Effect of Distal Cavity Mutations on the Binding and Activation of Oxygen by Ferrous Horseradish Peroxidase*

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Mutations have been introduced at residues Arg-38 or His-42 in horseradish peroxidase isoenzyme C (HRPC) in order to probe the role of these key distal residues in the reaction of ferrous HRPC with dioxygen. The association and dissociation rate constants for dioxygen binding to His-42 → Leu, His-42 → Arg, Arg-38 → Leu, Arg-38 → Lys, Arg-38 → Ser, and Arg-38 → Gly variants have been measured using stopped-flow spectrophotometry. Replacement of His-42 by Leu or Arg increases the oxygen binding rate constant by less than an order of magnitude, whereas changing the polar distal Arg-38 causes increases of more than 2 orders. These results demonstrate that His-42 and Arg-38 impede the binding of dioxygen to ferrous HRPC, presumably by steric and/or electrostatic interactions in the distal heme cavity. Recombinant HRPC oxyperoxidase reverted slowly to the ferric state with no spectrophotometrically detectable intermediates and with an apparent first-order rate constant of 9.0 × 10−3 s−1, which is essentially the same as that for the native, glycosylated enzyme. This reaction was accelerated when His-42 was replaced by Leu or Arg (k_{decay} = 0.10 and 0.07 s−1, respectively) presumably due to the loss of the hydrogen bond between the His-42 imidazole and the bound dioxygen. Substitution of Arg-38 by Leu, Lys, or Gly also produced a less stable oxyperoxidase (k_{decay} = 0.22, 0.20, and 0.58 s−1, respectively). However, with the Arg-38 → Ser variant, a transient intermediate, proposed to be a ferric-superoxide complex, was detected by rapid-scan stopped-flow spectrophotometry during the conversion of oxyperoxidase to the ferric state. This variant also exhibits an unusually high affinity for dioxygen. It is proposed that Arg-38 interacts with the bound dioxygen to promote superoxidede character, thereby stabilizing the oxyperoxidase state and making the binding of dioxygen to ferrous HRPC essentially irreversible. We conclude that Arg-38 and His-42 not only promote the heterolytic cleavage of bound hydrogen peroxide to form compound I but also decrease the lability of the ferrous enzyme-dioxygen complex in order to suppress the formation of the inactive ferrous state.

Horseradish peroxidase (HRP; donor:H2O2 oxidoreductase) is a member of the plant peroxidase superfamily (1). It is able to utilize hydrogen peroxide to catalyze the one-electron oxidation of a wide range of aromatic substrates. Peroxidase action involves e− oxidation of the enzyme by hydrogen peroxide to give an intermediate known as compound I. This then reverts to the resting enzyme via two successive 1e− reactions with reducing substrate molecules, the first yielding a second enzyme intermediate, compound II (2). A third enzyme intermediate, compound III, is also observed in the reaction of HRP with an excess of hydrogen peroxide (3). This compound, the peroxidase analogue of oxymyoglobin and oxyhemoglobin, does not normally participate in the peroxidatic cycle of HRP (2). The spectrum and reactivity pattern of compound III correspond to those expected for an Fe(III)-O2 adduct or oxyperoxidase (4). Oxyperoxidase can be formed in four different ways: (i) addition of a large excess of H2O2 to the native ferric enzyme, (ii) reduction of native ferric enzyme to ferroperoxidase followed by the addition of dioxygen, (iii) reaction of H2O2 with compound II, and (iv) addition of superoxide anion to native ferric peroxidase (2). The formation of oxyperoxidase from ferrous peroxidase and dioxygen follows second-order kinetics with a calculated rate constant of 5.8 × 104 M−1 s−1 at pH 7.0 and 20 °C (4). The mechanisms of oxy-HRPC (5, 6) and ligninoxyperoxidase (7) decomposition have also been studied. In both cases the oxyperoxidase form reverted slowly to the native ferric state with no spectrophotometrically detectable intermediates. This contrasts with the reactivity of ferrous yeast cytochrome c peroxidase (CcP) with dioxygen, which differs dramatically from that of other peroxidases. Photolysis of the CcP(II)-CO complex in the presence of oxygen converts the enzyme to a product with an absorption spectrum and an EPR radical signal at g = 2.00 that were identical to those of compound I formed by the reaction of ferric CcP with hydrogen peroxide (8, 9).

