The Stereochimstry of Peroxidase Catalysis* 

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Thomas L. Poulos and Joseph Kraut

From the Department of Chemistry, University of California, San Diego, La Jolla, California 92037

A stereochemical mechanism is proposed for the peroxidase-catalyzed heterolytic cleavage of the RO—OH bond. It is based on the 2.5 Å structure of cytochrome c peroxidase, model building experiments, and an extensive body of literature on peroxidase biochemistry. The essential features of this mechanism are acid-base catalysis by an invariant distal histidine (His-52 in cytochrome c peroxidase), charge stabilization by an invariant arginine residue (Arg-48), and stabilization of higher oxidation states of the heme iron atom by means of interaction between an invariant proximal histidine (His-174) and a nearby buried glutamine-glutamate pair (Gln-239, Glu-187).

The indole ring of Trp-51 on the distal side of the heme ring is probably the site of radical formation in compound I of cytochrome c peroxidase.

The essential role of the catalytic arginine in the proposed mechanism offers an explanation for why oxygen-binding globins are poor peroxidases. Quite simply, the globins possess no homolog to the peroxidase catalytic arginine and thus are unable to promote heterolysis of peroxides by stabilizing a developing negative charge on the leaving group of the substrate in the transition state.

Structural differences in the vicinity of the proximal histidine also explain why the midpoint potential of cytochrome c peroxidase (−194 mV) is much lower than that of myoglobin (+50 mV). In myoglobin the proximal histidine is hydrogen bonded to a backbone carbonyl oxygen atom, while in cytochrome c peroxidase the proximal histidine contacts and probably hydrogen bonds with the side chain of Gln-238, which in turn interacts with the buried carboxylate group of Glu-187. This latter set of interactions in cytochrome c peroxidase may impart sufficient anionic character to the proximal histidine to stabilize higher oxidation states of the heme iron during the peroxidase catalytic cycle.

Peroxidases are widely distributed throughout the biological world. They are found in many plants, in some animal tissues, and in microorganisms (Saunders et al., 1964) and they carry out a variety of biosynthetic and degradative functions calling for the use of peroxides, especially \( \text{H}_2\text{O}_2 \), as an oxidant. In so doing the peroxidases also protect the cell against accumulation of these dangerously reactive compounds. Indeed the enzyme catalase, the sole function of which appears to be just that, the destruction of excess \( \text{H}_2\text{O}_2 \), is also considered to be a specialized peroxidase which uses \( \text{H}_2\text{O}_2 \) as both oxidant and reductant and thereby converts it into \( \text{H}_2\text{O} \) and \( \text{O}_2 \).

Some recent reviews of peroxidase biochemistry are to be found in the following references: Williams et al., 1977; Morrison and Schonbaum, 1976; Yonetani, 1976; Schonbaum and Chance, 1976.

The overall net reaction catalyzed by many peroxidases may be written most simply as

\[
\text{ROOH} + 2\text{D} + 2\text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + 2\text{D}^*
\]

where \( \text{ROOH} \) is either hydrogen peroxide or an organic hydroperoxide and \( \text{D} \) the electron donor. The oxidized species \( \text{D}^* \) is often a highly reactive free radical which can enter into a variety of further nonenzymic reactions, including reaction with itself. Additionally, certain two-electron donors can also be utilized, and the overall peroxidase reaction is sometimes written

\[
\text{ROOH} + \text{H}_2\text{D} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{D}
\]

The prosthetic group in the well studied plant and yeast peroxidases is noncovalently bound heme. In this respect they resemble the globins, the \( \beta \) type cytochromes, and the P-450 cytochromes. Moreover, as in the globins, the fifth coordination position of the heme iron in the peroxidases is occupied by a proximal histidine side chain and the sixth coordination position by an aquo ligand. In contrast to the globins, however, \( E_0^c \) for the peroxidases is about −200 mV instead of +50 mV, and correspondingly the normal oxidation state of peroxidase is Fe(III) instead of Fe(II). In addition, the amino acid side chains forming the ligand binding pocket on the distal side of the heme are different in cytochrome c peroxidase and myoglobin. Although both proteins contain a similarly situated distal histidine, in myoglobin a valine and a phenylalanine contact the distal surface of the heme while the corresponding residues in cytochrome c peroxidase are tryptophan and arginine (Poulos et al., 1980). A very fundamental question to be addressed by structural investigations, then, is how such differences in the surrounding protein can confer peroxidase activity in one case and reversible oxygen binding capability in the other.

