Roles of Water in Heme Peroxidase and Catalase Mechanisms*

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A water molecule is coproduced with the Compound I intermediate in the reactions of native heme peroxidases and catalases with hydrogen peroxide. As a result of water release/rebinding from/to the coproduct formation site the Compound I intermediate may exist in two forms: a "wet" form, Compound I(H₂O), in which a water molecule is present at or near the site of coproduct water formation, and Compound I, in which the coproduct water formation site is "dry." It is postulated that the absence or presence of a water molecule at this site provides the structural basis for a redox pathway switching mechanism, such that the transition states for 2-electron equivalent reduction of Compound I intermediates are accessible in the dry form, but that in the wet form only 1-electron equivalent processes are possible, unless release of water can be stimulated. This concept provides the basis of a general mechanism in which the classical functional distinction between catalases and peroxidases, as well as the more complex behavior observed in halide oxidation and halogenation reactions, appear as particular cases in which variations in the degree of retention of water at the coproduct formation site influence Compound I reactivity.

The functional diversity of the enzymes of the heme peroxidase and catalase superfamily is well documented (1). At the same time marked similarities in the form of the catalytic mechanisms of the enzymes have emerged, notably the invariable involvement of a Compound I intermediate, which stores both oxidizing equivalents of hydrogen peroxide. A puzzling contrast, which still defies interpretation, is presented by the classical distinction in behavior of the Compound I intermediates of horseradish peroxidase and catalase. The former react preferentially by 1-electron equivalent oxidation of a wide range of donor substrates and show no significant ability to oxidize hydrogen peroxide, whereas the latter show a near-monofunctional catalatic behavior, rapidly oxidizing hydrogen peroxide directly to oxygen in a 2-electron equivalent redox process but possessing only rudimentary peroxidatic activity. Despite the availability of a considerable number of crystal structures for both types of enzyme (1), no structural bases for these redox pathway "preferences" have been assigned. Although it is true that in some cases (e.g. yeast cytochrome-c peroxidase) there are variations in the structures of the intermediates that may profoundly affect reactivity (2), more generally the data present the paradox of oxidizing intermediates with very similar (ferryl porphyrin π-cation radical) core structures (admittedly in different protein environments), which possess very different redox reactivities. In this report, the possibility is examined that a factor in the formation of Compound I, which may be of crucial importance, has been overlooked, namely that water, which is coproduced with Compound I intermediates, may profoundly influence the subsequent redox reactivity of these species, depending on the extent to which it (or a replacement provided via a reversible water exchange process) is retained at, or near, its site of formation.

THEORY AND DISCUSSION

Compound I Formation

Coproduct Water Release—In the first phase of the catalytic cycles of the enzymes of the heme peroxidase and catalase superfamily the native, Fe(III), enzyme is oxidized by hydrogen peroxide to a Compound I intermediate, and a water molecule is formed (1). The process is commonly expressed as an elementary bimolecular reaction.

\[ E(\text{native enzyme}) + H_2O_2 \rightarrow \text{Compound I} + H_2O \]

REACTION 1

The Compound I species are key oxidizing intermediates in the enzymatic reactions and Reaction 1 is in accord with the established retention in a Compound I heme of one oxygen atom from its parent hydrogen peroxide molecule, which resides in a ferryl (Fe(IV)=O) center. The second oxidizing equivalent of H₂O₂ is present, commonly (1) but not invariably (2), as an associated porphyrin π-cation radical. Although the formation, structures, and reactivities of the Compounds I have been extensively studied, the formation of the coproduct water molecule has been regarded as merely a stoichiometric requirement of the reaction and possible mechanistic consequences have not been considered. When the kinetics of water release and rebinding in Compound I formation are examined in detail, the possibility becomes evident that the fate of the product water may play a significant role in influencing the reactivity of the Compound I species as oxidants.