Hemoproteins show great diversity in their biological functions while retaining an essentially unaltered prosthetic group, iron protoporphyrin IX. Although key catalytic residues in the active site of peroxidases are highly conserved (10), in the context of the present paper it is useful to compare HRPC with CcP. The complete three-dimensional structure of CcP is known from crystallographic data at 1.7-Å resolution (11). Recombinant native CcP and several variants have also been available for structural and mechanistic studies (12, 13). Although the primary structure of CcP has only 18% identity with

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1 The abbreviations used are: HRP, horseradish peroxidase; HRPC, horseradish peroxidase isoenzyme C; HRPC*, recombinant horseradish peroxidase isoenzyme C; h42l, His-42 → Leu HRPC* mutant; h42r, His-42 → Arg HRPC* mutant; r38l, Arg-38 → Leu HRPC* mutant; r38k, Arg-38 → Lys HRPC* mutant; r38s, Arg-38 → Ser HRPC* mutant; r38g, Arg-38 → Gly HRPC* mutant; CcP, cytochrome c peroxidase; o2-HRP, horseradish oxyperoxidase; o2-CcP, cytochrome c oxyperoxidase.

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that of HRPC (14), over 80% of this sequence identity is centered around two highly conserved residues, the distal and the proximal histidines. An arginine residue in the distal heme pocket is also conserved in peroxidases. Distal and proximal histidines are also conserved in the globins, which react with hydrogen peroxide much slower than do peroxidases. This differential reactivity with hydrogen peroxide has been ascribed to the distal arginine, which has no equivalent in the globins (15). Recent studies with CcP showed that the guanidinium side chain of Arg-48 is not absolutely required for the heterolytic cleavage of peroxide (12, 13, 16), although we have shown that in HRPC this residue does play an important role in facilitating the binding of peroxide and modulating its subsequent reactivity (17, 18). Miller et al. (19) determined the crystal structure of the Fe(II)-O₂ complex formed by a yeast CcP mutant (Trp-191 → Phe). The refined structure showed that dioxygen forms a hydrogen bond with the imidazole side chain of the conserved distal histidine, but not with the guanidinium group of the conserved distal arginine. The recent availability of site-directed mutants of HRPC has now allowed us to investigate the role of Arg-38 and His-42 in the formation of oxy-HRPC. The results also provide further insights into the origins of the reactivity differences of HRP and CcP oxyperoxidases (9, 19) and the relationship between oxyperoxidases and oxyglobins (15, 19).

**Materials and Methods**

**Reagents—**HRPC (type 4B) was purchased from Biozyme Ltd., United Kingdom (Blaenavon, Gwent, UK) and used without further purification. Construction of the gene, expression and purification of HRPC* H₄₂L, H₄₂R, R₃₈L, and R₃₈K have been described previously (17, 20–22). Construction of R₃₈S and R₃₈G genes was carried out as described previously for the R₃₈L mutant (17), except that a Ser(TCA) was inserted at position 126. The spectral properties of the enzymes used in this study are given in Table I. The specific activities (units mg⁻¹) of the HRP preparations used were 920 for HRPC, 930 for HRPC*, 0.2 for H₄₂L, 5.0 for H₄₂R, 5.2 for R₃₈L, 47.7 for R₃₈G, 7.1 for R₃₈S, and 2.9 for R₃₈K. Activities were measured as described previously with 0.3 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 2.5 mM H₂O₂ (20). Reagent grade H₂O₂ (30% v/v) was obtained from BDH/Merck (Poole, UK) and its concentration determined by iodide titration with HRPC (23). All other chemicals were of analytical grade and supplied by BDH/Merck.