As a contribution toward answering this question, the main purpose of the present communication is to describe those features of the cytochrome c peroxidase structure that we believe are responsible for its characteristic reaction with peroxides, and to propose a detailed stereochemical mechanism based on that structure.

The peroxidase reaction is a two-electron oxidation-reduction, but the reaction normally occurs (excluding catalase) in three distinct steps:

\[
P + \text{ROOH} \rightarrow \text{P(II)} + \text{ROH} + \text{H}_2\text{O}
\]

\[
\text{P(II)} + \text{D} + \text{H}^+ \rightarrow \text{P(III)} + \text{D}^*
\]

\[
\text{P(III)} + \text{D} + \text{H}^+ \rightarrow \text{P} + \text{D}^*
\]
In this formalism P represents the native peroxidase molecule, P(I) is the so-called compound I, which is two oxidation equivalents above the native enzyme, and P(II) is compound II, which is one oxidation equivalent above the native enzyme. The electron donor substrate D may in general be a wide variety of organic and inorganic compounds, but in the special case of cytochrome c peroxidase with which we shall be concerned the enzyme exhibits a high degree of specificity for the reduced cytochrome c molecule.

In this paper we shall consider only the stereocchemical basis for catalysis of the first of the foregoing reaction steps, the formation of compound I. Clearly, however, now that the crystal structure of cytochrome c peroxidase is known, we should soon be in a position to illuminate the long standing question of how an electron is transferred between hemes, the process represented by the second and third of these reaction steps.

Yonetani and his colleagues, in a painstaking and sophisticated investigation extending over many years (Yonetani, 1976) have thoroughly characterized the cytochrome c peroxidase reaction sequence. They have shown that compound I (compound ES in their terminology) is rapidly formed by reaction between cytochrome c peroxidase and hydroperoxides, with a rate constant of 4.5 × 10^8 M^-1 s^-1, and contains two oxidation equivalents more than the native Fe(III) enzyme (Loo and Erman, 1975). From this information, together with the fact that ROH is released, it appears likely that the reaction involves initial heterolytic cleavage of the RO—OH bond to give RO^- as a leaving group and OH^+, or some species in the equivalent oxidation state, bound to the enzyme in compound I (Schonbaum and Lo, 1972).

Spectral methods have been the principle tools employed in elucidating the nature of compound I. Low temperature magnetic susceptibility measurements (Iizuka et al., 1971) and Mossbauer spectroscopic data (Lang et al., 1976) suggest that the heme iron in compound I is in the Fe(IV) oxidation state, and electron spin resonance spectroscopy reveals the presence in compound I of a stable free radical at a spin concentration of 1 mol/mol of cytochrome c peroxidase (Yonetani et al., 1966). The obvious inference is that one of the two extra oxidation equivalents in cytochrome c peroxidase compound I resides on the iron atom and the other on some amino acid side chain. As will be seen below, we can now identify that side chain as the indole ring of a tryptophan residue in intimate contact with both the heme group and the hydroperoxide substrate molecule.

THE MODEL

In a previous publication we have described the overall structure of cytochrome c peroxidase and the arrangement of certain amino acid side chains close to the heme as determined from a 2.5 Å electron density map (Poulos et al., 1980). Since then the primary sequence of cytochrome c peroxidase has been established (Takio et al., 1980) and we can now refer to residues in the structure by their location within the primary sequence. Thus, the proximal histidine occupying the fifth coordination position is His-174 and the distal histidine is His-52. The exact geometry of these and other important amino acid side chains interacting with the heme group is depicted in Fig. 1. A small sphere represents the aquo ligand occupying the sixth coordination position. NE2 of His-52 is approximately 3.2 Å from the aquo ligand and is therefore within hydrogen bonding distance. However, the hydrogen bonding geometry is not good, as can be seen by inspection of Fig. 1.

