Mechanism of Reaction of Hydrogen Peroxide with Horseradish Peroxidase: Identification of Intermediates in the Catalytic Cycle

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Abstract: The mechanism of the reaction of horseradish peroxidase isoenzyme C (HRPC) with hydrogen peroxide to form the reactive enzyme intermediate compound I has been studied using electronic absorbance, rapid-scan stopped-flow, and electron paramagnetic resonance (EPR) spectroscopies at both acid and basic pH. The roles of the active site residues His42 and Arg38 in controlling heterolytic cleavage of the H_2O_2 oxygen−oxygen bond have been probed with site-directed mutant enzymes His42 → Leu (H42L), Arg38 → Leu (R38L), and Arg38 → Gly (R38G). The biphasic reaction kinetics of H42L with H_2O_2 suggested the presence of an intermediate species and, at acid pH, a reversible second step, probably due to a neutral enzyme−H_2O_2 complex and the ferric−peroxoanion-containing compound 0. EPR also indicated the formation of a protein radical situated more than ∼10 Å from the heme iron. The stoichiometry of the reaction of the H42L/H_2O_2 reaction product and 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) (ABTS) was concentration dependent and fell from a value of 2 to 1 above 0.7 mM ABTS. These data can be explained if H_2O_2 undergoes homolytic cleavage in H42L. The apparent rate of compound I formation by H42L, while low, was pH independent in contrast to wild-type HRPC where the rate falls at acid pH, indicating the involvement of an ionizable group with p$K_a \approx 4$. In R38L and R38G, the apparent pK_a was shifted to ∼8 but there is no evidence that homolytic cleavage of H_2O_2 occurs. These data suggest that His42 acts initially as a proton acceptor (base catalyst) and then as a donor (acid catalyst) at neutral pH and predict the observed slower rate and lower efficiency of heterolytic cleavage observed at acid pH. Arg38 is influential in lowering the pK_a of His42 and additionally in aligning H_2O_2 in the active site, but it does not play a direct role in proton transfer.

Introduction

Peroxidases are ubiquitous oxidative heme-containing enzymes that are usually isolated from plants, fungi, and bacteria[1] but also increasingly from mammalian sources.[2] They have been implicated in diverse biological processes that include both cell wall synthesis and degradation, stress response, signaling during oxidative stress, and removal of xenobiotics. An example of their increasing importance is the identification of over 70 peroxidase isozymes in the *Arabidopsis thaliana* genome of which over half are considered to be involved in stress responses (K. Welinder, personal communication). An early step in the catalytic cycle following the binding of H_2O_2 to the heme in the Fe(III) state, is the heterolytic cleavage of the oxygen−oxygen bond of H_2O_2. A water molecule is released during this reaction with the concomitant two-electron oxidation of the heme to form an intermediate (compound I) comprising a ferryl species (Fe(IV)═O) and a porphyrin radical cation.[3] Compound I is then converted back to the resting enzyme via two successive single-electron transfers from reducing substrate molecules. The first reduction, of the porphyrin radical cation, yields a second enzyme intermediate, compound II, which retains the heme in the ferryl (Fe(IV)═O) state.[3]

Two key catalytic residues in the distal heme pocket, an arginine and a histidine, are involved in peroxide activation and compound I formation by catalyzing proton transfer from the α- to the β-oxygen atom of heme-bound H_2O_2 and by polarizing the oxygen−oxygen bond.[4,5] Recently, the crystal structure of isoenzyme C of horseradish peroxidase (HRPC) was determined at 2.15-Å resolution.[6] The nature and positions of the distal heme pocket residues of HRPC, in particular His42 and Arg38, are entirely consistent with a two-step mechanism for compound I formation.[5,7−9] Two essential features of this mechanism are

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acid−base catalysis by His42 and charge stabilization of an enzyme−substrate precursor complex by Arg38. Site-directed mutagenesis studies have suggested that Arg38 is not only involved in promoting the cleavage of the O−O bond of peroxide but also has an important role in facilitating the rapid binding of H_2O_2 to HRPC.[8] Mutagenesis of Asn70, another distal pocket residue, suggested that this residue indirectly contributes to catalysis by modulating the position and basicity of His42 by hydrogen bonding between the Oδ1 of Asn70 and the Nδ1 of His42.[10] Structural confirmation of this hypothesis is provided by the HRPC crystal structure.[6]

Kinetic evidence for a precursor of HRPC compound I was first provided by low-temperature stopped-flow experiments in 50% v/v methanol/phosphate buffer.[11] The new intermediate, designated compound 0, was proposed to be a hyperporphyrin species formed by deprotonation of a H_2O_2−HRPC complex and had a spectrum with two intense absorptions at 330 and 400 nm. The replacement of Arg38 of HRPC with a nonpolar leucine residue inhibited O−O bond cleavage and allowed the observation of an intermediate at 10 °C in aqueous buffer.[8] The spectrum of the species, however, resembled that of the ferric state, and no intermediates having a spectrum similar to that of compound 0 were detected. Recent calculations using the INDO/ROHF/CI quantum chemical method suggested that a hyperporphyrin spectrum, or a split Soret, would originate from the peroxoanion−iron(III) complex.[12] The authors further proposed that the spectrum of a neutral H_2O_2−HRPC complex would be similar to that of the resting enzyme. Taking this together with our results, the new intermediate observed for R38L HRPC is best described as a neutral peroxy−iron complex. Recent work on polyethylene glycolated HRPC in organic solvents has suggested the involvement of an H_2O_2−HRPC complex in the catalytic cycle.[13]

Although there is no doubt that an acid−base mechanism operates in HRPC compound I formation, the roles of several residues of its distal pocket remain unclear. Thus, it has recently been suggested that at neutral pH there is no reason to invoke His42 as both proton donor and acceptor, implying that Arg38 is the only residue that can act as a proton donor to the peroxide β-oxygen (i.e., the oxygen atom not bound to the heme iron).[14] In this paper, we present additional spectrophotometric evidence, obtained with the H42L HRPC variant, for the presence of a neutral H_2O_2 adduct as an intermediate in HRPC compound I formation. We suggest that HRPC binds neutral H_2O_2 in preference to its anionic form and conclude that His42 plays a crucial role in H_2O_2 activation at all pH values. We also present data that suggest that the positively charged side chain of Arg38, in concert with Asn70, regulates the pK_a of the Nϵ proton of His42 in HRPC, a key parameter in the activation of H_2O_2.

