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On the Structure–Function Relationship of Peroxidases and Peroxygenase

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The structure–function relationship of two kinds of hemoproteins, peroxidases and peroxygenase, is discussed and a tentative model for the active site (heme vicinity) structure of each hemoprotein is proposed. The mechanism of Compound I formation from peroxidases is presumed to involve an electrophilic attack of hydroperoxide, the electrophilicity of which is increased by forming a hydrogen bond to a distal acid group (with β-equatorial arrangement) on the heme iron, the basicity of which is being increased by electron donation from the anionic fifth ligand. On the other hand, the mechanism for peroxygenase is presumed to involve a nucleophilic attack of hydroperoxide, the nucleophilicity of which is increased by forming a hydrogen bond to a distal base group (with α-axial arrangement) to the heme iron ligating the neutral fifth ligand. It is presumed that Compound I of peroxidases, which consists of porphyrin π cation radical and ferryl iron, is stabilized by a π–π type charge transfer interaction between the radical, and stacking imidazolate group (not necessarily different from the distal group) which then ionizes, and by electron donation from the anionic fifth ligand. On the other hand, Compound I of peroxygenase, which is postulated to be an oxene complex, is presumed to be stabilized by an electrostatic interaction with a strongly negative environment, and by ionization of the fifth ligand, if such can happen.

INTRODUCTION

In recent years, the structure–function relationship of hemoproteins has been receiving extensive attention. The present article discusses this problem with respect to two kinds of hemoproteins, peroxidases (*1*) and peroxygenase (*2*), the functions of which are to catalyze hydroperoxide-dependent oxidation (electron transfer reaction) and hydroxylation (oxygen atom transfer reaction) of substrates, respectively. Although several types of peroxidases are known (*1*), this discussion will be limited to typical peroxidases, such as horseradish peroxidase (HRP), whose primary compounds with hydroperoxide (Compound I) are thought to consist of ferryl iron and porphyrin π cation radical (*3–5*).

A model for the active site (heme vicinity) structure of each hemoprotein enzyme is proposed herein. The proposal of a model seems to be indispensable for such a discussion. The prerequisites adopted in designing the models are as follows: (i) The model must not be incompatible in essence with any experimental

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[2] Abbreviations used: HRP, horseradish peroxidase; Hb, hemoglobin; Mb, myoglobin; metHb, methemoglobin; metMb, metmyoglobin; ROOH, hydroperoxide.

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result (not interpretations) available at this time. (ii) The model must be able to explain reasonably the function of each enzyme.

Much is known about peroxidases (*1, 6, 7*) and in particular about HRP,[2] while little is known about peroxygenase. In spite of a lack of knowledge about peroxygenase, a considerable resemblance between peroxygenase and hemoglobin (Hb) or myoglobin (Mb) (*8, 9*) enables us to guess at the active site structure of peroxygenase.

The reaction mechanism of peroxidase catalyzed reaction (*10, 11*) is ordinarily viewed as follows:

$$\text{Ferric enzyme} + \text{ROOH} \rightarrow \text{Compound I} + \text{ROH}, \quad [1]$$

$$\text{Compound I} + \text{SH} \rightarrow \text{Compound II} + \text{S}\cdot, \quad [2]$$

$$\text{Compound II} + \text{SH} \rightarrow \text{ferric enzyme} + \text{S}\cdot, \quad [3]$$

where ROOH and SH denote hydroperoxide and a substrate, respectively. On the other hand, the mechanisms of peroxygenase-catalyzed reactions, as suggested previously (*2*), would be essentially described as follows:

$$\text{Ferric enzyme} + \text{ROOH} \rightarrow \text{Compound I} + \text{ROH}, \quad [4]$$

$$\text{Compound I} + \text{SH} \rightarrow \text{ferric enzyme} + \text{SOH}. \quad [5]$$

As can be recognized from the Eqs. [2] and [5], it appears that the functional difference between the two classes of enzymes is predominantly derived from the difference in the reactivity (namely, electronic structure) of Compound I. It is hard to suppose that the function of each enzyme is determined by the particular manner of substrate binding to the enzyme, since the substrate specificity of each enzyme is very low (*1, 2*). Therefore, the problem can be reduced essentially to the electronic structure of the heme in Compound I of each enzyme, and to the factors which determine and stabilize that structure.

ACTIVE SITE STRUCTURE OF PEROXIDASES

The Model

The model tentatively proposed for the active site structure of peroxidases (limited to typical peroxidases) is described as follows:

1. In the ferric state, the sixth ligand of the heme iron is absent (pentacoordinated structure). This does not mean the absence of any interaction such as collision between the iron and bulk water.