Such an arrangement of a proximal histidine at coordination position 5, a water molecule at position 6, and a distal histidine within hydrogen bonding distance of the latter is highly reminiscent of the metmyoglobin and methemoglobin structures and indeed a close resemblance between the globin and peroxidase heme coordination has been expected for a long time. It is strongly suggested by the similarities in their spectral properties especially of the oxygenated ferrous derivatives (Wittenberg et al., 1967) and the ability of the ferric proteins to bind various anions like CN^-, N^2-, and F^- (Yonetani, 1976).

In addition, Yonetani et al. (1972) predicted on the basis of electron spin resonance studies of peroxidase and globin nitric oxide complexes that peroxidases also contain a proximal histidine heme ligand as was later substantiated by x-ray crystallography (Poulos et al., 1980). However, there is reason to believe the water ligand at position 6 in peroxidases is less strongly bound and in a somewhat different environment than it is in metmyoglobin. For example, results from both electronic absorption (Tamura et al., 1980) and nuclear magnetic resonance spectroscopy (Lanir and Schejter, 1975) indicate that in horseradish peroxidase the axial aquo ligand is either absent or loosely bound. This difference may be related to the fact that the distal imidazole in metmyoglobin is normally hydrogen bonded to the bound water molecule while, as mentioned above, His-52 in cytochrome c peroxidase is not properly oriented for that task.

Also close to the sixth coordination site are two amino acid side chains, those of Arg-48 and Trp-51, which we believe are intimately involved in the function of cytochrome c peroxidase.

The side chain of Arg-48 extends over the distal surfaces of pyrroles III and IV with its partially buried guanidinium group 3.6 Å above and nearly parallel to the heme. A buried charge group of this kind situated in the active site always suggests some sort of involvement in the enzymatic mechanism. Interestingly, the guanidinium is about 3.5 Å from the buried...
carboxylate group of pyrrole IV, but the local geometry indicates a charge-charge interaction rather than hydrogen bonding.

The indole ring of Trp-51 is also about 3.6 Å above and approximately parallel to the distal surface of the heme. It is situated above pyrrole II, well within the heme crevice. The indole ring NE1 is about 2.7 Å from and appears to hydrogen bond with the water ligand at coordination position 6.

**PEROXIDE BINDING SITE**

In order to explore the possible binding geometry of a hypothetical substrate peroxide molecule, the heme and the distal residues described above were displayed on an Evans and Sutherland Picture System simultaneously with a representation of the O—O group of a peroxide. The latter consisted of two small spheres rigidly connected at an O—O distance of 1.48 Å. The peroxide group was first positioned with one oxygen atom, identified as O2 in subsequent discussion, anchored at the site of the water ligand and then the O1—O2 bond of the peroxide model was moved through all possible orientations. It quickly became obvious that there is little room for maneuvering, and in fact just one clearly preferable orientation for the peroxide group stands out. This is shown in Fig. 2, where it will be seen that the O1 atom is neatly positioned to accept hydrogen bonds from both NE and NH2 of Arg-48, with distances of 2.7 and 2.9 Å, respectively. Notice also that NE2 of His-52 and NE1 of Trp-51 remain, respectively, 3.2 and 2.7 Å away from the peroxide O2 atom, as of course they must since O2 still occupies the site of the heme-bound water molecule. The Fe—O2—O1 angle is approximately 135° as might be expected for an oxygen ligand.

Let us now go back and consider the geometry of distal His-52 in relation to the model peroxide. As mentioned earlier, NE2 of His-52 is within hydrogen bonding distance (3.2 Å) of the water molecule coordinated to the heme iron at position 6, but the hydrogen bonding geometry is poor. Bearing that in mind, we now find upon examining our peroxide-containing model that in fact His-52 appears to be better oriented to make a hydrogen bond to the O1 atom of our hypothetical peroxide substrate than to O2. The distance between NE2 of His-52 and O1 is 2.7 Å and the geometry is essentially normal. We shall return to this important observation in the following section.