Reaction 1 encapsulates some remarkable enzyme chemistry and yet it is an inadequate mechanistic description of Compound I formation in the following two respects: (i) The product water molecule is formed within the enzyme active site, near the heme Fe center. Release of this water molecule to the body of the aqueous solvent is not instantaneous and, in different enzymes, involves journeys of different lengths and complexity, very likely different rates and possibly different mechanisms. In the simplest representation, we can consider a two-state model comprising two distinguishable Compound I species; a "wet" form, Compound I (H₂O), in which the coproduct water molecule is present at, or near, its site of formation and a "dry" form, Compound I, in which the coproduct water molecule has left its site of formation and has either joined, or is on its way to joining the bulk solvent. These two species are connected by the process of product water release shown in Reaction 4 (see...
below). Reaction 4 is an intrinsically reversible process and the reverse, water-rebinding reaction is considered later. (ii) In an earlier discussion, Jones and Dunford (3) argued for the need to include a classical, reversibly formed enzyme-H$_2$O$_2$ complex as a precursor of Compound I (Reaction 2 below). Compound I is then formed via an intramolecular transformation of this complex (Reaction 3).

Thus the apparently simple bimolecular process of Reaction 1 conceals a more complex situation involving (at least) three, kinetically distinct stages, as in Mechanism I as follows.

**Mechanism I: General Mechanism for Compound I Formation**

\[
E + H_2O_2 \rightleftharpoons E_1H_2O_2
\]

\[
E_1H_2O_2 \rightarrow \text{Compound I (H}_2\text{O)}
\]

\[
\text{Compound I (H}_2\text{O)} \rightleftharpoons \text{Compound I} + H_2O
\]

**REACTIONS 2–4**

**Displacement of Resident Water Molecules**—Crystallographic studies have revealed the presence of a number of water molecules in the distal active site cavities of peroxidases and catalases. These water molecules are often referred to as “structural,” but “resident” may be a more suitable term, because, although possessing some positional stability, they are not (with one apparent exception (4)) ligands to the heme iron. Moreover, in several cases some, or all of them, are either known (5) or believed (6) to be displaced in the course of Compound I formation. Dunford (1) concludes that the consensus of a variety of techniques is that penta-coordinate heme Fe(III) is usually, perhaps invariably, present in the active forms of the native peroxidases. Inactive hexa-coordinate forms, with water or hydroxyl ion as the sixth, distal ligand to iron, may be induced by pH or thermal stress, by the absence of essential metal-ion cofactors, or, in the case of yeast cytochrome-c peroxidase (CCP),\(^1\) by a little understood aging process. However, the active forms invariably possess a water molecule, which is not liganded to but resides nearby the heme Fe(III) and is held with some tenacity in a hydrogen bonding network involving the catalytically important His and Arg residues and usually at least one more water molecule. The situation with the catalases is by no means clear-cut.

The only direct evidence of resident water molecule displacement in the process of Compound I formation comes from a comparative crystallographic study of native CCP and Compound I intermediate (5), which showed that three water molecules were resident in the heme pocket of the active, native enzyme but only one remained in the Compound I crystal. This study provides no information on the kinetics of the process. The comment by Fita and Rossmann (6) that Lanir and Schejter (7) had observed the rapid release of a water molecule accompanying catalase Compound I formation is erroneous; unfortunately this interesting experiment was not attempted.\(^2\)

**Formation with Pseudo-substrates**—Taking the above result for CCP, and the crystal structure for native beef liver catalase, Fita and Rossmann (6) used computer graphics techniques to model the reaction of beef liver catalase with the pseudo-substrate ethyl hydroperoxide. Two resident water molecules were included in the model of the heme pocket of the native enzyme, although they had not been observed in the crystal structure (but two appropriately located water molecules were subse-

\(^1\) The abbreviations used are: CCP, cytochrome-c peroxidase; HRP, horseradish peroxidase; MPO, myeloperoxidase; CPO, chloroperoxidase; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

\(^2\) Abel Schejter, personal communication, August 2000.
show the same behavior, identical to Compound I formed with hydrogen peroxide.

