-methoxyaniline occurs *in vitro*. But further *in vivo* studies are required to support the assumption that peroxidases may be important in the activation of this carcinogenic aromatic amine also *in vivo* (ref.[122]).

Polycyclic aromatic hydrocarbons (PAH) are other carcinogens which are also activated by the peroxidase reaction which is needed for their carcinogenic responses. For PAH, the peroxy radicals form the same metabolites as cytochrome P450 monooxygenases but the stereochemistry of the metabolites is different[125]. The mechanistic difference between the peroxidase/peroxy radical-dependent systems and monooxygenases serves as a rational basis for the development of unique markers which can be used *in vivo* to test the biological importance of bioactivation occurring in peroxidase reactions[6,7]. For example, benzo[*a*]pyrene-7,8-diol (BP-7,8-diol) is epoxidized predominantly to *anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene-7,8-diol (anti-BPDE) with peroxy radicals formed by PHS in the metabolism of arachidonic acid[126,127]. Mouse skin, a target organ of BP-induced carcinogenesis, possesses besides the cytochrome P450 activity, also the activity of peroxidase (PHS) (ref.[128]). Cells of such tissue (keratinocytes) were used to investigate which system was epoxidizing (+)-BP-7,8-diol (ref.[129]). The anti-BPDE was the predominant product in keratinocyte cells indicating peroxy radical mechanism of epoxidation[129].

A similar mechanism was also suggested for another carcinogen, natural compound aflatoxin B_1 (AFB)[6]. It was proposed[130] that AFB is epoxidized to the AFB-2,3-epoxide with peroxy radicals *via* a mechanism described for BP-7,8-diol (refs[6,7,130]).

Phenolic compounds are excellent reducing cosubstrates for peroxidases, and are oxidized by these enzymes to reactive derivatives which covalently bind to protein and DNA (ref.[6]).

Phenolic antioxidant BHT has a variety of toxic, tumor-modulatory and carcinogenic effects in laboratory animals[131,132]. BHA is extensively oxidized with PHS peroxidase while BHT is only marginally oxidized with this peroxidase. However, in incubations where both BHA and BHT are included, oxidation of BHT is significantly enhanced due to a direct interaction of BHT with the BHA-derived phenoxy radical generated by the peroxidase. Hence, BHT is converted by this indirect peroxidase oxidation

to a quinone methide metabolite, a reactive two-electron oxidation product[109]. In PHS peroxidase-catalyzed reaction[109], covalent binding of BHT to protein was observed although only a relatively small percentage of parent BHT (23%) was metabolized. In contrast, in the presence of BHA, the metabolism of BHT leading to quinone methide, the ultimate toxic metabolite of BHT and its protein binding, were greatly enhanced (85% metabolized). The phenoxy radical of BHA therefore facilitates the activation of BHT *via* electron transfer[133]. Moreover, several other phenolic and amine compounds also stimulate the oxidation of BHT by a similar mechanism, including endogenous compounds present in tissues where BHT-mediated toxicity occurs[7].

Myelotoxicity and carcinogenicity of benzene metabolites such as the phenols catechol and hydroquinone are also thought to be enhanced by peroxidase in target tissues (*e.g.* bone marrow, Zymbal gland)[97]. Peroxidases present in these tissues oxidize hydroquinone to highly reactive benzoquinone, which is known to react directly with DNA *in vitro* (ref.[134]). The DNA adducts formed seem to be the same as hydroquinone-derived DNA adducts found in Zymbal gland cell cultures. DNA adducts of hydroquinone and 1,4-benzoquinone were also detected in intact HL-60 cells (human promyelotic cell line, having significant myeloperoxidase activity)[135]. Similarly, benzene metabolically activated in B6C3F1 mice leads to the formation of peroxidase-mediated DNA adducts in bone marrow and white blood cells[136]. Oxidation due to peroxidase compound III is assumed to be the predominant mechanism of the hydroquinone and quinone activation[103,104].