As mentioned in the introduction, cytochrome c peroxidase and other peroxidases also utilize organic hydroperoxides, and in particular Yonetani (1976) has shown that ethyl alcohol is released in the reaction between cytochrome c peroxidase and ethyl hydroperoxide. We are lead therefore to ask whether the geometry just described can accommodate the ethyl group or other R groups of an alkyl hydroperoxide, which in conformity with the oxygen-numbering convention we have been using above, must be represented as R—O1—O2—H. Inspection of the model on the Picture System showed that indeed it can, with the alkyl carbon chain extending out over pyrrole IV and thence into the surrounding solvent. Moreover, the spatial arrangement of the four orbitals surrounding O1 is very nearly tetrahedral. More precisely, the directions defined by the O1—O2 bond, the O1—O1 bond, the O1—His-52 hydrogen bond and the remaining lone pair orbital involved in hydrogen bonding to the guanidinium group of Arg-48 are all four tetrahedrally arranged around oxygen atom O1 of the peroxide molecule. This is exactly the geometry one would have anticipated, but we should emphasize that it was not “put into” the model, so to speak, at the beginning.

**PROPOSED MECHANISM**

Having just described the geometry of cytochrome c peroxidase in the vicinity of our hypothetical peroxide binding site, we now present a probable mechanism for the reaction between a peroxide molecule and cytochrome c peroxidase leading to compound I. This mechanism is of course based on a large body of biochemical, kinetic, and spectroscopic evidence in addition to our own structural data, but for the sake of clarity we shall present the mechanism first and defer discussion of some of that evidence until the following section.

Fig. 3 depicts the proposed mechanism in a highly schematic form. The resting cytochrome c peroxidase molecule is shown in Fig. 3a, with the side chain of Trp-51 represented as TRPH for later convenience. The side chains of Arg-48 and distal His-52 are also included, together with the heme, proximal His-174, and a water molecule bound at the heme’s 6th coordination site. The distal histidine is shown as making no hydrogen bonds in the native enzyme, in accordance with the discussion above.

In the first step a peroxide substrate R—O1—O2—H enters the heme crevice, displaces the bound water molecule, and forms a short-lived intermediate (Fig. 3b). The most probable structure for this intermediate contains a singly ionized peroxide molecule covalently bound to the heme iron atom with the hydrogen ion transferred to His-52.

In the next step, the enzyme-peroxide complex passes through the activated transition state (Fig. 3c) on its way to compound I. In the transition state the covalent bond between peroxide atoms O1 and O2 is undergoing heterolytic cleavage to give a leaving group R—O1 and a single oxygen atom, O2, covalently bound to the heme iron, while the proton is being transferred to the leaving group from His-52. Here, His-52 serves as an acid-base catalyst by facilitating the transfer of a
Fig. 3. Schematic representation of the cytochrome c peroxidase-catalyzed heterolytic cleavage of the RO1—O2H bond. 

a, the resting enzyme with a water molecule bound at the sixth coordination position; b, a hypothetical enzyme-substrate complex with the substrate bound as a hydroperoxyl anion, the O2 proton having transferred to the distal histidine; c, the transition state. The hydroperoxide O—O bond is undergoing heterolytic cleavage and a proton from O2 to O1. One might at first expect His-52 to form hydrogen bonds with both oxygen atoms of the substrate. In fact, the hydrogen bonding geometry and distance (3.2 Å) between His-52 and O2 is poor while both the hydrogen bonding distance (2.7 Å) and geometry between His-52 and the leaving group, RO1–, are quite good. Such an arrangement actually favors proton transfer from O2 to O1 and is reminiscent of another well studied class of enzymes, the serine proteases. In that case the active site histidine forms a better hydrogen bond with the substrate's leaving group which is to be protonated than with the proton donating OG atom of the catalytic serine residue (Matthews et al., 1977).