**Reduction of Compound I**

The vast body of experimental studies (1) show that a wide variety of redox reaction types occur in the reactions of Compound I species with reducing (donor) substrates: 1-electron equivalent and 2-electron equivalent processes; atom and electron transfers; reaction via the ferryl center and via a porphyrin π-cation radical heme edge; reactions involving a protein radical or a redox active cofactor; and reactions within the distal active site cavity or via electron transfer from a remote proximal donor. All Compounds I support reaction with a wide range of reducing substrates, but in some cases an overwhelming reducing substrate specificity in vitro suggests likely biological function. The relatively recent accumulation of enzyme crystal structures has provided valuable suggestions as to the structural origins of substrate specificity but no firm conclusions about the factors influencing redox mechanisms and kinetics. Although it would be absurd to suggest that coproduct water release/rebinding kinetics in Compound I formation could be the sole determinant of redox diversity; it is argued here that this process may have a particular and important significance.

If release of coproduct water to the bulk solvent in Compound I formation (the forward reaction in Reaction 4) is dissociative, it is a unimolecular process governed by a rate constant, \( k_r \). The reverse reaction is a bimolecular process, which is pseudo-first order (rate constant, \( k_i \)), because the bulk concentration of water is effectively invariant. The equilibrium constant for Reaction 4 may then be expressed by \( K = k_r/k_i = [\text{Compound II}]/[\text{Compound I} (H_2O)] \).

At the moment of formation of Compound I, the system in Reaction 4 is entirely on the left side of the process. Unless other perturbing factors intrude, the reaction will approach equilibrium with an effective rate constant, \( k = k_r + k_i \). At present we have no quantitative information about the values of these rate constants. If it is supposed that equilibrium in this process is rapidly established during the steady-state cycling of the enzymes, two extreme cases can be distinguished: (i) \( K \gg 1 \); so that the intermediate is overwhelmingly present in the dry form, Compound I; (ii) \( K \ll 1 \); the wet form Compound I (H_2O) predominates.

In the interaction of a reducing substrate molecule with the intermediate the transition states formed with Compound I (H_2O) and Compound I are “pseudo-isomeric” in the sense that, in the latter case, the water molecule produced in Reaction 3, although remaining in the system, is no longer interacting with the enzyme active site. Even if it fulfills no other role, the water molecule in Compound I(H_2O) is occupying space within a crucial region of the active site, so that activated complex geometries, which are accessible in reactions of Compound I, may either be inaccessible or not immediately accessible in reactions of Compound I (H_2O). The potential for redox reaction diversity is evident.

**Catalases**

**General Mechanism**—Dunford (1) has compared a “side-by-side” mechanism (proposed by Jones and Suggett (17)) and the “linear mechanism” of Fita and Rossmann (6) and concluded that there was little to choose between them, except that the space demands in a constrained active site were smaller for the latter mechanism. Both models were based on the, much earlier, formal “peroxidatic” mechanism of Britton Chance and are very much products of the influences of their times—in 1968 kinetics and UV-visible spectroscopy dominated; in 1985 the advent of enzyme crystal structures was making a profound impact. In fairness to the side-by-side model, it was proposed when Compound I was still thought of as a classical (albeit peculiar) enzyme-substrate complex. If it is adapted to the ideas presented above, it essentially suggests that the redundant H_2O_2 molecule in the “catalatic” process reacts with Catalase I(H_2O). In contrast, in the Fita and Rossmann model (6) the emphasis is on the influence of the enzyme molecular structure. The water molecules resident in the distal heme pocket and the coproduct water molecule simply vanish from the model as Compound I is formed and, therefore, could not be considered as influences on its reactivity. It is suggested below that a model, which retains the essential features of Fita and Rossmann’s model but which also takes into account the concept of water release kinetics described above, is more satisfactory than either earlier model.

