Chemistry & Biology, Vol. 9, 555–565, May, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00149-7

Suicide Inactivation of Peroxidases and the Challenge of Engineering More Robust Enzymes

Review

Brenda Valderrama,[1] Marcela Ayala, and Rafael Vazquez-Duhalt
Instituto de Biotecnología
Universidad Nacional Autónoma de México
AP 510-3 Cuernavaca
Morelos 62250
México

As the number of industrial applications for proteins continues to expand, the exploitation of protein engineering becomes critical. It is predicted that protein engineering can generate enzymes with new catalytic properties and create desirable, high-value, products at lower production costs. Peroxidases are ubiquitous enzymes that catalyze a variety of oxygen-transfer reactions and are thus potentially useful for industrial and biomedical applications. However, peroxidases are unstable and are readily inactivated by their substrate, hydrogen peroxide. Researchers rely on the powerful tools of molecular biology to improve the stability of these enzymes, either by protecting residues sensitive to oxidation or by devising more efficient intramolecular pathways for free-radical allocation. Here, we discuss the catalytic cycle of peroxidases and the mechanism of the suicide inactivation process to establish a broad knowledge base for future rational protein engineering.

Introduction

Redox reactions require a small but steady supply of oxidative species. In particular, physiological amounts of hydrogen peroxide, and of other reactive oxygen species, are generated during normal cellular activity either as a toxic by-product of respiration [1, 2] or from other oxidative reactions [3, 4]. Oxidative species attack a variety of cellular constituents, but proteins are well known to be especially sensitive, with the most susceptible amino acids being methionine, cysteine, tryptophan, tyrosine, and histidine [5, 6], although damage to other residues has also been observed [6, 7]. Protein oxidation is considered a cause or contributory factor to many diseases, such as Refsum's disease and Alzheimer's disease, and has also been related to the aging process [8–12]. Besides the global effects of oxidative damage to proteins, as evidenced by the overall increase of protein carbonyl content [13], certain enzymes have been found to be specifically inactivated by the irreversible oxidation of catalytically important residues [14–18]. For example, cysteine residues, once oxidized, disrupt the overall protein structure and facilitate further damage [19]. Additionally, protein glutathione adducts can form through cysteine residues [20]. In particular, the reversible oxidation of methionine residues has been suggested to play an important role in the regulation of various cellular processes [21, 22]. Although protein oxidation is generally independent of the catalytic activity of any given protein, the oxidative inactivation of hemeperoxidases is mechanism based. Here we present an integrative review of the literature related to the oxidative inactivation of different groups of peroxidasic hemeproteins with emphasis upon the general nature of this phenomenon and the proposal of a consensus mechanism.

Peroxidases can be divided into three classes that are defined according to their specific active center: hemeperoxidases are a subset of the hemeproteins, which contain the prosthetic group iron porphyrin and control a wide variety of fundamental biological processes in most living organisms [23]; vanadium peroxidases contain a vanadate ion at their active site and are most commonly found in marine environments [24, 25]; and finally, non-metal peroxidases require acetate or propionate buffer for activity and are found in bacteria [26].

The crystal structures of twelve hemeperoxidases and seven cytochrome P450 hemeproteins have been reported to date (see Table 1 and references therein). In each of these structures, a coordinated heme prosthetic group is present in the form of iron-protoporphyrin IX, generally coordinated by a histidine residue as the proximal ligand, with the exception of chloroperoxidase from *Caldariomyces fumago* and cytochrome P450 from all species examined to date, in which the proximal ligand is a cysteine residue [27]. The hemeproteins in Table 1 are distributed within five different folding groups according to structure-structure alignments [28]. The chloroperoxidase from *Caldariomyces fumago* is the only member of its group. Fold similarity is highly significant among members of the same group, despite the low identity of their amino acid sequences, which can be as little as 20% identical.

The classical reaction catalyzed by hemeperoxidases is oxidative dehydrogenation, although they also catalyze a variety of related reactions, including oxygen transfer, hydrogen peroxide cleavage, and peroxidative halogenations. These reactions are described in more detail in the following paragraphs.