2. The fifth ligand (proximal[3] group) is anionic (imidazolate is a probable candidate).

3. There is a distal[3] acid group (either an imidazole or an amino group is a possible candidate) which can readily form a hydrogen bond with coordinating

[3] The term "proximal group" represents the fifth ligand of hemoproteins. The term "distal group" represents the group of hemoproteins which links to the heme irons via the sixth ligands.

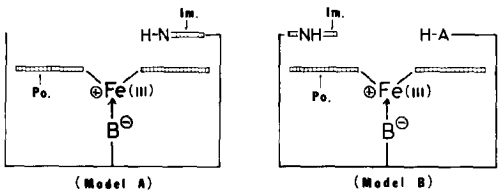

FIG. 1. The models tentatively proposed for the active site (heme vicinity) structure of peroxidases. AH denotes the distal acid group (amino group is a possible candidate); B^-, the anionic fifth ligand (imidazolate is a probable candidate); Po., porphyrin; Im., imidazole group stacking on the porphyrin plane. A stacking interaction between the imidazole group and the porphyrin ring is represented.

hydroperoxide at the β position (see Fig. 2 about the meaning of terms α and β).

4. There is an imidazole group stacked onto the porphyrin plane, which is not necessarily different from the distal group if it is an imidazole.

The model can be drawn schematically as shown in Fig. 1.

Grounds for the Model

A pentacoordinated structure of the heme iron in ferric HRP has recently been suggested by several workers (*12–14*). The structure can be reconciled with the view of Felton *et al.* (*4*), who have suggested a large out-of-plane displacement of the heme iron in ferric peroxidases and catalase. This structure also appears to be consistent with the very high formation rate of Compound I of peroxidases (*1*, *6*).

In regard to factors which stabilize the pentacoordinated structure, the occurrence of an anionic fifth ligand in peroxidases is primarily postulated. It seems reasonable to suppose that the presence of anionic fifth ligand in hemoproteins considerably decreases the coordination bonding energy of the sixth ligand, if it is a hard base such as water. Anionic characteristics of the fifth ligand in HRP have already been suggested (*15*). There is an increasing consensus that the imidazole ring of histidine is the proximal ligand in many peroxidases (*7*). The imidazolate is thus a probable candidate for the fifth ligand or peroxidases. The anionic fifth ligand might be responsible for the remarkably low affinities of anionic ligands for peroxidases (*16*, *17*).

An additional factor stabilizing the pentacoordinated structure is likely. In this regard, ferric peroxidases, unlike metHb (*18*) and MetMb (*19*), have little ability to form a hydrogen bond to the water molecule which enters into the sixth coordination position. According to the model, the distance between the distal group and such a water molecule appears too long for formation of a hydrogen bond between them. This may explain the very high reactivity of peroxidases toward H_2O_2 in aqueous solution, whose physical properties are similar to those of water. That is, the distal group can easily form a hydrogen bond to the coordinating hydroperoxide, but not water.

A probable configuration of the hydroperoxide coordinating to the heme iron may be as indicated in Fig. 2. One notices that the orientation of the lone pair electrons ($2p_x$ orbital) of the β oxygen atom is approximately parallel to the porphyrin plane. This would determine the spatial arrangement of the distal group if the formation of a rather strong hydrogen bond between the distal acid group

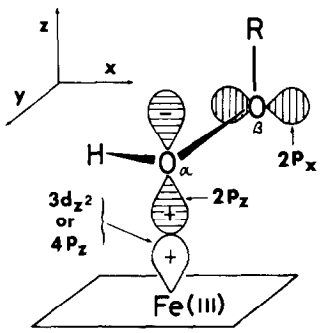

FIG. 2. A probable configuration of hydroperoxide (ROOH) coordinating to ferric high-spin heme iron. Terms α and β indicate the position. The x and y axes used are chosen for convenience.

and the β oxygen atom of the hydroperoxide is demanded. The arrangement may conveniently be termed "β equatorial."[4] If the distal group is histidylimidazole (model A), it would stack on the porphyrin plane. Whereas if it is an amino group (model B), various arrangements would be possible. In the latter case, it is additionally assumed that an imidazole group stacks on the porphyrin moiety, for reasons discussed later. It has been suggested (*20–23*) that the distal group of HRP is histidylimidazole. The former would thus be rather more likely than the latter for the case of HRP.