**Reaction of Ferrous Enzymes with Dioxygen—**Fully reduced native HRPC and its variants were prepared by incubation of enzyme with sodium dithionite for several minutes in a buffer of 0.1 M sodium phosphate at pH 7.0 until no further change in the reduced Soret band could be detected. Since ferroperoxidase reacts rapidly with dioxygen, the following procedures were carried out in an anaerobic glove box operating under N₂ with less than 1 ppm of O₂. The excess sodium dithionite was removed from the ferroperoxidase by passing the solution through a Sephadex G-25 column that had been equilibrated with deoxygenated buffer. Anaerobic rapid-scan stopped-flow studies were performed using a Hi-Tech SF-51 stopped-flow spectrophotometer equipped with a xenon lamp, 360 nm filter, and a diode-array detection system (MG-6000, Hi-Tech Scientific, Salisbury, UK). Single wave experiments were carried out with the same instrument equipped with a tungsten lamp. Enzyme stock and buffer solutions (0.1 M sodium phosphate, pH 7.0) were deoxygenated for 30 min by repeated evacuation and back filling with nitrogen on a vacuum line before transfer into the glove box. Air- or oxygen-saturated buffers were transferred into the anaerobic box in hermetically sealed serum vials. Oxygen concentrations were varied from 20 to 600 μM by premixing the air- or oxygen-saturated buffer with the deoxygenated buffer in syringes with no gas head space to avoid loss of O₂ from the solution. Data were recorded through a RS232 interface on a microcomputer and analyzed by fitting absorbance versus time curves to exponential functions using a least-squares minimization program supplied by Hi-Tech Scientific Ltd. Temperature was controlled at 25 °C using a Techne C-400 circulating bath with a heater-cooler also installed in the anaerobic box.

**Decomposition of Oxyperoxidases—**Oxyperoxidase solutions were prepared as described above. The disappearance of wild-type and mutant oxyperoxidases and the appearance of native ferric enzymes were followed at their respective Soret maximum indicated in Tables II and I, respectively.

**Results**

**Binding of Oxygen to Ferrous Wild-type HRPC—**Addition of molecular oxygen to ferrous HRPC resulted in the rapid formation of oxyperoxidase with absorption maxima at 417, 543, and 577 nm (Table II) as described previously by Wittenberg et al. (4). The reaction of ferrous HRPC with oxygen was followed at 417 nm. The observed first-order rate constant was directly proportional to the initial oxygen concentration (results not shown). At pH 7.0 and 25 °C, the oxyperoxidase subsequently reverted over a period of minutes to the native ferric state (Reaction 1). To study the kinetics of this reaction, the disappearance of oxy-HRPC and the appearance of native ferric enzymes were followed at 417 nm. The initial concentration of oxygen with kₘ (O₂) and kₐff was determined by fitting the absorbance versus time curves to a single exponential function and was found to be independent of oxygen concentration (Table III).

**Reactor 1**

The dependences of the pseudo-first-order rate constants on the initial concentration of oxygen with kₘ(O₂) and k₉ff in theory should yield the values of kₘ and k₉ff. The slope of the line should give the value of kₘ and its reciprocal with the ordinate axis kₐff. However, the intercepts are very near to zero, leading to inaccuracy in the determination of kₐff (data not shown). Similar results were obtained for the reaction of oxygen with the non-glycosylated recombinant HRPC*. The kinetics of formation and decay of the oxyperoxidase formed from recombinant enzyme yielded essentially the same rate constants as obtained for the glycosylated native enzyme (Table III). The lack of glycosylation in the recombinant enzyme therefore did not affect the kinetic parameters for the reaction of HRPC with the small ligand oxygen.
experiments at different oxygen concentrations.

The distal histidine of heme proteins such as myoglobin and hemoglobin is thought to influence the affinity of diatomic ligands bound at the sixth coordination position of the heme. Structural and kinetic studies indicate that CO and O₂

atomic ligands bound at the sixth coordination position of the bin and hemoglobin is thought to influence the affinity of diatomic ligands bound at the sixth coordination position of the reason dioxygen (19). We have therefore prepared the H42L and H42R variants of HRPC* and studied the effect on the kinetics of dioxygen binding to the ferrous forms of the enzyme.

The spectrum of the oxyperoxidase form of H42L was similar to that of the wild-type enzyme with absorbance maxima at 416, 541, and 575 nm (Table II). This compound autoxidized to the ferric form with isosbestic points at 408, 467, 524 and 598 nm (Fig. 1) and with a decay constant approximately 12 times faster than for the wild-type oxyperoxidase. The decay constant was also independent of the oxygen concentration (Table III).