In formal terms the proposed mechanism is shown in Mechanism II, in which the fate of the water molecules initially resident in the active site of the native enzyme is ignored for simplicity (but see below). Mechanism II most notably provides a rationale for the observation that Compound I reacts with hydrogen peroxide in two distinct ways: (i) a 2-electron equivalent reaction (the catalatic reaction, Reaction 8 below), leading to completion of the enzymatic cycle; (ii) a 1-electron equivalent reaction (Reaction 9) yielding the inert Catalase Compound II and hence resulting in enzyme inactivation (18). The mechanism also accounts for the observed limiting steady-state catalatic kinetics at high [H_2O_2] and, more particularly, for the finding that the same value of \( K_m \) = 1.1 M applies to both the catalatic and inactivation reactions (19).

**Mechanism II: Proposed Catalase Mechanism**

\[
\begin{align*}
\text{Cat I} + \text{H}_2\text{O}_2 & \rightleftharpoons \text{Cat I} \left( \text{H}_2\text{O}_2 \right) \\
\text{Cat I} \left( \text{H}_2\text{O}_2 \right) & \rightarrow \text{Cat I} \left( \text{H}_2\text{O} \right) \\
\text{Cat I} \left( \text{H}_2\text{O} \right) & \rightarrow \text{Cat I} + \text{H}_2\text{O} \\
\text{Cat I} + \text{H}_2\text{O}_2 & \rightarrow \text{Cat I} + \text{O}_2 + \text{H}_2\text{O} \\
\text{Cat I} \left( \text{H}_2\text{O} \right) + \text{H}_2\text{O}_2 & \rightarrow \text{Cat II}
\end{align*}
\]

**REACTIONS 5–9**

It is suggested that equilibration in Compound I coproduct water release and rebinding (Reaction 7) lies almost completely in the direction of release (\( K \gg 1 \)) so that: (i) the major catalatic reaction is carried by the dry form of the Compound I intermediate, Cat I; (ii) very occasionally the transition state for Reaction 8 is inaccessible to an incoming hydrogen peroxide molecule, because it encounters the wet form of Compound I, with a water molecule at or near the Compound I coproduct water formation site, and the alternative 1-electron equivalent Reaction 9 occurs.

Catalases are very efficient but not perfect enzymes. With Micrococcus lysodeikticus catalase inactivation via Reaction 9 occurs about once in every 10^6 cycles (19). For mammalian catalases inactivation by this reaction may be repaired by reduction of Compound II by the NADPH cofactor (20, 21). Enzyme selectivity is controlled partly by the narrow, deep channels leading to the heme site, so that larger substrates (both oxidizing and reducing) are much less favored reactants. Most remarkable is the overwhelming preference for hydrogen peroxide as reducing substrate. Fita and Rossmann (6) examined the structure of the main channel into the heme site in beef liver catalase and concluded that it can be described in terms of five sections; the outermost section (I) formed primarily by polar residues, sections II–IV showing gradually increas-
ing hydrophobicity, and section V a hydrophobic neck. In the present context this suggests that a water molecule leaving the active site would find its motion constrained toward release to the bulk solvent by a hydrophilicity gradient. Dunford (1) has noted that “existing” mechanisms do not explain the necessity for the tetrameric enzyme structure for catalatic activity. As Fita and Rossmann point out (6), R-axis-related subunit residues contribute substantially to the channel lining and hence, in the present interpretation to the release of water from the equivalent active sites of the tetrameric enzyme molecules.

**Water Traffic Management: A Priming Cycle Hypothesis—**If, at equilibrium, the active site cavities of native catalase each contain two resident water molecules (which are expelled by hydrogen peroxide as precursor complex is formed) and the water molecule coproduct of Compound I formation is then involved in the exchange Reaction 7, Reaction 1 must be revised to

\[ E + H_2O_2 \rightarrow \text{Compound I} + (3-a)H_2O \]

**REACTION 10**

where \( a \) (near zero) is the fraction of product water molecules remaining within the active site at release/rebinding equilibrium for Compound I. Further water traffic is involved in the second phase of the catalytic cycle (reduction of Compound I to native enzyme, Reaction 8). If all product water molecules are expelled as they are formed, the final process of the cycle must be the rehydration of the active site of dry Fe(III) enzyme to form the equilibrium native enzyme.

\[ E(\text{dry}) + 2H_2O = E(\text{native}) \]