The model can reasonably provide possible factors stabilizing the higher oxidation states of peroxidases. The anionic fifth ligand would neutralize the electron deficiency of Compounds I and II to some extent. The stacking imidazole group may explain the stabilization of the porphyrin π cation radical in Compound I. That is, the formation of the radical would be followed by the formation of a strong π–π type charge transfer interaction between the radical and the imidazole anion, which then ionizes. The occurrence of facile photoreaction of Compound I (*24, 25*) appears to indicate the presence of such an interaction. If such an interaction is present, it must result in an appreciable decrease and localization of the spin density of the radical in the prophyrin ring. This view may interpret the nmr spectrum of Compound I of HRP (*26*), which exhibits rather ordinary signals of heme peripheral methyl protons, which are not expected from the occurrence of an ordinary prophyrin π cation radical in the compound. The involvement of a base group in stabilizing the radical has already been suggested (*27*).

Hydroperoxide Activation Mechanism Based on the Model

Several workers (*28–31*) have proposed that the redox process in Compound I formation mechanism from peroxidases involves a nucleophilic attack of hydroperoxide anion (ROO^-) formed from hydroperoxide (ROOH) within the active site. It seems, however, that there has been no reasonable explanation regarding

[4] The term "equatorial" or "axial" means that the direction of the hydrogen bond formed between the sixth ligand and the distal group of hemoproteins is approximately parallel or perpendicular to the porphyrin plane, respectively.

the remarkably low reactivity of the anion in bulk solution (*28, 31*). Davies *et al.* (*28*) have proposed the "negative gate" model to explain this, but the model seems incompatible with the very high reaction velocity of Compound III formation from ferric peroxidases and superoxide anion (*32, 33*). An alternative interpretation, by Jones and Dunford (*29*), is that the proton from ROOH is necessary to convert RO^- formed in the active site to ROH, which is the better leaving group. According to this mechanism, however, the sixth ligand of the ferryl iron in Compound I is O^{2-} (but not OH^-). This would be unfavorable in the hydrophobic environment of the active site. Furthermore, such a structure seems incompatible with the Mössbauer spectra of Compounds I and II (*34–36*), which indicate that the electronic structures of the iron in both compounds are almost the same (ferryl low spin), suggesting that the same sixth ligand occurs in each compound, since OH^- is recognized to be the most plausible candidate for the sixth ligand in Compound II (*27, 37, 38*). The very poor reactivity of ROO^- in bulk solution leads directly to a proposal that the redox process in the Compound I formation from peroxidases is an electrophilic attack of ROOH but not a nucleophilic attack of ROO^-, even if which is formed within the active site.

A reasonable mechanism for ROOH activation by the model A is presented schematically in Fig. 3. The processes are described as follows.

Step 1. Coordination of ROOH (formation of precursor complex). In the complex, a hydrogen bond is formed between the distal acid group and the β oxygen atom of ROOH. This kind of ROOH binding to peroxidases has already been suggested (*39*).

Step 2. Electrophilic attack of ROOH on the iron. The electrophilicity of ROOH is increased by withdrawing of the lone pair electrons of the β oxygen through the hydrogen bond mentioned above. The basicity of the iron is being increased by electron donation from the anionic fifth ligand. The attack is followed probably in a concerted fashion by the cleavage of the O–O bond of ROOH, and the formation of ROH and the conjugate base of the distal group. The iron may then move toward an in-plane position.

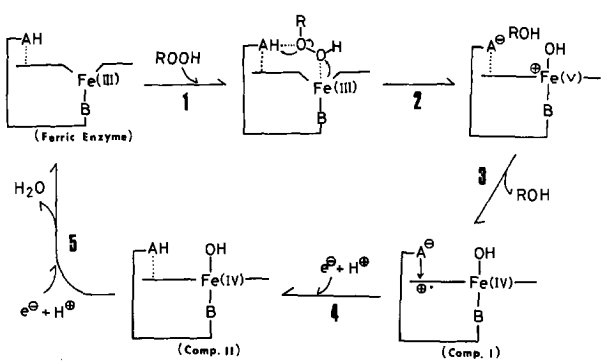

FIG. 3. A presumed mechanism of hydroperoxide (ROOH) activation by the model (Model A) for the active site of peroxidases. AH denotes distal imidazole group stacking on the porphyrin; B, proximal base group.

Step 3. Formation of Compound I. Ferryl iron and porphyrin π cation radical are formed by a redistribution of electrons in the oxidized heme. The formation of the radical may be facilitated by the presence of the stacking imidazolate which would increase the energy levels of porphyrin π electrons. The radical is stabilized by forming a strong π–π type charge transfer interaction with the imidazolate. The departure of ROH from the active site may follow.