We have proved participation of peroxidases in activation of another phenolic compound, Sudan I [(1-(phenylazo)-2-naphthol, Solvent Yellow 14]. This is a non-aminoazo dye containing a hydroxy group in its molecule. It causes tumors in the liver or urinary bladder of experimental animals[137–140]. Using ^{32}P-postlabelling analysis of DNA adducts with the carcinogen formed *in vivo* in Fisher 344 rats, we determined that peroxidases themselves or in combination with cytochromes P450 participate in the initiation phase of Sudan I carcinogenesis in the urinary bladder. The two DNA adducts found *in vivo* are namely the same as those formed from Sudan I (refs[141,142]) or its cytochrome-P450-mediated metabolite 1-(phenylazo)-naphthalene-2,6-diol (6-OH-Sudan I)[143,144] activated by peroxidases *in vitro*. Peroxidase (HRP as a model) directly oxidizes the Sudan I to radicals which covalently bind to DNA (refs[141,145,146]). The first mechanism of peroxidase-dependent bioactivation of xenobiotics (the conventional reaction cycle), which is discussed above, should be implicated in Sudan I

peroxidase-dependent carcinogenesis. However, a cytochrome- P450-mediated Sudan I metabolite (6-OH-Sudan I) is oxidized with another peroxidase (PHS) also to the two-electron oxidation product, 1-phenylazo-2,6-naphthoquinone. Formation of this two-electron oxidation product is probably a prerequisite for DNA binding in this case[144].

The synthetic estrogen diethylstilbestrol (DES) is another example of substances activated by peroxidases[147–152]. This compound is carcinogenic in experimental animals and human[147,153]. The final product of peroxidase-catalyzed oxidation of DES is β-dienestrol (Z,Z-DIES)[149,150]. This metabolite was found in urine of mice treated with DES. Z,Z-DIES is formed *via* the intermediacy of DES semiquinone and DES quinone, the one- and two-electron oxidized products of DES (ref.[148]). The DES quinone then tautomerizes to the final product Z,Z-DIES. The DES quinone is also a reactive species as suggested by its nonenzymatic binding to DNA (ref.[150]).

Nitro compounds. 5-Nitrofurans form a class of potent bladder carcinogens[154]. Several nitrofuran analogs, including *N*-[4-(5-nitro-2-furyl)-thiazol-2-yl]formamide (FANFT) and 4-(5-nitro-2-furyl)thiazol-2-amine (ANFT), are cosubstrates for PHS peroxidase[155–158]. ANFT is oxidized with PHS of rabbit renal medulla and dog bladder epithelium to an intermediate which binds protein and DNA (refs[156–158]). Since PHS-peroxidase-catalyzed metabolism and binding to protein of ANFT is greater than that of FANFT, carcinogenesis induced by FANFT is proposed to require deformylation to ANFT followed by peroxidase oxidation[158]. A two-stage carcinogenesis study of FANFT strongly suggests that PHS peroxidase has also a role in the *in vivo* activation of this carcinogen. However, the mechanism of peroxidase-dependent bioactivation of these carcinogens has not been explained yet.

We have recently found that peroxidases [HRP, lactoperoxidase[159] and PHS (ref.[160])] bioactivate two carcinogenic nitrophenanthrenecarboxylic acids, aristolochic acids. Surprisingly, peroxidases which are known to catalyze oxidative reactions led to the formation of the same deoxyguanosine and deoxyadenosine adducts in DNA as are those formed by reductive reactions[159,160]. Therefore, the peroxidase systems show nitroreductase activity in these nitro compounds under certain conditions. As yet we can only speculate about the chemical mechanism of this peroxidase-mediated reductive activation of aristolochic acids. The radicals, which are formed in oxidative reactions of aristolochic acids, are very reactive and may act as both strong reductants and oxidants depending on circumstances[159,161,162]. It is important to note that the same DNA adducts as mentioned above are also formed by aristolochic acids in humans and experimental animals *in vivo*, being responsible for initiation of carcinogenic processes[159,163–166].

5. CONCLUSIONS

Great attention is now focused on the extra-hepatic transformation of various carcinogens. It can be concluded from the review that several peroxidases participate in this transformation. It is evident from the work cited that peroxidase activation of carcinogens can occur not only with pure peroxidases, but also in subcellular fractions, cell culture systems and *in vivo* being implicated in initiation phase of carcinogenesis.

A large and diverse number of carcinogens are oxidized with peroxidase-mediated reactions, which are described and discussed in the review in detail. The radical nature of these oxidations is a common feature. The consecutive reactions that these chemicals undergo are determined by the nature of the radical and the environment in which the radical is generated. A direct conventional peroxidase reaction forming reactive species is generally responsible for the carcinogen bioactivation. For chemicals which are not substrates for peroxidase, the occurring epoxidation reactions are dependent on the subsequent formation of peroxy radicals. Moreover, other mechanisms of peroxidative reactions, even those which have not been explained as yet, cannot be excluded. Future detailed studies on enzyme kinetics of reactions of carcinogen bioactivation catalyzed by peroxidases should be carried out to better understand the reaction mechanisms (in particular the unknown), in which peroxidase reactions are implicated in chemical carcinogenesis.

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