Materials and Methods

Reagents. HRPC (type 4B) was purchased from Biozyme Ltd., United Kingdom (Blaenavon, Gwent, U.K.) and used without further purification. Construction of the gene, expression, and purification of nonglycosylated recombinant wild-type HRPC (HRPC*) and HRPC* mutants H42L, R38L, and R38G have been described previously.[5,8,15,16] Reagent grade H_2O_2 (30% v/v) was obtained from BDH/Merck (Poole, U.K.) and its concentration determined by iodide titration with HRPC.[17] ABTS was from Sigma (Poole, Dorset, U.K.). All other chemicals were of analytical grade and supplied by BDH/Merck. For the pH dependencies, stock buffer solutions were prepared using deionized water from a Milli-Q system (Millipore). McIlvane and acetate buffers were used between pH 3.0 and 5.5, Mops and phosphate buffers between pH 6.0 and 7.5, and bicine, Tris-HCl, and borate buffers between pH 8.0 and 10.

Equipment. Transient kinetics were monitored with a stopped-flow spectrophotometer (model SF-51, Hi-Tech Scientific, Salisbury, U.K.). Stopped-flow rapid-scan spectrophotometry was carried out with the same sample-handling unit equipped with an MG-6000 diode array system. Ultraviolet/visible absorption spectra were recorded in quartz cuvettes (1 cm) on a Shimadzu UV-2101PC spectrophotometer with a spectral bandwidth of 1 nm and a scan speed of 120 nm min^{-1}. Temperature was controlled at 25 °C using a Techne C-400 circulating bath with a heater−cooler. EPR spectra were recorded on a modified Bruker ER200D-SRC spectrometer with an Oxford Instruments ESR900 cryostat.

Pre-Steady-State Kinetics. Compound I formation was monitored at 395 nm (isosbestic point for compounds I and II) for wild-type enzymes (HRPC and HRPC*) and the H42L mutant. The time courses for the conversion of R38L and R38G to their respective compound I states at different pH values were followed at 401 nm, the isosbestic point between compounds I and III.[8] In a typical experiment, one syringe contained 1 μM ferric enzyme in 0.2 M ionic strength buffer (from pH 3.0 to 11.0), and the other contained various concentrations of H_2O_2 in aqueous solution. Pseudo-first-order rate constants (k_{obs}) were determined using an exponential curve-fitting program and then plotted against H_2O_2 concentration.

Spectrophotometry. The detection of enzyme intermediates and the characterization of the final state of the enzyme after the reaction of ferric enzyme with H_2O_2 (at 10 mM ionic strength buffer) was done by spectrophotometry. Transient and final spectra from rapid-scan stopped-flow experiments were deconvoluted using the SPECFIT Global Analysis computer program (Spectrum Software Associates, Chapel Hill, NC).

EPR Spectroscopy. EPR experiments for the reaction of H42L with H_2O_2 (5 mM) were carried out in phosphate buffer (10 mM, pH 7.0) using a manually operated rapid-mixing device comprising a Perspex T mixing block with two female Luers to receive two × 1 mL plastic drive syringes whose plungers were yoked together and a male Luer fitted with a stainless steel needle (15 cm, 21 gauge) for insertion to the bottom of a quartz EPR tube. A threaded front-stop located between the drive syringes allowed 0.15 mL of solution from each syringe to be rapidly injected into the EPR tube, which was then plunged into isopentane (−130 °C) precooled with liquid N_2. A minimum reaction plus freezing time of 3.0 ± 0.5 s was achieved with this simple, manually operated system.

Determination of the Stoichiometry between $ABTS^{\bullet+}$ Formation and H_2O_2 Consumption. To determine the stoichiometry between ABTS cation radical ($ABTS^{\bullet+}$) formation and H_2O_2 consumption in

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(16) The spectral properties of the enzymes used in this study are given in Rodriguez-Lopez et al.[15c] The specific activities (units mg^{-1}) were 920 for HRPC, 930 for HRPC*, 0.2 for H42L, 5.2 for R38L, and 47.7 for R38G. Activities were measured as described previously with 0.3 mM 2, 2′-azinobis(3-ethylbenzothiazolinesulfonic acid) (ABTS) and 2.5 mM H_2O_2.[15a]

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reactions with wild-type HRPC and the R38L, R38G variants, a known amount of H_2O_2 (20 μM) was oxidized in the presence of 0.2 μM HRPC at different concentrations of ABTS. Under these conditions, HRPC quickly consumed all the H_2O_2 present in the reaction medium. The concentration of the $ABTS^{\bullet+}$ radical cation formed in the reaction was calculated from the increase in absorbance at 414 nm ($\epsilon = 31\ 100\ M^{-1}\ cm^{-1}$). The slow reaction of H42L with H_2O_2 prevented an accurate determination of this stoichiometry due to the instability of the $ABTS^{\bullet+}$ radical cation product over the extended period of the assay. To avoid these problems, ascorbic acid (AH_2) was added to reduce the product radical cation $ABTS^{\bullet+}$ product back to ABTS as rapidly as it is generated by the peroxidase. The stoichiometry of the reaction between ascorbate and the ABTS radical is well known:[18]

$$2ABTS^{\bullet+} + 2AH_2 \rightarrow 2ABTS + 2AH^{\bullet+}$$

$$2AH^{\bullet} + \rightarrow AH_2 + A$$

The overall reaction is $2ABTS^{\bullet+} + AH_2 \rightarrow 2ABTS + A$, where A represents dehydroascorbic acid. A lag period (τ) is induced in the ABTS assay when AH_2 is present. Therefore, from the initial concentration of AH_2, the concentration of $ABTS^{\bullet+}$ formed at the end of the lag phase (τ) can be calculated. The $[H_2O_2]$ consumed in the reaction of H42L with ABTS ($[H_2O_2]_C$) was calculated by the difference between the initial $[H_2O_2]$ ($[H_2O_2]_0$) and that remaining at the reaction end point ($[H_2O_2]_R$). $[H_2O_2]_R$ was calculated by measuring the absorbance increase at 414 nm ($ABTS^{\bullet+}$) produced after the addition of 0.2 μM HRPC.

Results

Reaction between H42L and H_2O_2. (1) Kinetics of the Reaction. The reaction of this mutant with H_2O_2 is much slower than for HRPC*, and we have recently proposed that the redox step in compound I formation with this variant is rate-limiting with reducing substrates such as guaiacol or dopamine.[5] The reaction of H42L with H_2O_2 gave complex biphasic kinetics at neutral and acid pH. The fast phase comprised the first ~15 s of the reaction, during which well-defined products were formed (see below), followed by a slower phase (~10-min duration) during which an inactive form of the enzyme, P670 accumulated.