Step 4. Formation of Compound II. One electron enters from the substrate to the cation radical site, resulting in the protonation of the imidazolate.

Step 5. Reproduction of ferric enzyme. One electron enters from the substrate in the ferryl iron, resulting in the protonation of OH^-, the sixth ligand in Compound II. The iron may then move toward an out-of-plane position. The step is completed by the departure of H_2O from the sixth coordination position.

Supplements. The mechanism based on model B would need only a slight modification from that of model A, described above. The latter mechanism appears rather more simple than the former. This simplicity seems to imply that model A is preferable to model B, but it is unclear at this time whether model A is valid in general.

Peroxidases are known to have an ionizable group with a pK_a value around 4, which plays a crucial role in the formation of Compound I (*6, 30*). In the present model, the group may be assigned to the fifth ligand because its protonation must result in a substantial decrease of the basicity of the iron.

ACTIVE SITE STRUCTURE OF PEROXYGENASE

The Model

The model tentatively proposed for the active site (heme vicinity) of peroxygenase is described as follows:

1. In the ferric state, a water molecule coordinates to the heme iron as the sixth ligand.
2. There is a neutral fifth ligand (imidazole is a possible candidate).
3. There is a distal base group with an axial[4] orientation (imidazole is a possible candidate) capable of forming a hydrogen bond to the sixth ligand at the α position. The conjugate acid form of the distal group can form a hydrogen bond to the ligand at the β position.
4. There is a strongly negative environment.

The model can be drawn schematically as shown in Fig. 4.

Grounds for the Model

It has been reported (*8, 9*) that peroxygenase bears a considerable resemblance to Hb and Mb in some aspects. This analogy enables us to use the structure in the heme vicinity of either Hb or Mb (*40*) as a starting model for the active site of peroxygenase. From the starting model, it is assumed that peroxygenase has a water molecule as the sixth ligand in the ferric state, a neutral fifth ligand and an (α) axial distal base group. Such a structure is the case for Hb and Mb, and seems

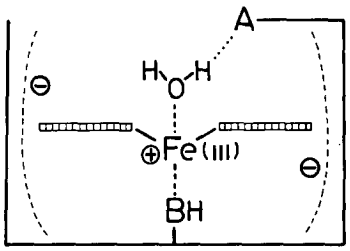

FIG. 4. The model tentatively proposed for the active site (heme vicinity) structure of peroxygenase. A, the distal base group (imidazole is a possible candidate); BH, the neutral fifth ligand (imidazole is a possible candidate).

consistent with a presumed mechanism of hydroperoxide activation by peroxygenase. This mechanism demands the ability of the distal group (conjugate acid form) to form a hydrogen bond to the sixth ligand at the β position, as discussed later.

The presence of a strongly negative environment in the heme vicinity of peroxygenase has been suggested previously (*8*). Such an environment may act to stabilize electrostatically Compound I of the enzyme, which is deficient in electrons. The environment, furthermore, would provide a restriction on the mechanism of ROOH activation. That is, the occurrence of anionic intermediates such as ROO^- and RO^- would be ruled out.

Hydroperoxide Activation Mechanism Based on the Model

In regard to the mechanism of ROOH activation by peroxygenase it is of crucial importance to know the electronic structure of Compound I of the enzyme. It is assumed to be an oxenoid species, $Fe(IV)^+$-$\dot{O}$, as suggested previously (*2*), which is substantially the same as that proposed for Compound I of cytochrome *P*-450 (*41–44*). A presumable mechanism of hydroperoxide activation by the model for the active site (heme vicinity) of peroxygenase is presented schematically in Fig. 5. The processes are described as follows.

Step 1. Formation of precursor complex. A ROOH molecule is substituted for the coordinating water molecule. In the complex, a hydrogen bond is formed between the distal base group and ROOH at the α position.

Step 2. Nucleophilic attack of ROOH on the iron. The nucleophilicity of ROOH is increased by forming the hydrogen bond mentioned above. The attack results in the formation of an Fe–O bond as well as proton transfer from ROOH to the distal base group. The processes may proceed in a concerted fashion to avoid the formation of the anionic intermediate, ROO^-. The iron may then move toward an in-plane position.