Oxidative dehydrogenation involves one-electron transfer processes between an oxo-iron(IV)porphyrin-based π-free radical (pathway 2 in Figure 1) or an oxo-iron(IV)porphyrin (pathway 3 in Figure 1) and a diversity of organic and inorganic substrates, with hydrogen peroxide, organic hydroperoxides, peracids, or inorganic oxides, such as periodate and chlorite, as electron donors. An example of this reaction is the spontaneous polymerization of phenol and aniline free radicals [29].

$$2\ RH + H_2O_2 \rightarrow 2\ R\cdot + 2H_2O \rightarrow R\text{-}R$$

Oxygen transfer is, from a synthesis point of view, the most interesting oxidative transformation catalyzed by peroxidases. This oxidation is comparable to those performed by monooxygenases, such as cytochrome P450. Such oxidations include hetero-atom oxidation (S-oxi-

[1]Correspondence: brenda@ibt.unam.mx

Table 1. Peroxidases and Cytochromes P450 with Available Crystal Structures Grouped by Fold Similarity

Folding Group Hemeprotein Source	Function	PDB Account Number	Reference
Plant peroxidases			
Soybean	peroxidase	1FHF	[153]
Arabidopsis	peroxidase A2	1PA2	[154]
Barley grain	peroxidase	1BGP	[155]
Horseradish	peroxidase	1ATJ	[156]
Peanut	peroxidase	1SCH	[157]
Arabidopsis	peroxidase N	1QGJ	[158]
Manganese and lignin peroxidases			
Phanerochaete chrysosporium	Mn-peroxidase	1MNP	[159]
Arthromyces ramosus	peroxidase	1ARV	[160]
Phanerochaete chrysosporium	lignin-peroxidase	1LLP	[130]
Cytochrome c peroxidases			
Yeast	cytochrome c peroxidase	1RYC	[161]
Pisum sativum	ascorbate peroxidase	1APX	[162]
Chloroperoxidase			
Caldariomyces fumago	chloroperoxidase	1CPO	[163]
Cytochromes P450			
Mammalian microsomal	cytochrome P450 monooxygenase	1DT6	[164]
Bacillus megaterium	cytochrome P450	1BU7	[165]
Pseudomonas sp.	cytochrome P450	1CPT	[166]
Fusarium oxysporium	nitric oxide reductase	1ROM	[167]
Mycobacterium tuberculosis	α-sterol demethylase	1EA1	[168]
Sulfolobus sulfactaricus	cytochrome P450	1F4T	[169]
Pseudomonas putida	cytochrome P450cam	1PHD	[170]

dation and N-oxidation), epoxidation, and C-H bond oxidation (pathways 9 and 10 in Figure 1) [30].

$$RH + H_2O_2 \rightarrow ROH + H_2O$$

Hydrogen peroxide decomposition is achieved through the heterolytic cleavage of H_2O_2 to form water (pathways 1 and 8 in Figure 1). In particular, chloroperoxidase exhibits a substantial catalase activity when hydrogen or organic peroxides are the only reductants present in the reaction mixture [31, 32]. In recent years, a new class of peroxidase-related enzymes, the catalase-peroxidase group, has been discovered. In addition to catalase activity, they exhibit a significant classical-peroxidase activity [33–36].