In our original analysis, we assigned the first process to the formation of compound I.[5] We have now reinvestigated this fast process over a wide pH range. The data were biphasic at all pH values and could be fitted to a two-exponential function $A(t) = A_A \exp(-k_A t) + A_B \exp(-k_B t)$. Under pseudo-first-order conditions with H_2O_2 in large excess, k_A increased linearly with increasing H_2O_2 concentration. The second exponential, k_B, exhibited a hyperbolic dependence on the concentration of H_2O_2. The dependencies of both k_A and k_B on H_2O_2 concentrations at both pH 7.0 and 4.0 are shown in Figure 1. Scheme 1 represents the simplest mechanism consistent with these data:

Scheme 1

$$E + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{-}H_2O_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X$$

where E is ferric H42L, $E{-}H_2O_2$ represents an enzyme$-H_2O_2$ complex, and the species X could be either compound I[5] or another enzymatic species. At neutral pH, the plot of k_B versus $[H_2O_2]$ passes through the origin (Figure 1a), and therefore, the second step must be essentially irreversible. However, at acidic pH, the plot of k_B versus $[H_2O_2]$ intercepts the ordinate axis with a value of $\sim 4.0 \times 10^{-3}\ s^{-1}$ (Figure 1b), indicative of a reversible second step in Scheme 1. The values of the rate constants

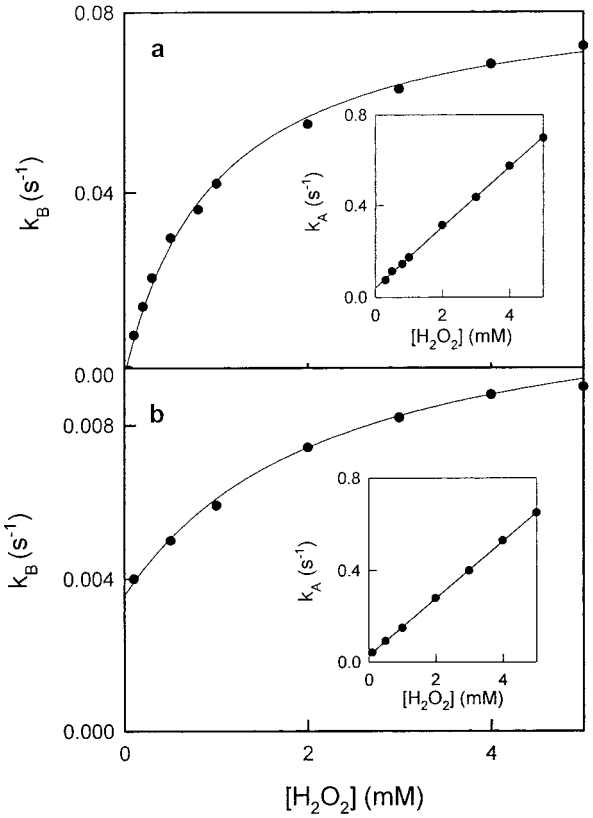

Figure 1. Dependence of apparent first-order rate constants k_A and k_B for the reaction of H42L with H_2O_2 at (a) pH 7.0 and (b) pH 4.0.

Table 1. Kinetic Parameters for the Reaction of H42L with Hydrogen Peroxide[a]

rate constant	value at pH 7.0	value at pH 4.0
k_1 ($M^{-1}\ s^{-1}$)	$(1.3 \pm 0.1) \times 10^2$	$(1.2 \pm 0.1) \times 10^2$
k_{-1} (s^{-1})	$(8.0 \pm 1.0) \times 10^{-2}$	$(3.0 \pm 0.2) \times 10^{-2}$
k_2 (s^{-1})	$(7.0 \pm 1.0) \times 10^{-2}$	$(9.0 \pm 0.7) \times 10^{-3}$
k_{-2}^{app} (s^{-1})		$(3.6 \pm 0.2) \times 10^{-3}$
k_{-2} ($M^{-1}\ s^{-1}$)[b]		$(3.6 \pm 0.2) \times 10^1$

[a] Parameters defined in Scheme 1. [b] The value of k_{-2} was calculated using $k_{-2}^{app} = k_{-2}[H+]$ (see Scheme 4).

defined in this scheme at pH 7.0 and 4.0 are given in Table 1.[19] To understand the kinetics occurring, during the first seconds of the reaction of H42L with H_2O_2 at neutral pH, we decided to characterize the kinetically identified intermediate(s) spectroscopically.

(2) Characterization of Intermediates. Rapid-scan stopped-flow spectrophotometry was used to detect transient intermediates in the reaction of H42L with H_2O_2 at pH 7.0. Mutation of the distal histidine to leucine in HRPC* results in an increase in the amount of the 6-coordinate high-spin form of the heme assigned by Howes et al.[9] using resonance Raman spectroscopy to the coordination of a water molecule. The visible spectrum of H42L was characterized by a Soret maximum at 403 nm, peaks at 504 and 643 nm, and shoulders at 372 and 530 nm

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(19) The values of k_1 and k_{-1} were calculated from the slope and the ordinate intercept of a plot of k_A versus $[H_2O_2]$, assuming $k_A = k_1[H_2O_2] + k_{-1}$. The value of k_2 at neutral pH was calculated directly from the saturation value in Figure 1a where $k_B = k_2[H_2O_2]/(K_m + [H_2O_2])$ and $K_m = (k_{-1} + k_2/k_1)$.[20] The values of k_2 and k_{-2} at pH 4.0 were calculated from a plot of k_B versus $[H_2O_2]$ (Figure 1b) with $k_B = (k_2[H_2O_2]/(K_m + [H_2O_2])) + k_{-2}$ and $K_m = (k_{-1} + k_2/k_1)$.[20] k_2 was calculated from the limiting value and k_{-2} from the ordinate intercept.

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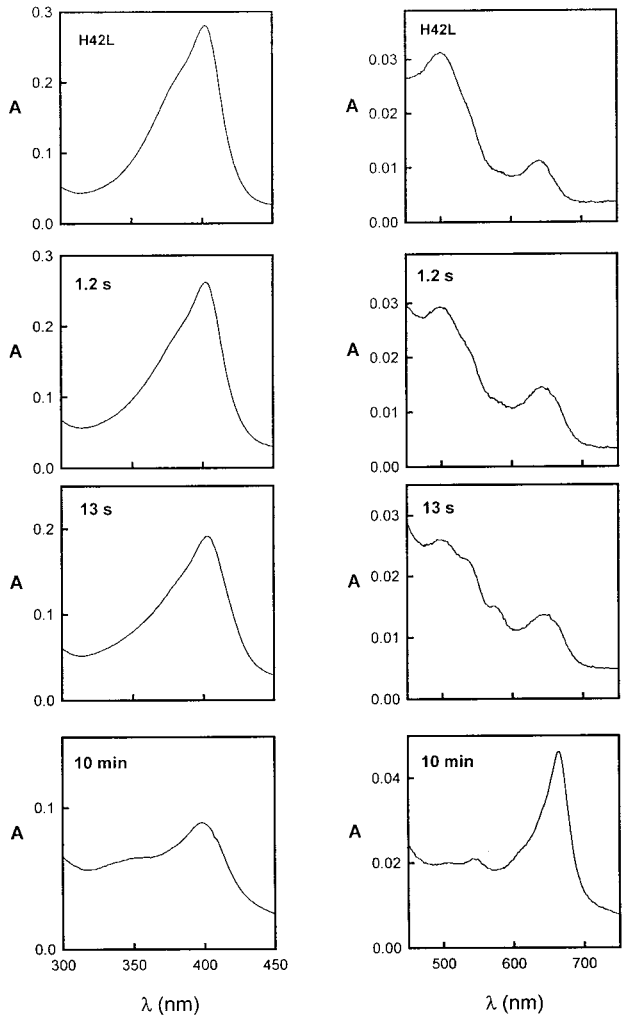

Figure 2. UV/visible spectrum of H42L (3 μM) in the Soret and visible regions, and changes observed at 1.2, 13, and 600 s during the reaction of H42L with a large excess of H_2O_2 (5.0 mM) in 10 mM phosphate buffer, pH 7.0.