**REACTION 11**

Could catalases use a process of water traffic management to ensure catalytic efficiency? An intriguing possibility is that the first cycle of equilibrium, native enzyme may be a priming cycle in which the resident water molecules are cleared from the active site, allowing further cycles to occur with minimum water traffic through the active site until hydrogen peroxide is exhausted. The selection of enzyme molecules with two (or more) resident water molecules in the active site cavity may be advantageous according to the following argument: (i) Equilibration in the rehydration of dry enzyme may be attained only slowly, because binding involves a ternary process, or more likely, consecutive bimolecular processes where there is little stabilization for the intermediate state,

\[ E(\text{dry}) + H_2O \rightleftharpoons E(\text{H}_2O) \]

\[ E(\text{H}_2O) + H_2O \rightleftharpoons E(\text{H}_2O)_2 \]

**REACTIONS 12 and 13**

but, in the equilibrium form, the water molecules may achieve positional stability both by their mutual interactions and their interactions with the active site components. Equilibrium will be established in a well-incubated enzyme molecule in solution or in a crystal bathed in mother liquor. (ii) The binding of hydrogen peroxide to equilibrium native enzyme in the first cycle, with expulsion of resident water molecules, involves the disruption of a hydrogen bonding network and a significant energy demand. If, at the end of this cycle, the substrate level is sufficiently high that its bimolecular binding to dry native enzyme competes with ternary rehydration, the second and subsequent cycles will occur without rehydration and with minimal water traffic through the active site, until the substrate level has reduced sufficiently to allow rebinding of resident water to the native enzyme. It is not clear that currently available data permit a test of the priming cycle hypothesis. If it is valid, an interesting corollary is that single turnover, pre-steady state (stopped-flow) studies of Compound I formation examine the enzyme in a different “native” state from that which persists during most of steady-state turnover.

**Peroxidases**

**Horseradish Peroxidase—**The failure of horseradish peroxidase (HRP), and most peroxidases, to display catalatic activity has presented a longstanding and unsolved puzzle for mechanistic interpretation. The problem is not thermodynamic; HRP shows a pseudo-catalatic activity in the presence of iodide, which involves the extra-enzymic reaction of hypiodous acid with hydrogen peroxide (1). Lactoperoxidase and thyroid peroxidase show similar activities (22, 23). Both catalatic (24) and peroxidatic (25–27) activities are intrinsic properties of protein-free Fe(III) heme complexes in aqueous solution. The crystal structure of native HRP (28) does not suggest that the problem for oxidation of hydrogen peroxide by HRP Compound I is one of protein constraint on substrate accessibility to the heme. It seems likely that the absence of catalatic activity with HRP arises from a suppression effect, which is kinetic in origin.

An explanation must be sought that allows rapid reaction of hydrogen peroxide at the Fe(III) site of native HRP in Compound I formation but which prohibits access of hydrogen peroxide to the transition state, which leads to 2-e equivalent catalatic reduction of Compound I. A satisfactory hypothesis must also comprehend the effectiveness of HRP I as a 1-e equivalent oxidant, in a wide range of classical donor substrate reactions, and also the clearly demonstrated (29) stimulation of 2-e equivalent oxidation of iodide and hydrogen sulfite anions by conversion of HRP I to an acid form. These requirements are met if HRP I is a coproduct water-retenive species, existing essentially completely in the wet form HRP I (H_2O), in which the transition state for 2-e equivalent reaction is not immediately accessible. It is not the present purpose to attempt to identify the geometries of the transition states for 1-e and 2-e reductions of Compound I species but merely to suggest that the binding or absence of water at the Compound I coproduct formation site provides a mechanism for switching the redox reactivities of the intermediates between these pathways.

On this basis Mechanism III offers a modified version of the classical mechanism of HRP catalyzed 1-e equivalent oxidation of donor substrates (AH_2).