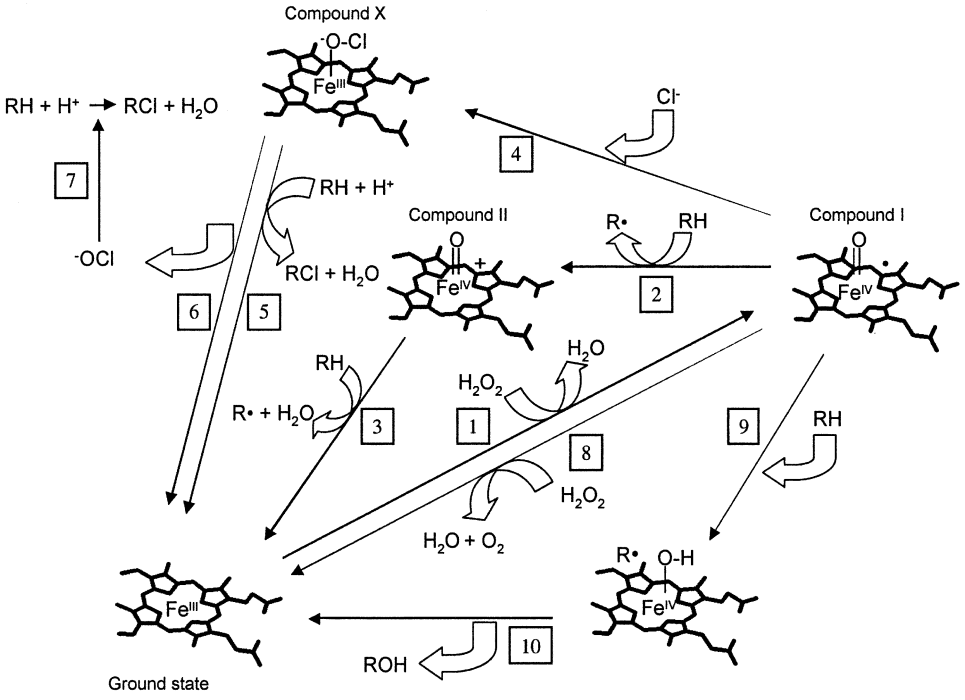

Figure 1. Summary Mechanistic Cycle of Peroxidases

$$2\ ROOH \rightarrow 2\ ROH + O_2$$

Finally, peroxidative halogenation is catalyzed by a special class of peroxidases called haloperoxidases, which mediate the halogenation of organic substrates (pathways 4–7 in Figure 1). Peroxidative halogenation is not limited to hemeperoxidases but is also catalyzed by vanadium haloperoxidases and other non-heme enzymes [24–26, 37]. Chloroperoxidase from *Caldariomyces fumago* is the most active heme-containing haloperoxidase [31].

$$RH + H_2O_2 + H^+ + X^- \rightarrow RX + 2\ H_2O$$

Non-enzymatic hemeproteins are also able to catalyze peroxidase-like reactions. Hemoglobin [38, 39], myoglobin [40], cytochrome *c* [41], and microperoxidase, a heme-bound octapeptide derived from the enzymatic proteolysis of cytochrome *c* [42], are able to oxidize organic substrates. Hydrogen peroxide enables the iron atoms of these proteins, whether high- or low-spin and whether hexa- or penta-coordinated, to perform one-electron oxidations, most likely by the peroxidase cycle.

The development of ecoefficient technological innovations will be critical in this new century and will propel us toward sustainable industrial practices [43]. Green chemistry seeks to develop and deploy chemical products and processes that reduce or eliminate the use and generation of hazardous substances [44]. Valid concerns about the effects of current practices on the environment and energy sources are the driving force behind these unavoidable and necessary changes. Biotechnology will, without a doubt, play an important role in this transformation. The industrial application of enzymes is growing, and the industrial processes in which they are involved are considered clean and low in energy demand. Peroxidases have potentially interesting applications in diverse fields. The most important application so far is in the analytical diagnostic field, where peroxidases are used as a key component of biosensors and immunoassays [45–47]. Peroxidases are currently extensively studied for their use in industrial processes such as Kraft pulp bleaching [48–50], in which they can substitute for the large amounts of chlorine that are currently used and thus prevent the formation of toxic halogenated compounds during the process. Further, these enzymes are involved in the degradation of aromatic compounds and other xenobiotics, including pesticides, polycyclic aromatic hydrocarbons, and dioxins [51] and thus can be developed for the removal of phenolic and aromatic pollutants [52, 53], as antioxidants [54], as indicators for food processing [47], in bioelectrodes [55], in the production of pharmaceuticals [56], and in the synthesis of conducting plastics [29]. In addition, peroxidases could also be used in the synthesis of fine chemicals and optically and biologically active compounds [30, 57].

Despite the obvious value of peroxidases, their present commercial uses are limited, primarily by the low stability of peroxidases in the presence of their natural substrate, hydrogen peroxide. All hemeproteins, including peroxidases, are inactivated in the presence of catalytic concentrations of hydrogen peroxide. This process, which can be described as a suicide inactivation, is especially important in the absence of reducing substrates, and its mechanism has not been fully elucidated.

Suicide Inactivation of Peroxidases

Classical Peroxidases

Extensive investigations into the mechanism of function of classical peroxidases resulted in a consensus catalytic network (Figure 1) that proceeds via the establishment of a sixth-coordination bond between hydrogen peroxide and the heme iron and yields Compound I, a high-valent oxo-iron(IV)porphyrin-based π-free radical (pathway 1 in Figure 1). Electron paramagnetic resonance (EPR) studies established that the second oxidation equivalent in Compound I is initially present as a porphyrin-based free radical, but in some cases, electron abstraction from the protein results in formation of a second Compound I species with an unpaired electron based in a residue close to the porphyrin [58, 59]. The presence of 0.5 equivalents of a two electron-reducing agent, such as an aromatic compound, generates Compound II, which is an oxo-iron(IV)porphyrin without the associated porphyrin π-free radical (pathway 2 in Figure 1). Compound II oxidizes a second molecule of substrate via the peroxidase shortcut to form the resting-state iron(III)porphyrin (pathway 3 in Figure 1). Classical peroxidases are irreversibly inactivated by exposure to high concentrations of hydrogen peroxide [60–63].