Similar results were obtained for the decomposition of the H42R oxyperoxidase (Tables II and III). The reactions of ferrous H42L and H42R with oxygen gave single exponential absorbance changes with pseudo-first-order rate constants that exhibited a linear dependence on the initial oxygen concentration (Fig. 2). The bimolecular rate constants for H42L and H42R were 6- and 2-fold higher, respectively, than that of the wild-type enzyme (Table III). Unlike the data for the native enzyme forms, the ordinate intercepts for the mutants were statistically significant due to higher $k_{off}$ values (five determinations at each of the oxygen concentrations). This allowed the values of $k_{off}$ and the equilibrium constant for oxygen binding to both mutants to be calculated (Table III).

The substitution of His-42 by Leu or Arg produced an increase in both the rates of binding and dissociation, resulting in a net decrease in the affinity for oxygen, i.e. less stable oxyperoxides.

**The Binding of Oxygen to Ferrous HRPC Arginine 38 Mutants**—Two pieces of evidence suggest that the distal arginine impedes the reaction of ferroperoxidase with diatomic ligands such as O₂ and CO. First, proteins designed to react with oxygen such as myoglobins and hemoglobins have an apolar residue at the position corresponding to that of the distal arginine in peroxidases (27, 28). Second, replacing Arg-38 by Leu in ferrous HRPC increases the affinity for CO by 3 orders of magnitude relative to native enzyme (22). In order to better understand the role of Arg-38 in the binding of oxygen to ferrous HRPC, we have replaced it by other amino acids with side chains of different lengths and/or polarity and determined the effect on the kinetics of oxygen binding (Table III).

Simple monophasic time courses were observed for oxygen binding to each of these mutant enzymes. Replacement of Arg-38 by Leu, Lys, or Gly had similar effects on both $k_{on}$ and $k_{off}$ (Fig. 3). There was a progressive increase in the observed bimolecular rate constant for oxygen binding in the order wild-type < R38K < R38L < R38G. The value for the R38G mutant was increased by nearly 2 orders of magnitude relative to the wild-type enzyme. However, a more than 500-fold compensating increase in the dissociation constants ($k_{off}$) for each of the mutants resulted in a net lower affinity for O₂ than the native enzyme (Table III). The spectra of the Arg-38 variant oxyperoxides were all similar to that obtained for the native enzyme (Table II).

The R38S mutant bound oxygen 50 times tighter than any one of the other Arg-38 variants (Fig. 3). The low $K_P$ results from both an increase in the second-order association rate constant ($k_{on}$) and a decrease in the dissociation rate constant ($k_{off}$) (Table III). Although R38S oxyperoxidase has a similar absorbance spectrum to that of native oxy-HRPC, it does exhibit a 6-nm displacement of the Soret band maximum to 411 nm. This suggests that the environment of the bound oxygen has been significantly perturbed by the introduction of a serine residue at position 38.

Fig. 4 shows a linear decrease in the logarithm of the second-order rate constant for oxygen binding to each Arg-38 variant as a function of the calculated side chain volumes of each of the residues substituting for arginine at position 38. This behavior is entirely consistent with the side chains of the residues at position 38 sterically hindering oxygen binding. However, a

### Table II

**Absorption maxima for the oxyperoxidases used in this study**

| Protein | Absorption maxima |
|---------|-------------------|
|         | nm                |
| HRPC   | 417 543 577       |
| HRPC*  | 417 543 577       |
| H42L   | 416 541 575       |
| H42R   | 416 543 579       |
| R38K   | 415 542 578       |
| R38L   | 416 542 578       |
| R38S   | 411 541 575       |
| R38G   | 416 540 578       |