Other than the acid-base catalytic role of His-52, the most striking feature of this mechanism, and certainly the one for which the evidence so far comes exclusively from the present crystal structure, has to do with the role of Arg-48. Notice that the transition state depicted in Fig. 3c necessarily involves a developing negative charge on O1, and recall that O1 is precisely positioned to accept a pair of hydrogen bonds from NE and NH2 of Arg-48. In short, the buried and positively charged side chain of Arg-48 must function in a simple and obvious way to stabilize the transition state required for heterolytic cleavage of the peroxide group. Considering that the coordination sphere of the heme iron atom in cytochrome c peroxidase on the one hand and in metmyoglobin or met-hemoglobin on the other are almost identical, it may not be going too far to guess that the presence of such a strategically situated positively charged group is an essential structural feature that distinguishes a peroxidase from an oxygen-carrying globin.

We come next to Fig. 3d. His-52 delivers its proton to R—O1— which then dissociates from the enzyme as R—O1—H. Left behind is a single oxygen atom, an “oxene” (Hamilton, 1974), attached to a heme that was originally in the Fe(III) state. Formally, therefore, the iron is now at the Fe(V) oxidation level, that is, two oxidizing equivalents above the ground state. However the weight of spectroscopic evidence favors an electronic description of compound I with the iron atom in the Fe(IV) state, and so in Fig. 3e we include that presumably predominating canonical resonance structure.

As depicted in Fig. 3f, the indole ring of Trp-51 is oxidized to an aromatic radical. The edge of the Trp-51 ring is only 2.7 Å from O2 and is the only readily oxidizable amino acid side chain contacting the substrate. Thus, the reactive oxene intermediate should be able easily to abstract a hydrogen atom from the indole ring leaving an indole-free radical. The presence of such a radical is consistent with Yonetani’s interpretation of the cytochrome c peroxidase compound I electron spin resonance spectrum (Yonetani et al., 1966). However, on the basis of cytochrome c peroxidase compound I electron spin resonance and electron nuclear double resonance spectra obtained at 1.4°K, Hoffman et al. (1979) concluded that the
free radical in compound I resides on the sulfur atom of a methionine residue. Of the five methionine residues in cytochrome c peroxidase, the closest to the hydroperoxide binding site is Met-171 with its sulfur atom approximately 5.6 Å below the proximal surface of the heme and 8 Å from the heme iron atom. Thus, it is difficult to see how the oxygen atom could preferentially oxidize Met-171 rather than Trp-51. Unfortunately, we cannot for the present resolve this apparent inconsistency, although it should be emphasized that the magnetic resonance spectral results were interpreted without full benefit of the three-dimensional structure.

Finally, two one-electron reduction steps bring compound I back to the native enzyme to begin the catalytic cycle all over again. As stated at the outset, we will not attempt to discuss this part of the mechanism in the present communication.

DISCUSSION

Model Reactions—It is a matter of both reason and faith that any credible enzymic reaction mechanism will have some sort of counterpart in the realm of classical organic or inorganic chemistry. We are thus impelled to ask whether or not the preceding peroxidase mechanism is consistent with well established peroxide chemistry. More specifically, one might wish to know if there is any convincing evidence that peroxides are capable of heterolytic cleavage in the presence of Fe$^{3+}$ to produce an intermediate complex of oxygen with iron in a higher oxidation state. The question is easier to ask than to answer, unfortunately, but the attempt does shed some light on the matter.

As a generality, it is a well known characteristic of peroxides that they readily undergo homolytic cleavage under the influence of heat or radiation with production of RO· free radicals. This property is the consequence of a relatively low O—O bond energy, 34 kcal/mol, to be compared with, for example, an S—S bond energy of 63 kcal/mol or an O=O bond energy of 119 kcal/mol (Peruzzo and Kotz, 1977). On the other hand, several well characterized reactions of peroxides are known to proceed by nonradical mechanisms involving heterolytic cleavage of the O—O bond to yield an oxidized product plus OH$^-$ or H$_2$O. Examples are the oxidation by peroxides of halide ions to oxylhalides and of various organic sulfides to sulfoxides (Edwards, 1964). The problem with trying to find model peroxidase reactions among this class of reactions, however, is that they all involve attack on the peroxide by readily polarizable nucleophiles, and normally the Fe(III) center of the heme group is not considered to be in that category.