(Figure 2). This figure also shows the UV/visible spectra recorded at 1.2 and 13.2 s, for the reaction of H42L with a large excess of H_2O_2 in phosphate buffer at pH 7.0. The spectra obtained at 1.2 s resembled that of the resting ferric enzyme but with peaks at 403, 490, 576, and 639 nm and shoulders at 372 and 545 nm and was similar to that observed during the reaction of the R38L mutant of HRPC with H_2O_2, which was previously assigned to a neutral peroxy−iron complex.[8] This spectrum changed with time (13.2 s) to one with peaks at 404, 495, 540, 576, and 640 nm and a shoulder at 600 nm. There was no shoulder at 372 nm, characteristic of ferric H42L, and the spectrum was similar to that of resting ferric H42L at pH 12.0 as reported by Howes et al.[9] Similar results have been obtained for H42A at pH 7.0 by Newmyer and Ortiz de Montellano.[7] These authors identified compound I as the first species formed in the reaction of H42A with excess H_2O_2 and a second species, with a compound III-like spectrum, as verdoheme. None of the spectra obtained in our reaction medium were characteristic of compound I as defined by Schonbaum and Lo.[21]

To detect and characterize any radical species formed, we monitored the reaction of H42L with H_2O_2 by EPR spectroscopy with samples frozen at defined times and run at 10 and 40 K.

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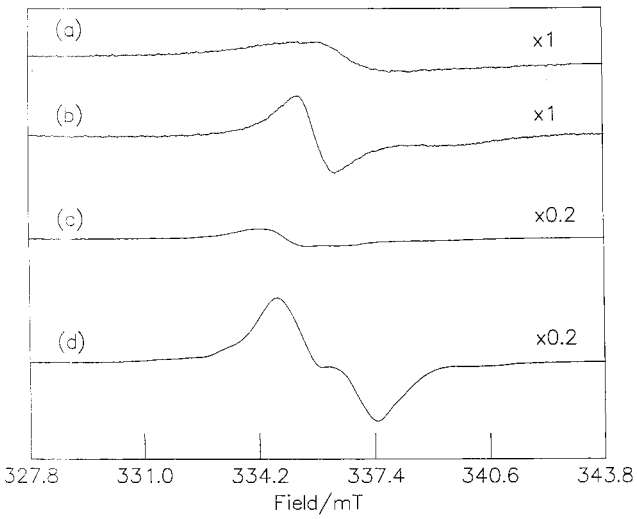

Figure 3. EPR spectra of (a) wild-type compound I formed in the dark after 2-s reaction of HRPC (25 μM) with stoichiometric H_2O_2 (25 μM); (b) the intermediate formed in the dark after 15-s reaction of H42L (23 μM) with an excess of H_2O_2 (5.0 mM) in 10 mM phosphate buffer, pH 7.0; (c) the same sample as (b) after illumination at 77 K; (d) the same sample as (a) after illumination at 77 K. EPR spectra were recorded at 10 K using 5.0-mW microwave power at 9.428 GHZ and field modulation of 0.23 mT at 100 kHz.

Wild-type HRPC with equimolar H_2O_2 showed a broad EPR signal characteristic of compound I, oxyferryl porphyrin π-cation radical (Figure 3a). The reaction of H42L with an excess of H_2O_2 showed the formation, within the first 15 s, of a sharper (peak-to-peak line width of 1.0 mT) EPR signal at $g = 2.005$. This signal must have arisen from a radical cation located more than ∼10 Å from the iron because it was relatively easily saturated and remained observable above 40 K (Figure 3b).[22a] It also did not generate significant new EPR signals on illumination at 77 K since the integrated intensity only increased by 50% (Figure 3c). In contrast, the compound I porphyrin π-cation radical was harder to saturate, could not be seen at 40 K, and generated a characteristic EPR signal on illumination at 77 K that had an integrated intensity of 500% of the nonilluminated sample (Figure 3d).[23] Therefore, the UV/visible spectrum, which resembled compound III, and the EPR data show that compound "X" in eq 1 is a novel type of compound I (compound I*) presumably with a protein residue radical cation located some distance from the iron.

H42L compound I* was reduced by ABTS ($k = 6 \times 10^5$ M^{-1} s^{-1}) at ∼20% of the rate for wild-type compound I. Addition of ascorbic acid regenerated the resting ferric enzyme. These experiments confirmed that this novel form of H42L was not compound III, consistent with it converting to a form with clear isosbestic points at 337, 450, and 539 nm. The process was accompanied by a large decrease in the Soret band intensity and an increase at 665 nm (P670) (Figure 2), both effects characteristic of irreversible inactivation of peroxidases.[24]

(3) Heterolytic versus Homolytic Peroxide Bond Cleavage by H42L. The observation of a novel protein radical in H42L could be explained by electron transfer from a protein residue

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to the porphyrin ring cation (cf. generation of the Trp radicals in C*c*P, ascorbate peroxidase (APX), and the HRPC Phe221 → Trp mutant).[22] Such a radical transfer would be facilitated by destabilization of the π-cation radical. Previous studies have proposed that high-valent porphyrin species, such as oxyferryl porphyrin π-cation radicals, are stabilized by electron donation from anionic axial ligands.[25] This also explains the correlation between the redox potential for the Fe^{2+}/Fe^{3+} couple and the stability of high-valent porphyrin species.[26] A higher redox potential is associated with decreased electron donation from the axial ligand, which leads to the destabilization of the oxyferryl π-cation radical species. However, we do not favor this explanation of our observations with H42L for two reasons. First, we have not detected by rapid-scan stopped-flow spectrophotometry or rapid-freeze EPR a species with spectroscopic features characteristic of a compound I porphyrin π-cation radical. Second, the midpoint potential for the Fe^{2+}/Fe^{3+} couple for this mutant (−280 mV)[27] is lower than for the wild-type enzyme (−230 mV), which ought to stabilize a porphyrin π-cation radical form. An attractive alternative mechanism for the formation of a protein radical cation ($R^{\bullet+}$) involves the homolytic cleavage of H_2O_2 on H42L to initially yield ferryl heme (Fe(IV)═O, compound II) and a hydroxyl radical:

$$(R)PorFe^{III} + H_2O_2 \rightarrow (R)PorFe^{IV}{=}O + HO^{\bullet} + H^+$$

$$(R)PorFe^{IV}{=}O + HO^{\bullet} + H^+ \rightarrow (R^{\bullet+})PorFe^{IV}{=}O + H_2O$$