**Mechanism III: Horseradish Peroxidase Mechanism with 1-e Reductants**

\[ 	ext{HRP} + H_2O_2 \rightarrow \text{HRP I} (H_2O) \]

\[ \text{HRP I (H}_2O) \rightarrow X \rightarrow \text{HRP I} + H_2O \]

\[ 	ext{HRP I (H}_2O) + AH_2 \rightarrow \text{HRP II} + \text{AH} \]

\[ 	ext{HRP II} + AH_2 \rightarrow \text{HRP} + \text{AH} + H_2O \]

**REACTIONS 14–17**

In Mechanism III the fate of native resident water molecules is ignored for simplicity, and the precursor complex HRP(H_2O) is omitted, because it is not kinetically significant under accessible conditions. Reaction 15 is shown in an extreme form as a nonreaction, which could be dynamic in nature reflecting an associative water interchange, but a reversible water exchange, involving dissociative water release, with \( K \ll 1 \) would also be acceptable. Commonly the rate-limiting step in these cycles under steady-state conditions is the reaction of reductant, AH_2, with HRP II (Reaction 17).
A satisfactory HRP mechanism must also be able to comprehend reactions where the oxidation of reducing substrate by Compound I is clearly established as a 2-electron process. The best characterized such reaction is iodide oxidation (29). The reaction is notably different from those involving 1-electron-reducing substrates such as phenols, in that it occurs only with an acid form of HRP I, formed by protonation of an enzyme group with \( pK_a \approx 4.6 \). According to the hypothesis of a water-retentive HRP I, the transition state for 2-electron equivalent oxidation of iodide is not immediately accessible to iodide in HRP I (\( \text{H}_2\text{O} \)).

**Mechanism IV: Horseradish Peroxidase-catalyzed Oxidation of Iodide**

\[
\begin{align*}
\text{HRP} + \text{H}_2\text{O}_2 & \rightarrow \text{HRP I (H}_2\text{O}) \\
\text{HRP I (H}_2\text{O}) + \text{I}^- & \rightleftharpoons \text{H}^+\text{HRP I (H}_2\text{O}) \\
\text{H}^+\text{HRP I (H}_2\text{O}) & \rightleftharpoons \text{H}^+\text{HRP I + H}_2\text{O} \\
\text{H}^+\text{HRP I + I}^- & \rightarrow \text{HRP + HOI}
\end{align*}
\]

**REACTIONS 18–21**

Mechanism IV proposes a mechanism in which the key feature is the stimulation of water release from protonated wet Compound I, Reaction 20, permitting the reaction of \( \text{I}^- \) at the ferryl \( \text{O} \) atom of a dry, acid form of HRP I and hence to direct formation of hypiodious acid.

A crystallographic study (5) of yeast cytochrome-c peroxidase Compound I at present provides the only direct evidence of water retentiveness in the distal heme cavity of a Compound I species. Three water molecules are resident in the cavity of the native enzyme but only one remains in Compound I. It is, of course, impossible to ascertain whether this water molecule is a persistent resident, the coproduct water, or a water molecule in exchange equilibrium with the bulk solvent, and we do not know whether this water molecule blocks access of a hydrogen peroxide molecule to the transition state for a 2-electron equivalent catalatic reaction, although its presence is consistent with this concept. It is also the case that CCP Compound I is an atypical species, which contains a proximal Trp-191 radical rather than a porphyrin \( \pi \)-cation radical, and is highly selective for remote 1-electron transfer from reduced cytochrome c. Nevertheless, suppression of catalatic activity would represent a useful gain in efficiency.

The crystal structure of HRP (isozyme C) (28) shows two water molecules resident in the distal heme pocket, with a third site partially occupied. Water 92, which is closest to the heme Fe(III), is involved in a hydrogen bonding network to the Ne2 of His-42, to the Ne of Arg-38, and to the second water molecule. The location of Water 92 may therefore be a prime candidate site for coproduct water retention in HRP I. Investigators have been cautious in assigning reactivity influencing protonation sites, because \( pK_a \) values of amino acid side chains within the active site can diverge substantially from those in free aqueous solution. Nevertheless, it seems very likely that the acid form of HRP I involves protonation of the imidazole of His-42, a process that would destabilize a water molecule retained at, or near, the Water 92 site and provide a molecular description consistent with Mechanism IV.