### Table III

**Rate and equilibrium parameters for oxygen binding to wild types and positions 42 or 38 mutants of horseradish peroxidase isoenzyme C at 25°C, pH 7.0**

| Protein | $k_{on}$ | $k_{off}$ | $K_P$ | $k_{decay}$ |
|---------|----------|-----------|-------|-------------|
|         | $M^{-1}s^{-1}$ | $s^{-1}$ | $M$ | $s^{-1}$ |
| HRPC   | (5.3 ± 0.4) × 10⁴ | <0.1 | <1.9  | (8.2 ± 1.0) × 10⁻³ |
| HRPC*  | (5.2 ± 0.4) × 10⁴ | <0.1 | <1.9  | (9.0 ± 1.0) × 10⁻³ |
| H42L   | (3.4 ± 0.1) × 10⁶ | 3.9 ± 0.1 | 12 ± 3 | (1.0 ± 0.2) × 10⁻¹ |
| H42R   | (1.2 ± 0.1) × 10⁶ | 1.2 ± 0.1 | 10 ± 2 | (7.1 ± 0.8) × 10⁻² |
| R38K   | (6.7 ± 0.2) × 10⁵ | 53 ± 6 | 80 ± 4 | (2.0 ± 0.3) × 10⁻¹ |
| R38L   | (8.4 ± 0.2) × 10⁵ | 48 ± 5 | 57 ± 4 | (2.2 ± 0.4) × 10⁻¹ |
| R38S   | (2.2 ± 0.6) × 10⁶ | 0.9 ± 0.1 | 0.4 ± 0.1 | (5.8 ± 0.6) × 10⁻² |
| R38G   | (3.0 ± 0.6) × 10⁶ | 50 ± 5 | 17 ± 2 | (5.8 ± 0.5) × 10⁻¹ |

* Rate constant for the conversion of peroxidase into the ferric-superoxy complex.

* Rate constant for the conversion of the ferric-superoxy complex into the native enzyme.
purely steric effect clearly does not explain the deviance of the point for the native enzyme with an arginine at position 38 and suggests electrostatic factors associated with this positively charged residue are important. We discuss below the structural and mechanistic significance of this observation.

Decomposition of Arginine 38 Mutant Oxyperoxidase—Table III shows the effect of various substitutions of Arg-38 on the stability of the oxyperoxidase complex. The R38L oxyperoxidase prepared by the reaction of ferrous enzyme with oxygen reverted directly to the native ferric enzyme with a half-life of 3 s. Similar results were observed for the R38K (half-life 3.5 s) and R38G (half-life 1.2 s) variants. However, the oxyperoxidase formed by the reaction of R38S ferrous enzyme with dioxygen reverted to native ferric enzyme with the accumulation of a spectroscopically detectable transient intermediate shown by the single wavelength stopped-flow trace in Fig. 5. Rapid-scan stopped-flow spectrophotometry reveals the formation of a transient species with a Soret maximum at 406 nm (the same as that for the resting enzyme) but with a higher extinction coefficient ($\varepsilon_{406\, nm} = 5160\, M^{-1}\, cm^{-1}$; cf. $133\, M^{-1}\, cm^{-1}$ for native enzyme) (Fig. 6A). Moreover, this transient species has peaks at 499, 573, and 630 nm and a shoulder at 537 nm (Fig. 6B). This new intermediate was converted to ferric enzyme, as indicated by the decrease in absorbance in the Soret region (Fig. 6C). Differences in the spectra between this new intermediate and that of the ferric enzyme are shown in Fig. 6B. A transient species, thought to be a ferric hydroperoxide complex (Fe(III)-OOH), with a spectrum similar to that shown in Fig. 6 (A–C) has been previously observed during the reaction of the R38L variant with hydrogen peroxide (17).