The presence of highly reactive hydroxyl radicals in the distal cavity would be expected to produce secondary protein radical cations ($R^{\bullet+}$) of variable reactivity and stability which, due to through protein electron-transfer events, could be located at some distance from the distal cavity. Examples of this are the radical mediated hydroxylation of Trp171 in lignin peroxidase[28] and formation of the thiyl radical during autoxidation of oxyhemoglobin.[29] In the absence of a protein environment, hydroperoxides in reaction with water-soluble iron(III)−porphyrin complexes undergo homolytic cleavage of the O−O bond to yield a ferryl porphyrin π-cation radical (compound I).[30]

To check the possibility of homolytic cleavage by H42L, we introduced ABTS as a trap for intermediate species formed in the reaction and determined the ratio between $ABTS^{\bullet+}$ formation and H_2O_2 consumption. Using wild-type HRPC, this ratio was independent of ABTS concentration with a calculated value of 2.0 ± 0.2 (Figure 4), in agreement with the well-known mechanism for the oxidation of ABTS by this enzyme.[3] However, the ratio was markedly dependent on the initial concentration of ABTS in the reaction of H42L, going from a value of 2.0 ± 0.1 to a value of 1.0 ± 0.2 at ~0.7 mM ABTS (Figure 4). These data indicated nontypical behavior in the H42L mutant.

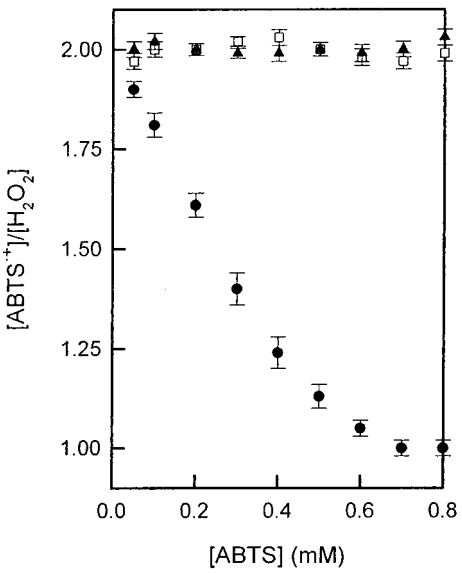


Figure 4. Stoichiometry between $ABTS^{\bullet+}$ formation and H_2O_2 consumption in the reaction of (□) HRPC*, (▲) R38L, and (●) H42L.

(4) Effect of pH on the Reaction. The pH dependence of the rate of compound I formation can be utilized to understand the role played in catalytic function by ionizable groups of the enzyme. Compound I is readily distinguished from the native form of the enzyme by its decreased absorbance in the Soret region. The pH dependence of the formation rate of compound I has been studied for several peroxidases such as lactoperoxidase, myeloperoxidase, lignin peroxidase, and HRPC.[31] These studies have indicated that there is an ionizable group (pK_a 3.0−5.3) in the distal cavity, the protonation state of which controls the rate of compound I formation. Thus, His52 in C*c*P, His47 in lignin peroxidase, and His42 in HRPC and APX have been proposed to be the distal residue.[32] We have determined the second-order-rate constant for compound I formation (k_1^{app}) as a function of pH for nonglycosylated recombinant HRPC (HRPC*). The pH dependence of k_1^{app} (Figure 5) suggested the existence of an acid−base group which, when protonated, prevents the reaction between HRPC and H_2O_2. Such a group cannot come from the substrate, H_2O_2, because its pK_a is around 12. Scheme 2 can account for this pH dependence, which leads to eq 1, where k_1 is the intrinsic or pH-independent second-order rate constant and k'_1 is the rate constant when the His42 is protonated.[33] A nonlinear least-squares analysis was performed to fit the data points in Figure 5 to eq 1. The fitting yielded a pK_E of 4.0 ± 0.2 for the acid−base group in the

$$k_1^{app} = (k_1K_E + k'_1[H^+])/(K_E + [H^+]) \qquad (1)$$

(24) (a) Bagger, S.; Williams, R. J. P. *Acta Chem. Scand.* **1971**, *25*, 976−982. (b) Nakajima, R.; Yamazaki, I. *J. Biol. Chem.* **1987**, *262*, 2576−2581. (c) Rodriguez-Lopez, J. N.; Hernandez-Ruiz, J.; Garcia-Canovas, F.; Thorneley, R. N. F.; Acosta, M.; Arnao, M. B. *J. Biol. Chem.* **1997**, *272*, 5469−5476.

(25) (a) Yamaguchi, K.; Watanabe, Y.; Morishima, I. *J. Am. Chem. Soc.* **1993**, *115*, 4058−4065. (b) Adachi, S.; Nagano, S.; Watanabe, Y.; Ishimori, K.; Morishima, I. *Biochem. Biophys. Res. Commun.* **1991**, *180*, 138−144.

(26) Gunsalus, I. C.; Meeks, J. R.; Lipscomb, J. D.; Debrunner, P.; Munk, E. *Molecular Mechanism of Oxygen Activation*; Academic Press: New York, 1974; p 559.

(27) Meunier, B.; Rodriguez-Lopez, J. N.; Smith, A. T.; Thorneley, R. N. F.; Rich, P. R. *Biochem. J.* **1998**, *330*, 303−309.

(28) Blonding, W.; Doyle, W. A.; Smith, A. T.; Winterhalter, K.; Choinowski, T.; Piontek, K. *Biochemistry* **1998**, *37*, 8832−8838.

(29) Balagopalakrishna, C.; Abugo, O.; Horsky, J.; Periakaruppan, T.; Mmanoharan, T.; Nagababu, E.; Rifkind, J. M. *Biochemistry* **1998**, *37*, 13194−13202.

(30) Almarsson, Ö.; Bruice, T. C. *J. Am. Chem. Soc.* **1995**, *117*, 4533−4544.

(31) (a) Maguire, R. J.; Dunford, H. B:; Morrison, M. *Can. J. Biochem.* **1971**, *49*, 1165−1171. (b) Bolscher, B. G. J. N.; Wever, R. *Biochim. Biophys. Acta* **1984**, *188*, 1−10. (c) Marquez, L. A.; Huang, J. T.; Dunford, H. B. *Biochemistry* **1994**, *33*, 1447−1454. (d) Glenn, J. K.; Gold, M. H. *Arch. Biochem. Biophys.* **1985**, *242*, 329−341. (e) Araiso, T.; Dunford, H. B. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1177−1182.