This argument extends naturally to another phenomenon that has bedeviled the study of the chemistry of HRP and other peroxidases, namely the protonation-induced binding of anions commonly used in buffers and for ionic strength adjustment (notoriously acetate and nitrate), which has required considerable re-evaluation of pH effects in, e.g. the kinetics of Compound I formation at low pH (30). In the present context the behavior may be interpreted as resulting from the opening of the anion binding site by protonation-induced water release. Again protonation of His-42 imidazole is likely, although, in the native enzymes the \( pK_a \) is remarkably low (\( \sim 2.5 \) in HRP). Araiso and Dunford (30) have pointed out that neglect of these complications distorts \( pK_a \) values estimated from kinetic effects. Similar distortion may result from neglect of protonation-induced water release as in Reaction 20.

In Fita and Rossmann’s modeling of catalatic action (6), hydrogen bonds are formed from a reducible hydrogen peroxide molecule to the ferryl oxygen of Cat I and Ne of the imidazole of the distal histidine. Overall, the process involves the transfer of two protons and two electrons from the reducible hydrogen peroxide molecule to Cat I, and a proton transfer mediation role of the distal imidazole, from outer peroxide oxygen to ferryl oxygen, is very plausible. According to the arguments developed above, in HRP I the site for 2-e oxidation of hydrogen peroxide is blocked by water at pH \( > 4.6 \). At pH \( < 4.6 \) the site for 2-e oxidation is accessible in dry acid HRP I, but proton transfer mediation by distal imidazole is now blocked, because the imidazole is completely protonated. Thus, in HRP I the two requirements for the catalatic reaction: (i) accessibility of the site for 2-e oxidation; (ii) available proton transfer mediation, are mutually exclusive. In principle, reaction of dry acid HRP I with the anion, \( \text{HO}_2^- \), might be possible (a reaction analogous to Reaction 21), but hydrogen peroxide is a very weak acid (\( pK_a \approx 11.6 \)) so that the anion concentration at pH \( < 4.6 \) would be vanishingly small.

**Chloroperoxidase and Myeloperoxidase—Myeloperoxidase (MPO) reactivity (31, 32) shows several features indicative of a coproduct water-retentive Compound I: (i) unusually, the formation of MPO I with hydrogen peroxide is a reversible reaction, that is water can undergo a 2-electron equivalent oxidation to \( \text{H}_2\text{O}_2 \); (ii) although MPO shows no significant catalatic activity, MPO I reacts in a 1-electron equivalent process with \( \text{H}_2\text{O}_2 \) to form MPO II (33). MPO I is a very powerful oxidant species, which can oxidize \( \text{Cl}^- \) and is thermodynamically capable of oxidizing water to \( \text{H}_2\text{O}_2 \). The wet form of MPO I is either identical, or very similar to the successor complex in the Compound I formation reodox reaction, which, by the microscopic reversibility principle, must be the precursor complex for the reverse reaction. Thus a coproduct water molecule, retained at or near its site of formation, is precisely located for reoxidation. However, the presence of the water molecule would inhibit binding of a \( \text{H}_2\text{O}_2 \) reductant molecule to MPO I in the transition state for its 2-electron equivalent oxidation to oxygen and water, but the alternative 1-electron pathway leading to MPO II would not be inhibited. Formation of MPO II is inhibited by protonation of MPO I, suggesting that mediation of proton transfer by the enzyme is also important in the 1-e reaction, which results in the formation of superoxide radical as coproduct (33).