In part because of their intrinsic biochemical relevance, the chemistry of several iron peroxide systems have been studied in some detail, but apparently there is still considerable controversy concerning mechanisms. The prototypical system is the Fenton reaction (Walling, 1975), in which catalytic concentrations of Fe$^{3+}$ ion promote the oxidation of a wide variety of organic compounds by H$_2$O. Although the Fenton reaction has been known for some 86 years (Fenton, 1894), it is not yet generally agreed whether the primary oxidant is the HO· radical (Walling, 1975) or an iron-oxygen complex, the ferryl ion [Fe(IV)=O]$.^+$ (Grosven and Van Der Puy, 1976). From the viewpoint point of the nonexpert, however, it seems that the detailed mechanism might well vary with reaction conditions, such as solvent, Fe$^{3+}$ ligation, etc.

Of perhaps greater relevance for the peroxidase mechanism, since peroxidases in their active state contain Fe$^{3+}$ rather than Fe$^{2+}$, is the catalysis of peroxide decomposition by Fe$^{3+}$ ion. Additionally, the Fe$^{3+}$—H$_2$O$_2$ system also can oxidize organic substrates in peroxidase and catalase like reactions. Again, two reaction schemes have been proposed. One involves intermediacy of the HO· radical (Barb et al., 1951) and the other a higher valence iron-oxygen complex analogous to that proposed in our peroxidase mechanism (Fig. 3d) (Kremer and Stein, 1959). However, later work by Walling and Goosen (1973) favors the radical mechanism.

An interesting extension of the Fe$^{3+}$—H$_2$O$_2$ reaction has been studied by Hamilton as a model for catalase and cytochrome P-450 (Hamilton, 1974). In Hamilton’s system aromatic compounds are hydroxylated by H$_2$O$_2$ in the presence of catalytic amounts of Fe$^{3+}$ and catechol. A detailed kinetic and product study led to the conclusion that the hydroxylating agent is a ternary complex of Fe(III), catechol, and a single oxygen atom derived by heterolysis of H$_2$O$_2$. The similarity between such an intermediate and the structure proposed for compound I of cytochrome c peroxidase is evident.

Hamilton has also focused attention on the resemblance between reactions of carbenes and nitrenes on the one hand and both enzymatic and model nonenzymatic hydroxylation reaction on the other, and refers to the latter as “oxenoid” mechanisms. In particular, Hamilton points out that a lone oxygen atom coordinated to an Fe$^{3+}$ center is isoelectronic with carbene CH$_2$ or nitrile NH, and might therefore be expected to participate in similar C—H bond insertions, double bond additions, and hydrogen abstractions (Hamilton, 1974). This is the rationale behind our labeling the species represented in Fig. 3d an “oxene intermediate.” Hamilton and others believe a species of this kind may be active in heme-containing oxygenases like cytochrome P-450. Presumably cytochrome c peroxidase and other peroxidases are not oxygenases because of restricted access to the site of “oxene” formation and rapid conversion of the latter to the ferryl state plus a radical.

Finally, the presence of the higher, Fe(IV), oxidation states is not without precedent in porphyrin chemistry. Felton et al. have demonstrated that one electron can be removed from the iron atom in ferric tetraphenyl and octaethyl porphyrins by cyclic voltammetry to generate a Fe(IV) porphyrin (Felton et al., 1971).