(32) (a) Poulos, T. L. *Adv. Inorg. Biochem.* **1987**, *7*, 1−36. (b) Edwards, S. L.; Raag, R.; Wariishi, H.; Gold, M. H.; Poulos, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 750−754. (c) Raven, E. L.; Celik, A.; Cullis, P. M.; Sangar, R.; Sutcliffe, M. J. *Biochem. Soc. Trans.* **2001**, *29*, 105−111.

(33) Initial estimates of k'_1 are difficult to obtain from our experimental data, but we assume they are similar to the rates of reaction of heme complexes with H_2O_2 (~10^2 M^{-1} s^{-1}).[34]

(34) Bruice, T. C. *Acc. Chem. Res.* **1991**, *24*, 243−249.

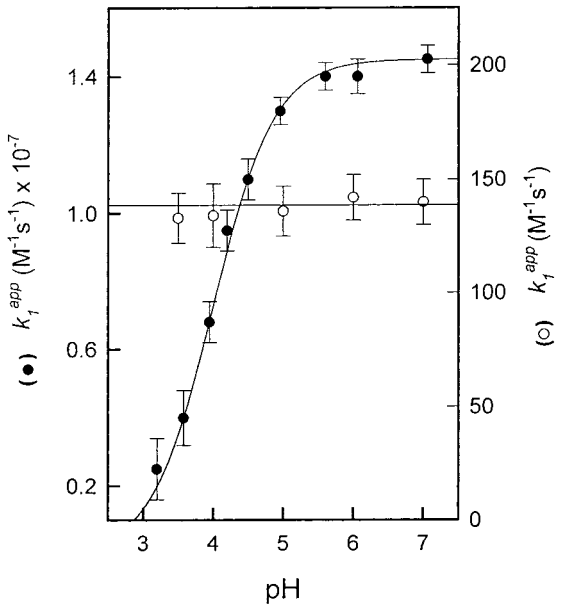
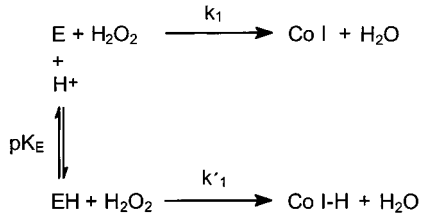


Figure 5. pH dependence of the apparent second-order rate constant of the reaction of (●) HRPC* and (○) H42L with H_2O_2.

Scheme 2



enzyme when acetate buffer was used. Within experimental error, similar results were obtained for glycosylated plant HRPC (data not shown) and are in agreement with the pK_a of 3.8 reported by Araiso and Dunford[31e] in the presence of acetate at low pH. The same study also extrapolated the effects of added electrolytes and buffers to zero showing a pK_a of 2.5.

The apparent second-order rate constant (k_1) for the reaction of H42L with H_2O_2 was only weakly dependent on pH between 3.0 and 11.0 (Figure 5). This contrasts with the marked pH dependence of k_1 for both wild-type and recombinant HRPC (Figure 5). These data provide additional evidence for a key role for His42 as an acid−base catalyst in HRPC. The effect of pH on k_2 and k_{-2} (Scheme 1) was also apparent, but it was difficult to calculate a pK_a from these data. These results suggested that the back reaction (k_{-2} in Scheme 1) was markedly dependent on the proton concentration.

Reaction of Arginine 38 Mutants with H_2O_2. (1) Kinetics. Contrary to the complex kinetics observed for H42L, Arg38 mutants exhibited much simpler behavior. The reaction between R38L and H_2O_2 to form compound I gave monoexponential absorbance versus time curves at all the pH values studied. Under pseudo-first-order conditions, with H_2O_2 in large excess, the observed first-order rate constants exhibited saturation behavior in a pH range from 5.0 to 7.5. However, at high pH, the kinetics were not hyperbolic and resembled wild-type HRPC's behavior. R38G showed similar H_2O_2 concentration dependence (Figure 6).[35] Additionally, the reactions of R38L and R38G with H_2O_2 were sensitive to both the concentration and nature of the buffer used. Thus, phosphate, borate, and Tris-HCl buffers differentially influenced k_1^{app} and k_2^{app}, whereas Mops and bicine buffers had no effect on either rate constant. The effects of phosphate concentration on k_1^{app} and k_2^{app} are shown in Figure 7.

(35) The data are consistent with biphasic kinetics with $k_A = k_2^{app}[H_2O_2]/(K_m + [H_2O_2])$, where $K_m = k_2^{app}/k_1^{app}$. Therefore, k_2^{app} is directly calculated from the limiting value, whereas k_1^{app} is calculated from the slope at low concentrations of H_2O_2.[8]

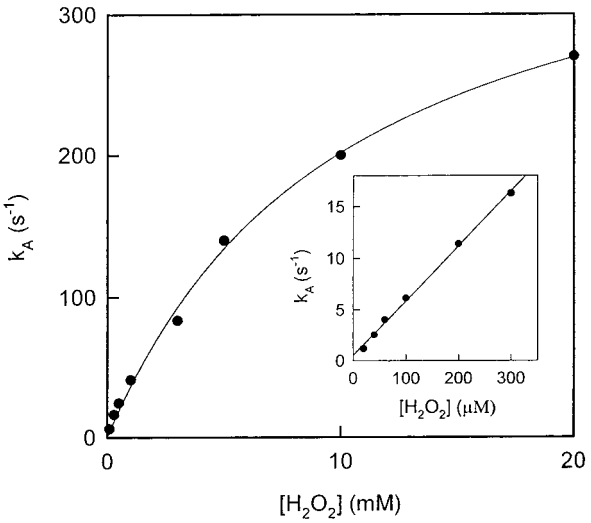


Figure 6. Dependence of k_A, the pseudo-first-order rate constant for R38G compound I formation, on the H_2O_2 concentration at pH 7.0 and 25 °C.

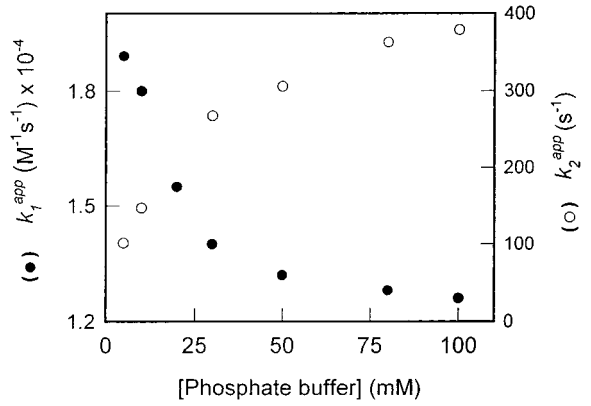


Figure 7. Effect of phosphate concentration on (●) k_1^{app} and (○) k_2^{app} in the reaction of R38L with H_2O_2 at pH 7.0 and 25 °C.