**DISCUSSION**

Effects of Distal Mutations on Oxygen Binding—Replacement of the distal His-42 by Leu or Arg produced a 6- and 2-fold increase in the association rate constants, respectively, whereas the replacement of the distal Arg-38 caused more dramatic increases of up to 100-fold (Table III). Since there do
not appear to be any previous reports describing the role of His-42 and Arg-38 in the binding of oxygen to ferrous HRPC, it is only possible to discuss our data in the context of other oxygen-binding heme proteins. In pig and sperm whale myoglobin, substitutions of the distal histidine (His-64) by leucine produced a 7-fold increase in $k_{on}$ and a greater than 100-fold increase in $k_{off}$, resulting in a dramatic loss of oxygen affinity in these variants (25, 29). The equivalent substitution in glycera hemoglobin induces significantly weaker oxygen binding (15, 30). Substitution of His-42 by Leu in HRPC* produces a similar increase in $k_{on}$ to that for myoglobin and hemoglobin variants. These observations suggest that the access of oxygen to the heme is hindered by the distal histidine of HRPC in a similar manner to that which occurs with the globins. Since a hydrogen bond between the bound oxygen and the distal histidine has been demonstrated in oxy-CrP (19) and oxymyoglobin (26), the instability of the His-42 oxyperoxidase variants is best explained by the presence of a similar interaction in oxy-HRPC. However, other residues in the protein must also contribute to the stabilization of oxy-HRPC because a more dramatic decrease in both oxygen affinity and oxyperoxidase stability was observed when Arg-38 was replaced. The high polarity of the distal pocket created by the invariant distal arginine has been proposed to be a major determinant of ligand binding rates for peroxidases (15). We have not only confirmed this in the present studies with oxygen but also recently for CO binding to HRPC* variants (22). Arg-38 is clearly both a steric and an electrostatic impediment to the binding of oxygen and CO to the ferrous iron in HRPC.

**Mechanism of Oxyperoxidase Decay**—The mechanism of oxyperoxidase decay has been studied previously in the presence of ferroperoxidase, which accelerates the formation of ferriperoxidase (6). Oxyperoxidase is relatively stable when there is no ferroperoxidase present with a half-life of 20 s. The present experiments were performed under these conditions. The mechanism of oxy-HRPC decay most likely involves the dissociation of a ferric-superoxide complex to yield ferric-HRPC and $O_2$. Peisach et al. (31) originally proposed that oxygen binding to the ferrous heme induces partial oxidation of the iron with electron density migrating to the oxygen. Resonance Raman studies have...
shown that oxygen bound to oxy-HRPC and lignin-oxyperoxidase have substantial superoxide character (32, 33). A hydrogen bond between the β-oxygen of oxyperoxidase and the NH of His-42 and an electrostatic interaction between the guanidinium group of Arg-38 and the bound oxygen would be expected to stabilize such a charge distribution, thereby increasing the affinity for oxygen (19). The increased rates of decay of the R38K, R38L, and R38G variants (k_{decay} Table III) must mean that the decreased affinity for oxygen (K_P) is more than compensated for by an increase in the rate of dissociation of superoxide anion from the ferric enzyme.

The Structure of Oxyperoxidase—In the absence of a high resolution crystal structure of HRPC (34), the data presented above allow some predictions on the structure of the oxyperoxidase form. (i) The NH of the imidazole group of the distal His-42 most likely hydrogen bonds (≈3.0 Å) with the bound oxygen. Such an interaction has been observed in the crystal structure of oxy-CcP (19) and in oxymyoglobin (26). (ii) The guanidinium side chain of Arg-38 is close enough to the β-oxygen of oxy-HRPC for hydrogen bonding and/or an electrostatic interaction. This contrasts with the situation in oxy-CcP, in which the guanidinium group of Arg-48 is some distance away from the bound oxygen with an intervening water molecule (water 648) (19). These structural differences explain the different reactivities of the oxyperoxidase forms of native CcP and HRPC. (iii) An interaction between the ε-amino group of Lys in the R38K variant and a group from the heme or from the protein is the most likely explanation for the 20-fold increase in dioxygen binding rates. The crystal structure of the corresponding CcP mutant shows that the side chain of Lys-48 is positioned between His-52 and the heme iron with the ε-amino group of Lys-48 2.7 Å distant from the iron occupying the position of water-595 in the wild type (13). The different positions and/or interactions of Lys in HRPC and CcP Arg → Lys variants can also explain their different reactivities with hydrogen peroxide (13, 35, 36). (iv) We expected a decrease in the second-order rate constant for O₂ association in the H42R mutant, but instead we observed a 2-fold increase similar to the 6-fold increase for the H42L variant. The presence of two arginines in the distal pocket should have increased the polarity of the oxygen access channel and caused a decrease in the rate of oxygen binding. Our data suggest that in the H42R mutant, the new arginine (Arg-42) adopts a conformation with the charged side chain pointing out of the distal pocket toward the solvent, thereby creating a less hindered pathway for oxygen binding. A similar structural adjustment was postulated to occur in β-chains of hemoglobin Zurich, in which the distal histidine was replaced by arginine. The guanidinium group swings out of the active center and interacts with one of the heme propionates, thereby removing a hydrogen bonding interaction with the sixth coordination position and facilitating oxygen binding (37).