The Role of Arg-49—Regardless of what mechanism is ultimately agreed upon for these potential model reactions, there is considerable direct evidence for the existence in compound I of an iron-oxygen atom complex. As mentioned earlier, the presence of two additional oxidation equivalents in cytochrome c peroxidase compound I (Coulson et al., 1971) and the release of ROH when ROOH is the peroxide substrate for horseradish peroxidase (Schonbaum and Lo, 1972) or cytochrome c peroxidase (Yonetani, 1976) certainly argues for binding of a single oxygen atom. In the case of chloroperoxidase, experiments with $^{18}$O-labeled substrates indicated that one substrate-derived oxygen atom is retained in compound I of that enzyme (Hager et al., 1973). Additionally, Coulson and Yonetani (1975) found that various hydroxylamine derivatives of the type RONH$_2$ react with cytochrome c peroxidase to form compound I only if the RO moiety is a good leaving group. These results are compatible only with heterolysis of the RO—OH bond.

In view of the foregoing, it is satisfying to find an arrangement of amino acid side chains at the distal side of the heme in cytochrome c peroxidase which is ideally suited to promote heterolysis of a peroxide molecule, namely Arg-48 which stabilizes a developing charge separation and His-52 which serves as an acid-base catalyst by facilitating proton transfer to the leaving group.

The need for some kind of enzymic machinery to promote charge separation and proton transfer in the catalase mechanism was recognized by Jones and Suggett (1968). They
suggested that an arginine side chain and a heme propionate side chain would meet this requirement, which, assuming the catalase active site turns out to resemble cytochrome c peroxidase, may be at least half right.

The importance of Arg-48 for peroxidase catalysis is underscored by comparing cytochrome c peroxidase with myoglobin. Even though the heme environment of metmyoglobin is superficially similar to that of cytochrome c peroxidase, myoglobin only weakly promotes cleavage of H2O2 (George and Irvine, 1956; King and Winfield, 1963). We propose that the chief reason for this difference between myoglobin and the peroxidases is that no analog of Arg-48 exists in myoglobin, where the only site that even roughly corresponds is occupied by phenylalanine CD1 instead (Poulos et al., 1980).

The charge stabilizing influence of Arg-48 is also reflected in another property of peroxidases. By suitable treatment horseradish peroxidase can be prepared in the Fe(II) state, as the so-called compound III (Yamazaki, 1974). Thus compound III should resemble myoglobin in its active Fe(II) state, and indeed compound III does contain bound molecular oxygen and exhibits an oxymyoglobin-like spectrum (George, 1953). In fact, it would appear from qualitative observations that O2 is bound exceptionally strongly in compound III. Now it is thought that O2 binding to transition metal centers involves a major resonance contribution from the M→O2− canonical structure (McLendon and Martell, 1976), and the same view has come to prevail regarding oxygen binding to heme proteins (Peisach, 1975). Thus it is reasonable to suppose that charge stabilization by Arg-48 (or its homolog in horseradish peroxidase) would strengthen the oxygen binding capability of Fe(II) peroxidase. By the same mechanism we would also expect ready reaction between Fe(II) peroxidase and O2 to form Fe(III) peroxidase and superoxide anion, O2−.

Comparison with Other Peroxidases—A rather obvious question that should be dealt with before proceeding is the extent to which the molecular machinery we have been describing is preserved in peroxidases other than cytochrome c peroxidase. Structurally the sequence of residues 48 through 52 in cytochrome c peroxidase, consisting of Arg-Leu-Ala-Trp-His, forms the middle of helix B with the carbonyl oxygen of Arg-48 hydrogen bonded to the backbone amido group of His-52. As one can see from Table I the homologs of Arg-48, Leu-49, and His-52 are present in all six peroxidases for which complete sequence data are available. Preservation of Arg-48 and His-52 is expected, of course, and implies that the mechanism we have proposed here for cytochrome c peroxidase is also applicable to other peroxidases. However the function of Leu-49 is not obvious. Probably it serves to maintain the overall positioning and orientation of helix B.