Ligninolytic microorganisms secrete two extracellular types of peroxidases, lignin peroxidase [64–67] and manganese peroxidase [68]. Although these enzymes are structurally very similar (see Table 1), their reaction mechanisms are significantly different. Lignin peroxidase presents an unusually low optimum pH and is able to catalyze the oxidation of a variety of compounds with reduction potentials exceeding 1.4 V [69], although the most important substrate is veratryl alcohol (3,4-dimethoxybenzyl alcohol) [70]. Like the classical peroxidases, ferric lignin peroxidase follows the peroxidase cycle (pathways 1–3 in Figure 1). In the absence of substrate, the addition of hydrogen peroxide results in the formation of Compound III (pathway 1 in Figure 2) [71]. Further addition of hydrogen peroxide to Compound III drives the enzyme toward bleaching, and irreversible inactivation [71]. Although not directly related to the inactivation process, the surface tryptophan 171 residue is hydroxylated and functions as the endogenous electron donor for Compound I reduction, revealing the existence of multiple electron transfer pathways between the protein and the porphyrin [67, 72]. In contrast, manganese peroxidase produces the oxidant Mn(III) ion from Mn(II), which behaves as a low-molecular-weight mediator that diffuses to remote regions into the lignin molecule and initiates its oxidation. In the presence of hydrogen peroxide, manganese peroxidase forms Compound I, which is in turn reduced by a bound Mn(II) atom to form Compound II (pathway 2 in Figure 1). Compound II then oxidizes another Mn(II) ion, driving the enzyme back to the ground state. As with other peroxidases, the addition of excess hydrogen peroxide drives manganese peroxidase into Compound III [73], which can be further oxidized until bleaching and irreversible inactivation [74]. This fungal peroxidase is, so far, the only known enzyme

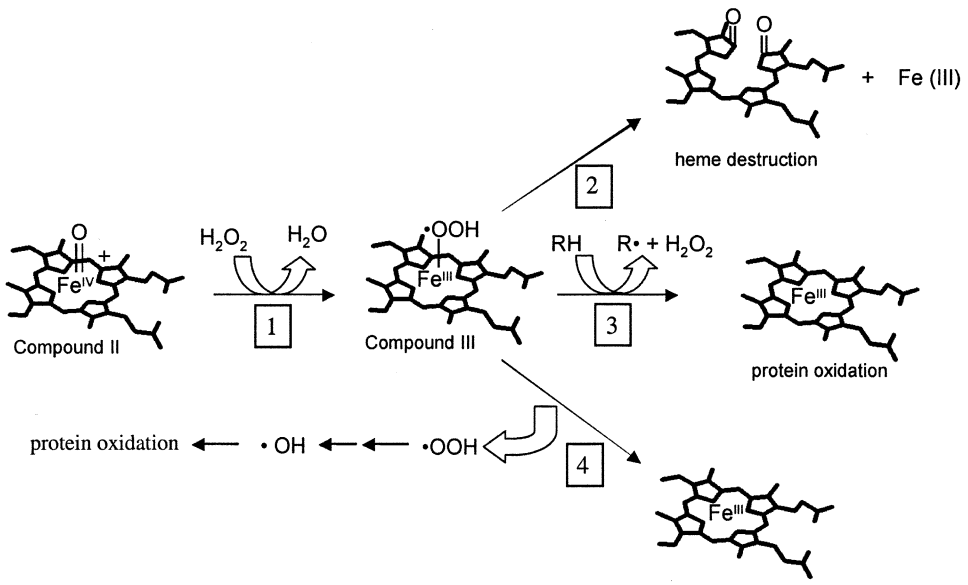

Figure 2. Alternative Inactivation Pathways from Compound III Intermediate

system that utilizes soluble Mn(II)/Mn(III) as a redox couple.