Oxyperoxidase and the Ferric Hydroperoxide Complex—A number of attempts have been made to detect and characterize the transient hydroperoxide intermediate (Fe(III)-OOH) formed in the reaction of ferric enzyme with hydrogen peroxide. Baecck and Van Wart (38, 39), working in methanol media at −26 °C, detected an intermediate with a hyperporphyrin optical spectrum that they called compound 0. A structure of oxy-CcP at 2.2-Å resolution has been proposed as a model for the transient ferric enzyme-peroxide complex (19). We have observed a new intermediate with neither a hyperporphyrin nor a peroxidase spectra formed transiently during the reaction of the HRPC-R38L variant with hydrogen peroxide at 10 °C (17). The spectrum obtained using rapid-scan stopped-flow spectrophotometry was similar to that of the ferric enzyme but with peaks at 397, 487, and 580 nm, a shoulder at 530 nm, and an increased extinction coefficient in the Soret region (17). We have now detected a similar spectrum to that proposed for the Fe(III)-OOH complex in the reaction of ferrous HRPC-R38S with oxygen (Fig. 6). This suggests a stabilization of the ferric-superoxide complex through a new hydrogen bond between the serine introduced at position 38 and the β-oxygen in oxy-HRPC. This structure is analogous to the Fe(III)-OOH intermediate, and the similarity of the absorption spectra is to be expected.

Oxyperoxidase and the Globins—Oxyperoxidase is analogous in many ways to the oxymyoglobins and oxyhemoglobins. They all contain low spin protoheme with histidine and dioxygen as the fifth and sixth ligands, respectively. However oxyperoxidases do not bind oxygen reversibly and are less photolabile. Oxyperoxidases because of their inherent instability are more difficult to study (33). Clearly the structures of both the distal and proximal pockets of oxyperoxidase and oxymyoglobins are optimal for their respective functions. The differences between oxyperoxidases and oxymyoglobins are analogous to the character of the σ-bond to the proximal histidine (33). A stronger σ-bond to the proximal histidine in oxyperoxidase may in part explain the greater degree of oxygen activation in oxyperoxidase compared to that for oxymyoglobin. This should weaken the O–O bond and activate toward reduction. The different reactivities of these two groups of proteins have also been ascribed to differences in the polarities of the distal heme pockets (15, 19). Our new data on HRPC variants provide additional evidence that these different polarities are essential for oxyperoxidases and oxymyoglobins to perform their specific functions. The mutations we have made at Arg-38 in HRPC cause the behavior of the oxyperoxidase forms to more closely resemble those of oxymyoglobins. The association rate constants for oxygen binding (k_{on}) are 2 orders of magnitude higher for the Arg-38 variants and closer to those reported for myoglobin or hemoglobin (15). The dissociation rate constants (k_{off}) are also of the same order as those of the oxymyoglobins (15). We have created peroxidase variants that closely resemble globins with respect to their oxygen binding kinetic parameters but with enhanced rates of decay to yield ferric enzyme and superoxide. Clearly other features of the protein environment on the proximal side of the heme group must contribute to the relative stability of oxymyoglobins.

The reversible binding of oxygen to myoglobin and hemoglobin is central to the metabolism of vertebrates and some invertebrates, providing a reservoir of O₂ that is available for respiration. In contrast, peroxidases are designed to react with peroxide and reducing substrates and they are involved in various biosynthetic pathways. The active center of peroxidases not only provides optimum reaction rates with hydrogen peroxide and their reducing substrates but also induces irreversible decay of oxyperoxidase to yield ferric enzyme, the active form of the enzyme in the peroxidic cycle. This is a physiologically important reaction in that, under aerobic conditions in vivo, it removes ferrous enzyme and/or compound III that have accumulated due to an excess of hydrogen peroxide or superoxide anion, or following peroxidase catalyzed indole-3-acetic acid oxidative decarboxylation (40, 41).

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