Apparently, then, cytochrome c peroxidase and the plant peroxidases catalyze the reduction of peroxides by virtually identical mechanisms. Nevertheless, cytochrome c peroxidase and horseradish peroxidase compounds I exhibit two markedly different spectral properties: (1) cytochrome c peroxidase compound I generates an easily discernable electron spin resonance signal while horseradish peroxidase compound I does not, and (2) cytochrome c peroxidase compound I is red while horseradish peroxidase compound I is green. Because compound I in both enzymes contains a ferrous iron, these spectral differences are most likely associated with the source of the second reducing equivalent. As discussed above, in cytochrome c peroxidase the second equivalent is probably removed from Trp-51, while examination of Table I shows that the corresponding residue in the plant peroxidases is phenylalanine. Since phenylalanine is more resistant to oxidation than tryptophan, the second equivalent in horseradish peroxidase compound I must be abstracted from some other neighboring group, most probably the heme ring itself. The presence of a heme radical in horseradish peroxidase compound I is consistent with both its green color (Dolphin et al., 1971) and a recently detailed analysis of its electron spin resonance spectra (Schulz et al., 1979).

Role of the Proximal Histidine—Thus far we have discussed only the function of the distal residues in cytochrome c peroxidase catalysis, but obviously the proximal histidine, His-174, and its surroundings must also be considered. Peisach (1975) and others (Nappa et al., 1977) have suggested that interaction between the proximal histidine and neighboring groups in the protein molecule will regulate the electronic properties and therefore the reactivity of the heme iron. If, for example, this interaction imparts enhanced anionic character to the proximal histidine, higher oxidation states will be stabilized and Eo will be lowered. Indeed, Mincey and Traylor (1979) have found that model heme compounds containing anionic axial ligands are more readily oxidized than those containing neutral ligands.

In the case of cytochrome c peroxidase, the oxidation-reduction potential of the ferrous/ferric couple is −194 mV (Conroy et al., 1978) which is to be compared with myoglobin at +50 mV (Cassatt et al., 1975). Mechanistically this requirement for a lowered oxidation-reduction potential in cytochrome c peroxidase is understandable, since the Fe5+ formal oxidation state of the FeFe2+ couple (Fig. 3d) must be stabilized sufficiently to permit it to be produced at least transiently in a two-electron oxidation of Fe5+ by peroxide. In intriguing question, then, is how can the structure of cytochrome c peroxidase explain its low oxidation-reduction potential in comparison with myoglobin. We had expected to find the proximal histidine, His-174, hydrogen bonded to a negatively charged carboxylate side chain. Now that the amino acid sequence of cytochrome c peroxidase is in hand, however, we find instead that His-174 is within hydrogen bonding distance of the side chain of Gln-239. Specifically, ND1 of His-174 is about 2.9 Å from O2E2 of Gln-239. In turn, Gln-239 is hydrogen bonded to the side chain of Glu-187 (see Fig. 1). All three side chains (His-174, Gln-239, and Glu-187) are buried and inaccessible to solvent. A preliminary sequence alignment of cytochrome c peroxidase and horseradish peroxidase is so far inconclusive concerning the possible conservation of this structural feature. However, it is noteworthy that both Gln-239 and cytochrome c peroxidase and Gln-245 in horseradish peroxidase are located in identical Leu-Ile-Gln sequences.

By way of comparison, the proximal histidine of myoglobin, His-8F, is hydrogen bonded to the backbone carbonyl of Leu-89.

Presumably this arrangement of a buried negative charge and hydrogen bonding to the proximal histidine in cytochrome c peroxidase is responsible for regulating its oxidation-reduc-
tion potential. However, it is not entirely clear if there would be sufficiently large inductive effect at the heme iron due to a negative charge on Glu-187 to explain the observed oxidation-reduction potential difference between cytochrome c peroxidase and myoglobin, but for the moment that seems to be the best working hypothesis. Moreover, the hydrogen bonding network in cytochrome c peroxidase is considerably more flexible than the carbonyl oxygen-His interaction in myoglobin. Therefore, it is intriguing to consider the possibility that heme reactivity is modulated by the dynamics of the His-174—Glu-239—Glu-187 interaction during the catalytic cycle. The recent observation (Hayashi and Yamazaki, 1979) that the oxidation-reduction potential of the two couples, compound I/compound I1 and compound II/native peroxidase in horseradish peroxidase, is nearly the same suggests that some such mechanism may be at work.

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