Chloroperoxidase is the most unusual type of peroxidase described so far, and its catalytic cycle has not been completely elucidated [23, 75–77]. The proposed mechanism includes a first activation step, in which hydrogen peroxide transforms the iron(III)porphyrin group to an oxo-iron(IV)porphyrin-based π-free radical (Compound I) (pathway 1 in Figure 1). Subsequently, Compound I can follow three alternative pathways: first, the oxidation of a substrate molecule to form an oxo-iron-(IV)porphyrin without the associated porphyrin π radical (Compound II) (pathway 2 in Figure 1); second, the reaction with a chlorine ion to form a ClO-iron(III)porphyrin group, called Compound X (pathway 4 in Figure 1), which seems to be the only enzymatic activity responsible for the enzymatic reaction of halogenation. In addition, this Compound X appears also to be able to perform oxidative reactions by liberating a chlorine ion [75, 78] (pathway 7 in Figure 1). After both reactions occur, Compound X returns to the ground iron(III)porphyrin state (pathways 5 and 6 in Figure 1). Finally, chloroperoxidase shows a significant catalatic activity (pathway 8 in Figure 1), in which Compound I reacts with a second peroxide molecule to form a three-oxygen-containing enzyme intermediate, which decomposes to yield stoichiometric amounts of oxygen, water, and ground-state chloroperoxidase [32]. Chloroperoxidase is significantly resistant to inactivation by hydrogen peroxide or by organic hydroperoxides because of its intrinsic catalase activity [79]. Nevertheless, exposure to high concentrations of hydrogen peroxide (30 mM) irreversibly inactivates chloroperoxidase with a half-life of about 1 min [80]. However, very high turnover numbers can be reached with fed-batch reactors coupled to a hydrogen peroxide-sensor controller [81]. No information is available about the chloroperoxidase intermediates during the hydrogen peroxide-mediated inactivation process.

Other Hemeproteins

Cytochrome P450 is widely distributed among living organisms and has been found in mammals, fish, yeast, bacteria, and plants [82]. This enzyme is part of multienzymatic systems called monooxygenases that catalyze the activation of dioxygen and the transfer of one of its oxygen atoms into substrates with the consumption of NADPH (or NADH). The monooxygenase cycle of cytochrome P450 has been thoroughly studied [27, 83, 84]. The low-spin hexa-coordinated ferric resting form of iron is converted into a high-spin penta-coordinated state upon substrate binding. Further reduction leads to a high-spin penta-coordinated ferrous form capable of binding dioxygen or carbon monoxide. One-electron reduction of the oxygen adduct, the last stable intermediate in the reduction cycle, leads to release of water and the hydroxylated product. Cytochrome P450 is also able to carry out oxidations with exogenous single-oxygen atom donors such as H_2O_2, alkyl-hydroperoxides, iodosobenzene, amine oxide, and peracids [85–87]. Oxidations with these single-oxygen donors are observed in vitro without any need for cytochrome P450 reductase or any other electron transfer protein and without consumption of NADPH [85, 86]. In this case, it seems possible that the enzyme follows a peroxidase-like catalytic cycle as showed in pathways 9 and 10 in Figure 1. The major products formed from the oxidation of benzo(α)-pyrene by the monooxygenase pathway with NADPH are phenols, whereas in the presence of cumene hydroperoxide, the products are quinones, supporting the existence of a peroxidasic activity for cytochrome P450 [85]. Treatment of cytochrome P450 with excess cumene hydroperoxide leads to protein inactivation and to destruction of the porphyrin into reactive fragments that irreversibly bind to the protein [86]. Self-inactivation of cytochrome P450 during benzphetamine oxidation is accompanied by heme degradation and apo-enzyme modification, followed by protein aggregation, probably

through cross-linked oligomers, as evidenced by the appearance of novel carbonyl groups [87].

The peroxidasic activity of cytochrome *c* has been previously reviewed [41], and the hydrogen peroxide-mediated production of peroxyl and alkoxyl radicals has been investigated by ESR-trapping techniques [88, 89]. Exposure of cytochrome *c* to excess hydrogen peroxide leads to heme bleaching and protein inactivation [90] by a mechanism presumably involving the formation of a reactive species equivalent to Compound III. Free-radical species such as tyrosine-based free radicals [91, 92], as well as alkoxyl and peroxyl reactive species [88, 93], have been detected after exposure of cytochrome *c* to an excess of hydrogen peroxide.

The metabolic role of myoglobin and hemoglobin involves the reversible binding of molecular oxygen to an iron(II)porphyrin group [94]. Part of the oxygenated iron(II)porphyrin form of the protein is spontaneously auto-oxidized into the iron(III)porphyrin state known as the met form, which is unable to bind oxygen, with the generation of a superoxide anion [94]. Auto-oxidation is pH dependent, and although the half-life of oxygenated myoglobin at physiological conditions was found to be 3.3 days, at low pH it became less than 30 min [95]. Although the met form could be reduced back to the ground iron(II)porphyrin state, the superoxide anion produced can easily be converted into hydrogen peroxide by spontaneous dismutation [96, 97]. Hydrogen peroxide can induce very rapid oxidation of the deoxyiron(II)-porphyrin into the met form through the formation of an iron(IV)porphyrin species. On the other hand, the iron(III)-porphyrin-met form further reacts with hydrogen peroxide via the cyclic formation of an oxo-iron(IV)porphyrin radical cation, analogous to Compound I of peroxidases. That species is very unstable and decays nearly immediately to form a tryptophanyl radical that rapidly reacts with oxygen to form a peroxyl radical [98–102]. It is unclear from the data if a second tyrosyl radical observed is formed simultaneously with the tryptophanyl radical or if it is formed by subsequent rescue of the former radical [98–102]. Reaction with hydrogen peroxide also results in covalent dimerization of sperm whale myoglobin [103] and in the oxidation of the porphyrin moiety [104, 105].