Chloroperoxidase (CPO) displays an extraordinary range of catalytic properties involving \textit{inter alia} the capacities to oxidize chloride and carry out substrate chlorinations, peroxidatically oxidize a wide range of typical 1-e equivalent donor substrates, and catalytically decompose both hydrogen peroxide and some alkyl and acyl hydroperoxides. This behavior suggests that, in CPO I, water release/rebinding at the coproduct water formation site is more evenly poised than in the cases discussed above. In accord with this, it has proved possible to study (34) the simultaneous catalytic decomposition of hydrogen peroxide and the peroxidatic oxidation of a typical 1-e equivalent donor substrate (TMPD). The two reductant substrates compete for CPO I, implying, in the present interpretation, that in the Compound I water release/rebinding equilibrium, significant
concentrations of both wet and dry forms are present, reacting with TMPD (a 1-e reaction) and hydrogen peroxide (a 2-e reaction), respectively.

From a previous study (35) of the chlorination of 2-chlorodimethane, using peroxycetic acid to suppress the catalatic reaction, the rate constant for the formation of the halogenating intermediate was found to increase with decreasing pH as would be expected if this reaction followed the pattern of halide oxidation seen with HRP and is promoted by water release from an acid form of CPO I.

The chemistry of CPO is unusual and complicated in another respect (36), because the enzyme contains a transition metal ion, Mn(II), in a binding site adjacent to the heme. Although the role of manganese is unknown, it could certainly be involved in aspects of the redox chemistry of the enzyme.

**Bacterial Catalases-Peroxidases**—The existence of a relatively new class of enzymes that displays substantial peroxidatic and catalatic activities is again readily accommodated by supposing that coproduct water retention in Compound I is incomplete. With a value of α = 0.5 the two activities would be half-maximal and therefore well within the range of variation displayed by each enzyme group. Detailed kinetic studies of the bacterial catalase-peroxidases are beginning to appear. The stopped-flow studies of the reduction of cyanobacterial catalase-peroxidase Compound I (formed with peroxycetic acid) reported by Obinger et al. (37), yielded reactivities much lower than HRP I with a series of phenols and anilines (sulfanilic acid providing a notable exception), which suggests that the major activity of this enzyme is catalatic and that the complexity of the structures of the catalase-peroxidases may, as in the classical catalases, reflect a mechanism for coproduct water removal from Compound I. More detailed studies are awaited with interest.

**Water in Enzyme Degradation**

Compound I derivatives have been formed by the oxidation of protein-free, monomeric ferriheme complexes in nonaqueous solvents such as dichloromethane. Under these conditions the intermediates are very stable in the absence of added donor but are markedly destabilized by the addition of water to the system (38). In aqueous solution at pH = 6, the monomeric coperoxidase forms a Compound I with peroxycacid (39). With initial [peroxycacid]:[ferriheme] = 1 and with no added donor present the Compound I decays rapidly and biphasically to reform only 75–80% of the initial ferriheme. From detailed studies (40) it was concluded that the Compound I undergoes nucleophilic attack by water to yield species that are susceptible to oxidation by unmodified Compound I, a process in which 4–5 molecules of Compound I are reduced to ferriheme for each molecule that is oxidatively destroyed.

Both types of study imply that the presence of uncontrolled water represents a threat to the stability of the species that forms the core structure of the crucial intermediate in peroxidatic enzyme cycles. Although the enclosure of the heme within a protein matrix affords protection, this cannot be complete, because access of substrate is also necessary and water is the inevitable coproduct of Compound I formation with hydrogen peroxide. There are two evident strategies to meet this situation: (a) select protein structures that can efficiently remove the water, or (b) select protein structures that immobilize the water molecule near its site of formation. According to the arguments presented above, strategy (a) leads to catalases, whereas strategy (b) leads to peroxidases.

**CONCLUSIONS**

The introduction of the concept of coproduct water release kinetics and the insertion of a simple model of this process into catalase and peroxidase mechanisms together with some additional concepts of water management in the enzyme systems provides an intriguing rationale for the selection of redox pathway in the reduction of the Compound I intermediates of the heme peroxidases and catalases. This offers a beginning in the attempt to formulate a coherent, structural basis for the historical, empirically assigned redox pathway reactivity “preferences” of different enzymes in these classifications.

Although the available evidence is broadly consistent with the proposals, it derives from investigations in which the evaluation of the roles of water in the reactions was not of primary concern and further, more directly targeted studies are highly desirable.

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