Step 3. Formation of Compound I. A hydrogen bond is formed between the distal group (conjugate acid form) and the β oxygen atom. The process is followed by a proton transfer to the β oxygen from the distal group, cleavage of the O–O bond, and the formation of Compound I, which may also proceed in a concerted fashion to avoid the formation of RO^-. In Compound I, the proximal group, if it has a dissociable proton, would ionize (not shown in Fig. 5).

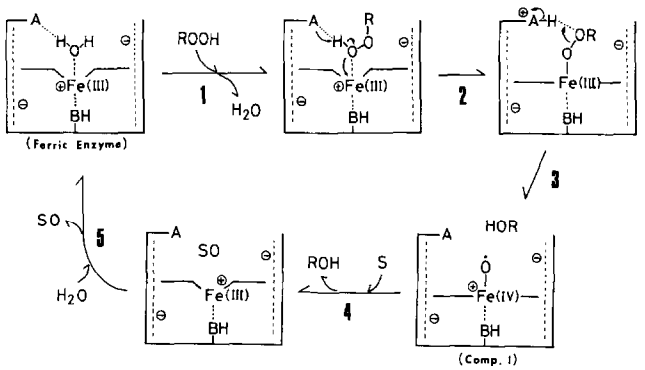

FIG. 5. A presumed mechanism of hydroperoxide (ROOH) activation by the model for the active site of peroxygenase. A and BH denote the distal and proximal base groups, respectively; S and SO, substrate and hydroxylated product, respectively.

Step 4. Hydroxylation of substrate. Electrophilic attack of the iron-borne oxygen atom on substrate, which has previously bound to the enzyme, results in the transfer of the oxygen atom from the heme to the substrate (formation of product and pentacoordinated ferric heme iron).

Step 5. Reconstitution of ferric enzyme. Departure of the product and recoordination of water to the vacant sixth coordination position occurs.

Supplements. The rate-limiting step of peroxygenase reaction appears to be Step 2, for the following reasons. (a) Only a small spectral change is observed at the steady state of the enzyme reaction (*2*). (b) The pH profile of enzyme activity (*2*) implies the participation of an ionizable group (base form is active) with a pK_a value at neutral pH region, in the rate-limiting step of the reaction. The decrease of the activity in alkaline pH region (*2*) is presumably due to the transformation of the active enzyme to its alkaline form, an inactive form of the enzyme.

It is to be noted that the mechanism presumed in this article for the formation of Compound I from peroxygenase (Steps 1–3) is closely similar to that from peroxidases, proposed by Jones and Dunford (*29*).

GENERAL DISCUSSION

In the preceding discussion I have tried to elucidate crucial factors controlling the catalytic functions of peroxidases and peroxygenase, and to propose active site (heme vicinity) structures, under the guiding principle that the function of each enzyme is essentially determined by the electronic structure of its Compound I (primary compound with hydroperoxide). The essence of the discussion is listed in Table 1. In conclusion, it is suggested that the catalytic function of each enzyme is predominantly determined by a particular combination of the proximal and distal groups of the heme prosthetic group of the enzyme, which operate synergistically. The combination seems to control the nature of redox process in the formation of Compound I of each enzyme. Reasonable mechanisms for

TABLE 1

COMPARISON OF STRUCTURE–FUNCTION RELATIONSHIP BETWEEN PEROXIDASES AND PEROXYGENASE

	Peroxidases	Peroxygenase
Catalytic function	Hydroperoxide-dependent oxidation	Hydroperoxide-dependent hydroxylation
The fifth ligand	An anionic group	A neutral group
The sixth ligand (in ferric state)	None	Water molecule
The distal group		
Its acid–base nature	Acid group (form)	Base group (form)
Its spatial arrangement	β Equatorial	α Axial
Redox process in Compound I formation	Electrophilic attack of hydroperoxide	Nucleophilic attack of hydroperoxide
Electronic structure of Compound I	Fe(IV)-OH + porphyrin π cation radical	Oxene complex ($Fe(IV)^{+}$-$\dot{O}$)
Environment of the heme vicinity	Hydrophobic	Strongly negative + hydrophobic
Factors stabilizing Compound I	π–π type charge transfer interaction between porphyrin π cation radical, and stacking imidazolate, and electron donation from the anionic fifth ligand	Electrostatic interaction between oxidized heme prosthetic group and strongly negative environment, and electron donation from the fifth ligand, if any, which then ionizes

stabilizing the electronic structure of Compound I of each enzyme are also suggested.

The discussion in this article, furthermore, might enable us to suggest that most, if not all, of high-spin hemoproteins could be classified by the combination of their proximal groups (neutral or anionic) and distal groups (base or acid, α or β interacting, and axial or equatorial).

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