A Consensus Inactivation Mechanism

The oxidative inactivation of hemeproteins is mechanism based. The molecular mechanism underlying this hydrogen peroxide-mediated inactivation is extraordinarily complex because of the fact that a multitude of reactions can occur subsequent to the reaction of the heme iron with the hydroperoxide (Figure 1). Despite peculiarities among different hemeproteins, a common inactivation mechanism comprising several stages can be proposed. In the absence of substrate, or when exposed to high concentrations of hydrogen peroxide, peroxidases show the kinetic behavior of a suicide inactivation, in which hydrogen peroxide is the suicide substrate that converts Compound II into a highly reactive peroxy-iron(III)porphyrin free-radical called Compound III (pathway 1 in Figure 2) [106]. Compound III is not part of the peroxidase cycle, but it is produced under excessive exposure of protonated Compound II to oxidative species in a reaction partially mediated by superoxide free radical [60, 106, 107]. ESR spin trapping and spectral analyses have demonstrated the occurrence of this species after the oxidative treatment of cytochrome *c* [89], horseradish peroxidase [60], prostaglandin H synthase [108], lignin peroxidase [71], and manganese peroxidase [73].

Despite representing different structural groups, the kinetic models for the hydrogen peroxide-mediated inactivation of horseradish peroxidase [109] and ascorbate peroxidase [61] are similar in that they are time dependent and show saturation kinetics. In both cases, the addition of a reducing substrate protected the enzyme from inactivation. From the stoichiometry of the inactivation, it was concluded that for ascorbate peroxidase only two molecules of hydrogen peroxide are required per active site to generate the inactive form [61] in contrast to 265 molecules required for horseradish peroxidase [109]. This difference arises from the fact that horseradish peroxidase exhibits a low, albeit significant, catalatic activity that is absent in ascorbate peroxidase [110]. For ascorbate peroxidase, inactivation correlated with enzyme bleaching, suggesting heme destruction [61].

The addition of excess substrate would preclude the suicide inactivation by competing with hydrogen peroxide for Compound II, as has been previously suggested [61, 109]. Once formed, Compound III might follow at least three alternative decomposition pathways (pathways 2, 3, and 4 in Figure 2). First, given the vicinity of the bound peroxyl radical of Compound III to the porphyrin ring, it is reasonable to suspect that once formed, this reactive species would potentially reach the tetrapyrrole structure and oxidize the porphyrin moiety (pathway 2 in Figure 2). This speculation is supported by the existence of an inactive species, different from but related to Compound III and characterized by heme bleaching, which has been observed after the treatment of ascorbate peroxidase [61], hemoglobin [111], myoglobin [112], horseradish peroxidase [62], prostaglandin H synthase-1 [108], microperoxidase-11 [113], cytochrome P450 [86], chloroperoxidase [114], and peroxidase from *Coprinus cinereus* [63], as well as in prostaglandin H synthase [115] with excess hydrogen peroxide. Heme compounds are particularly susceptible to the formation of biliverdin ring systems by oxidative attack at the meso positions, and the dependence of this process on exogenous peroxide has been recently demonstrated [116]. Such oxidation readily leads to rupture or elimination of the carbon bridges linking the pyrrole rings and results in cleavage of the porphyrin macrocycle and formation of an open-chain tetra-pyrrole structure [62, 117, 118]. The release of heme iron during the formation of these species confirms that they are associated with heme degradation. Second, Compound III might return to the ground state after catalyzing the oxidation of the surrounding protein, yielding an oxidized amino acid side chain group in a reaction similar to that previously described for lignin peroxidase (Pathway 3 in Figure 2) [119]. Alternatively, the electron donor might be a substrate molecule, in which case the porphyrin moiety would be repaired, and a ground state enzyme would

Table 2. Redox Potential of Some of the Reactions Involved in the Oxidative Inactivation of Peroxidases

Redox Reaction	E_m (mV)	Reference
$HO\cdot + H^+ + e^- \rightarrow H_2O$	2200	[151]
Mn (III) + $e^- \rightarrow$ Mn (II)	1540	[171]
$Met\cdot^+ + e^- \rightarrow$ Met	1500	[156]
$HOO\cdot + H^+ + e^- \rightarrow H_2O_2$	1480	[151]
$VA\cdot^+ + e^- \rightarrow$ VA	1400	[69]
$Trp\cdot^+ + e^- \rightarrow$ Trp	1200	[151]
$TyrO\cdot + e^- + H^+ \rightarrow$ TyrOH	930	[151]
$CysS\cdot + e^- + H^+ \rightarrow$ CysS	900	[151]

result [120]. Finally, the spontaneous liberation of free radicals by the unimolecular decay of Compound III is feasible because the peroxyl radical is not covalently bound to the porphyrin. This assumption is supported by experimental evidence demonstrating that in the presence of excess hydrogen peroxide and no reductant, Compound III decays irreversibly because of the formation of reactive oxygen species [109, 121–124]. Once released, two molecules of superoxide free radicals might undergo spontaneous rearrangement into a short-lived tetraoxide species that decomposes into two molecules of hydroxyl free radicals and one of oxygen [123]. Hydroxyl free radicals are more reactive than peroxyl free radicals (Table 2), and given their solubility, they are potentially able to oxidize remote amino acid side chains. Amino acid-based free radicals have been detected after the treatment of cytochrome *c* [92], metmyoglobin [99], prostaglandin H synthase [125, 126], microperoxidase-8 [127], ascorbate peroxidase [58], and lignin peroxidase [128–130] with excess hydrogen peroxide. In some cases the amino acid-based free radical is located in the vicinity of the porphyrin, whereas in other cases it is stabilized in the outskirts of the protein. It is well known that free-radical damage may be propagated within protein structures and that, in most cases, transient short-lived species react rapidly with a range of targets to yield other radicals [5]. Once formed, protein-based free radicals might travel back and forth between the protein backbone and proximal side chains until they reach the lowest reduction potential site available. The ultimate sink for oxidizing equivalents in proteins is cysteine residues (Table 2), although tryptophan- and tyrosine-based free radicals were observed after the examination of a number of peptide radicals [131]. Transfer reactions within hemeproteins have been observed in myoglobin [132], hemoglobin [133], and leghemoglobin [134]. These hemeprotein-derived radicals generate intermolecular crosslinks through the formation of di-tyrosine links. Tyrosine-mediated oligomerization has been observed during the oxidative inactivation of myoglobin [103], cytochrome *c* peroxidase [135], cytochrome P450 [87], lactoperoxidase [136], and myeloperoxidase [137].

Improving Stability through Protein Engineering

Protein engineering is generally understood as the use of site-directed or random mutagenesis to alter the properties of a protein. Due to the delicate balance between stabilizing and destabilizing interactions, proteins are only stable under physiological conditions, and two different approaches have been used to increase their stability under non-natural conditions: (1) random mutagenesis followed by selection, also known as directed or molecular evolution and (2) the rational introduction of possibly stabilizing amino acids based on the knowledge derived from the three-dimensional structure. Comprehensive reviews of these strategies are available elsewhere [138, 139]. It may be possible to harness the powerful tools of molecular biology to directly replace low-redux-potential residues around the active site. This would alter the intermolecular radical transfer pathways with the goal of reducing undesirable paths. As a consequence, the inactivation process would be delayed or even suppressed.

In an interesting structure-based molecular-modeling approach, ascorbate peroxidase variants with increased activity were recovered by engineering of the active site [140]. Stabilization of non-heme proteins has been obtained by substitution of amino acids, mainly methionine residues, that are susceptible to oxidative damage [141]. Significant results increasing oxidative stability in hemeproteins have resulted from site-directed mutagenesis. In the case of cytochrome P450 BM-3, the F87A substitution significantly increased the stability toward hydrogen peroxide [142], whereas in hemoglobin the formation of a stable thiyl radical decreased the rate of autoxidation and reduced heme degradation attributed to the reaction of superoxide with the heme [143]. Substitution of the active site residues N52I and Y67F abolished heme destruction but not protein inactivation in cytochrome *c* [90], whereas substitution of residue N81 significantly increased the resistance toward hydrogen peroxide apparently by reducing the protein afinity for this compound in the case of recombinant manganese peroxidase [144]. In light of the inactivation mechanism proposed here, these results can be interpreted as evidence of electron abstraction pathway reconfigurations that lead to an altered electron-allocation equilibrium between the substrate molecule and secondary sinks.

Despite the wide acceptance of directed-evolution methods in protein engineering, only a few hemeproteins have been subjected to this approach and, in most cases, the aim was to modifiy their catalytic properties. In some pioneering studies, a recombinant horse heart-myoglobin quadruple variant with increased peroxidasic activity was obtained after successive rounds of random mutagenesis and screening [145]. In a more recent publication, yeast cytochrome *c* peroxidase mutants with increased activity toward guaiacol were recovered after several rounds of DNA shuffling and screening [146]. Interestingly, all these mutants contained the multi-species conserved arginine 48 residue changed to histidine, in addition to other changes related to the general stability of the apo-protein, which is believed to play a key role in the active site of the enzyme as a gatekeeper controlling the access of small molecules to the ferryl oxygen and the distal histidine [146]. *Pseudomonas putida* cytochrome P450 was successfully evolved toward the hydroxylation of naphthalene with hydrogen peroxide as the electron acceptor. A first round of mutagenesis and a single round of recombination were followed by a second series of experiments, in which five first-generation variants were used as parents in a novel

shuffling reaction yielding mutants with as much as a 20-fold improvement in activity over the wild-type protein [147]. Finally, random mutagenesis has been used in the *Caldariomyces fumago* chloroperoxidase locus to develop a mutant that resisted the suicidal inactivation by allylbenzene [148] as well as a mutant with increased activity in organic cosolvents [149].

In the only example of molecular evolution of a hemeprotein being aimed to increase protein stability toward hydrogen peroxide, a combination of approaches were used to develop a fungal peroxidase for activity in the highly alkaline and oxidative conditions of laundry wash water [150]. Using the crystal structure as a guide, the researchers first used site-directed mutagenesis to target amino acid residues susceptible to oxidation by hydrogen peroxide. Three amino acids were identified and combined by site-directed mutagenesis to generate a variant with an oxidative stability 5-fold higher than that of the wild-type enzyme and thermostability that had impoved by more than 100-fold. Further random mutagenesis and selection identified a series of mutants with greater improvements in thermostability and peroxide stability, but such improvements came at the cost of reducing the overall activity of the enzyme. To overcome this obstacle, the authors then shuffled in vivo clones with improved thermostability with clones encoding enzymes with high activity. The output of these experiments was mutant enzymes with higher specific activity and greater stability than any of the input parental gene products. A final round of in vivo shuffling resulted in two distinct mutants with substitutions in the same position, I49. The best of these products was 174 times more thermally stable and 100 times more stable in the presence of hydrogen peroxide than the native enzyme. Most of the changes selected in the former mutant were located inside the active site, mainly in the contact point between the two helices that coordinate the binding of peroxide. The replacement of isoleucine at position 49 with amino acids that can establish hydrogen bonding was especially important. The I49S substitution increased oxidative stability 50-fold, probably by promoting the establishment of an alternative hydrogen bonding network. Protein-based free radicals are known to be allocated through covalent bonds but also through hydrogen bonding networks [151], and therefore the stabilizing effect of a novel hydrogen bonding architecture might allow alternative free-radical pathways, enabling other sources of electrons to provide the reducing power instead of the protein.

In cases in which neither the crystallographic structure nor a recombinant expression system are available, disruption of the electron tunnel pathway could be attained by chemical means. An example of this approach is the electron abstraction source switching from the substrate to the porphyrin ring, as observed in the hydrogen peroxide-treated prostaglandin H synthase upon the addition of cyclooxygenase inhibitors [152].

Future Prospects

From the evidence presented here, we conclude that in the case of hemeperoxidases substrate oxidation is a naturally imperfect process, and we hypothesize that to some extent, the porphyrin ring or the protein backbone may become the alternative electron sources. Protein destruction seems to arise ultimately as a consequence of the of nonproductive electron abstraction pathways in the reaction pathway, whereas protection by the substrate comes from the favorable partition of the oxidative equivalents toward the substrate. Although we do not know whether these alternative pathways operate sequentially or simultaneously, overall stabilization of peroxidases against hydrogen peroxide might be achieved through the rational reorganization of low-reduction-potential residues within the active site. The aim of this approach would be to orient the electron abstraction pathways toward the substrate instead of the porphyrin or the protein. We are confident that the systematic study of the mechanism-based inactivation of hemeperoxidases will undoubtedly provide the knowledge required for the rational design of site-directed variants to prevent suicide inactivation.

Acknowledgments

This work was funded by Conacyt grant 33611 and by Mexican Institute of Petroleum (IMP) grant FIES 98-110-VI. We thank Grant Mauk and Michael Pickard for their comments on the manuscript.

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