(2) Characterization of Intermediates. R38L and R38G reacted slowly with a 10-fold excess of H_2O_2 to yield a relatively stable (i.e., more stable than the wild type) compound I species (data not shown). The spectrum of this intermediate was exactly the same as that previously described for native HRPC compound I.[21] Recently, we reported the presence of a transient intermediate in the reaction of R38L with H_2O_2 at pH 7.0 with a spectrum consistent with a ferric−hydroperoxy complex.[8] The spectrum of this intermediate was reminiscent of that of resting enzyme but with peaks at 397, 487, and 580 nm and a shoulder at 530 nm. The same intermediate was detected in the reaction of R38G under the same conditions (data not shown). It has been proposed recently that Arg38 can act as a proton donor during HRPC compound I formation.[14] Therefore, the availability of HRPC Arg38 mutants gave us the opportunity to check for the existence of a peroxoanion form, like compound 0, in which HOO^- is coordinated to the heme iron. This enzymatic form is believed to have a hyperporphyrin-type spectrum.[12] Unfortunately, the reaction of R38L or R38G with high H_2O_2 concentrations (up to 50 mM) at various pHs (from 5.0 to 9.0)

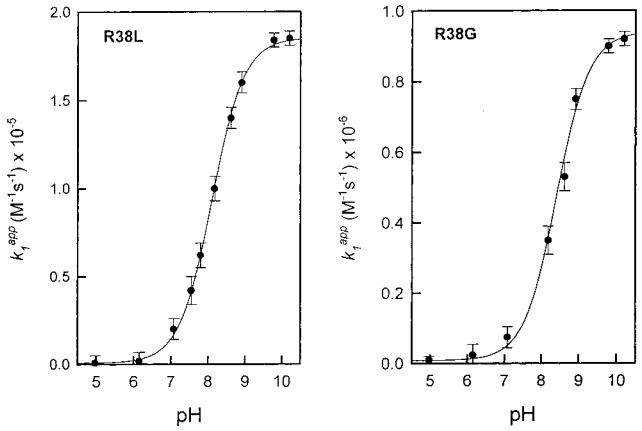


Figure 8. pH dependence of the apparent second-order rate constant (k_1^{app}) of the reaction of R38L and R38G with H_2O_2.

and temperatures (from 10 to 25 °C) did not provide any spectroscopic evidence for this type of enzyme intermediate.

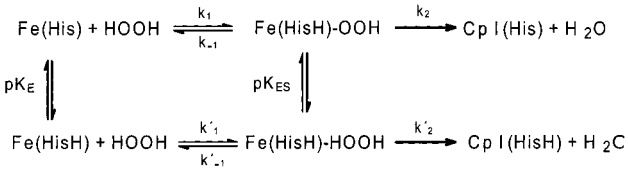
(3) Heterolytic versus Homolytic Peroxide Bond Cleavage by Arginine 38 Mutants. It has been reported that ~30% of the peroxide bond-breaking reaction in sperm whale myoglobin occurs via homolytic cleavage, compared to 100% heterolytic cleavage in peroxidases.[36] This suggests that the more polar pocket of peroxidases promotes charge separation, as is required for heterolytic cleavage, whereas in the nonpolar heme cavity of myoglobin, $OH^{\bullet}$ radical formation may be competitive with deprotonation of H_2O_2, the first step in heterolytic cleavage. To investigate the role of Arg38 in the heterolytic cleavage of HRPC we have looked for the presence of homolytic H_2O_2 cleavage in Arg38 HRPC mutants over a wide range of pH. As described above, R38L and R38G generate typical compound I species after their reaction with H_2O_2. Moreover, the presence of different concentrations of ABTS did not modify the ratio between $ABTS^{\bullet+}$ formation and H_2O_2 consumption (Figure 4). Therefore, HRPC catalyzes essentially complete heterolytic cleavage of H_2O_2 even in the absence of the polar distal arginine.

(4) Effect of pH on the Reaction. The pH dependencies of compound I formation by R38L and R38G were determined in Mops or bicine to avoid buffer effects. The value of k_1^{app} for R38L changes from a minimum value of 700 M^{-1} s^{-1} at pH 4.0 to a maximum of 1.9×10^5 M^{-1} s^{-1} at pH 10 with an apparent pK_a of 8.0 (Figure 8). k_2^{app}, when determined, also showed pH dependence, being higher at pH 7.4 ($k_2^{app} = 350$ s^{-1}) than at acid pH ($k_2^{app} \approx 100$ s^{-1} from pH 6 to 3). R38G compound I formation exhibited essentially the same pH-dependent kinetic profiles as those shown for the R38L variant (Figure 8). k_1^{app} for this mutant changes from a minimum value of 800 M^{-1} s^{-1} at pH 4.0 to a maximum of 9.1×10^5 M^{-1} s^{-1} at pH 10 with an apparent pK_a of 8.4. The simplest mechanism able to explain the pH dependence of the kinetic data is shown in Scheme 3.

Depending on the pH, H_2O_2 reacts with E, EH, or both, where the protonation state of His42 is determined by pK_E. Unprotonated His42 acts as a proton acceptor as H_2O_2 binds, and the species represented as EH−OOH in Scheme 3 has His42 protonated and a peroxide anion bound at the heme iron. The pH dependence of k_2^{app} leads us to consider two protonation states of the enzyme−peroxide complex. This complex can bind

(36) (a) Allentoff, A. J.; Bolton, J. L.; Wilks, A.; Thompson, J. A.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1992**, *114*, 9744−9749. (b) English, A. M.; Tsaprailis, G. *Adv. Inorg. Chem.* **1995**, *43*, 79−125.

Scheme 3



a proton (pK_{ES}) to generate the species EH−HOOH in Scheme 3. This enzyme peroxide complex can also be generated directly by reaction of H_2O_2 with the enzyme when His42 is in the protonated state. The rate of O−O cleavage is controlled by both k_2 and k'_2. CI and CHI represent compound I with His42 unprotonated and protonated, respectively. We assume that the ionization process is in rapid equilibrium and that the enzyme−peroxide complexes are in the steady state during compound I formation. Using the steady-state approximation, k_1^{app} has the expression described in eq 1 whereas k_2^{app} has the following expression:

$$k_2^{app} = (k_2 K_{ES} + k'_2[H^+])/(K_{ES} + [H^+]) \qquad (2)$$

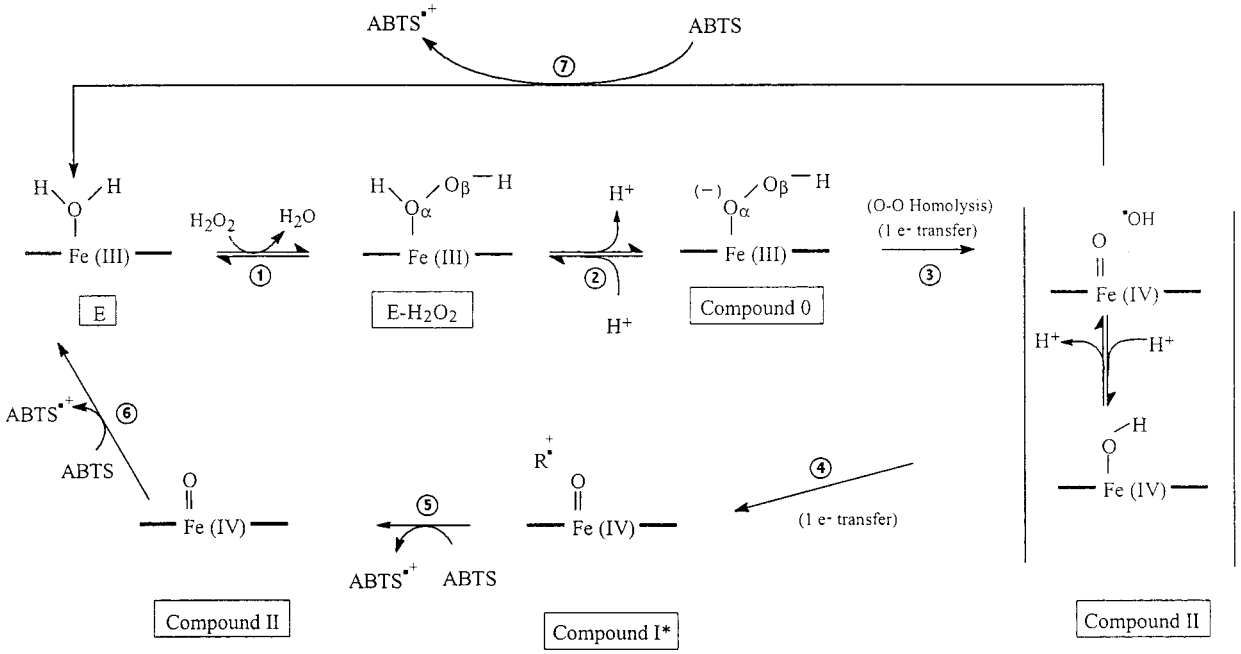
Equation 2 could be used with the pH dependence of k_2^{app} to determine the values of k_2, k'_2, and K_{ES}, where K_{ES} is related to the ionization of the bound peroxide. However, due to the lack of data for the pH dependence of k_2^{app} at pH >7.5, it was not possible to calculate the values of k_2 and K_{ES}. k'_2 was determined from the minimum value of k_2^{app} at acid pH and calculated to be around 100 s^{-1} for both R38L and R38G.

Discussion

Mechanism of H42L Compound I Formation. Substitution of His42 by leucine in HRPC* produces a profound change in the kinetics of compound I formation and allows the kinetic and spectroscopic detection of reaction intermediates. This effect could have been induced by an alteration in the three-dimensional structure of the heme cavity as a result of the introduction of the hydrophobic leucine side chain. However, similar kinetic and spectroscopic data have been obtained using the H42A mutant of HRPC[7] in which no significant structural changes were detected using ^1H NMR, suggesting that the dramatic effect on compound I formation in both these mutants is due to the lack of His42 in the distal pocket. The mechanism shown in Scheme 4 together with the rate constants in Table 1 can explain the kinetics of the reaction of H42L with H_2O_2. The apparent bimolecular rate constant (k_1^{app}) is 5 orders of magnitude slower than that for wild-type HRPC* at pH 7.0. The values for the H42L reaction with H_2O_2 are similar to those for the analogous reactions of water-soluble Fe(III)tetraphenyl−porphyrins.[34]

Different transient intermediates accumulate in the reaction medium to detectable levels at neutral pH. The spectrum of the first intermediate detected in the reaction of H42L with H_2O_2 is very similar to that observed in the reaction of R38L with H_2O_2 or R38S with dioxygen.[8,15c] This transient form of R38L has been proposed by Harris and Loew[12] to be a neutral peroxide complex. The absence of His42 would be expected to inhibit proton removal from the α-oxygen of H_2O_2, resulting in the accumulation of neutral H_2O_2−enzyme complex, E−H_2O_2 (Scheme 4). The UV/visible spectrum and EPR data indicate that H42L forms a novel type of compound I with a protein-based radical cation located more than ~10 Å from the iron (compound I* in Scheme 4). It resembles another species that has a compound III-like spectrum but with the Soret maximum

Scheme 4. Proposed Mechanism for Compound I Formation in H42L



at 404 nm. Moreover, it is also similar to the spectrum observed for H42L at alkaline pH.[9] The strong pH dependence of its rate of formation provides additional information on the rate-limiting step in its formation and justified the inclusion of a new intermediate, a peroxoanion species or compound 0, prior to the cleavage of the O−O bond of H_2O_2. The reversibility/irreversibility of its formation step can be explained if the deprotonation step governed by k_2/k_{-2} (step 2 in Scheme 4) is rate-limiting in compound I* formation. Similar conclusions were obtained for the reaction of compound I formation in a distal histidine mutant of human myoglobin.[37] k_{-2} represents the second-order rate constant for the reaction of compound 0 with protons. Thus, there is a pK_a in the E−H_2O_2 complex where $K_a = k_2/k_{-2}$ (Table 1).[38] Compound 0 does not accumulate in the reaction medium; therefore, the cleavage of the O−O bond (step 3 in Scheme 4) is not rate-limiting in this mechanism.

The presence of a protein radical in compound I* together with the radical trap experiments with ABTS favor homolytic cleavage of the O−O bond in H42L. Homolysis of H_2O_2 by H42L yields the hydroxyl radical and a ferryl heme (Fe(IV)═O; compound II) (step 3 in Scheme 4). Hydroxyl radicals would be expected to produce secondary protein radical cations ($R^{\bullet+}$) that could be located at some distance from the distal cavity (step 4 in Scheme 4). The introduction of the radical trap ABTS in the assay medium may compete with hydroxyl radical for the oxidation of compound II, explaining the change of stoichiometry between ABTS radical formation and H_2O_2 consumption (Scheme 4). The reaction of peroxides with wild-type peroxidases results exclusively in heterolytic cleavage of the dioxygen bond. In this mechanism, transfer of electrons from the prosthetic group to the oxygen is facilitated by electron release from the proximal histidine iron ligand and by proton transfer to the departing oxygen from the protonated distal histidine. Clearly, replacement of His42 by Leu in HRPC would be expected to increase the relative amount of homolytic cleavage of bound peroxide, giving a rational explanation for the formation of a novel protein radical that our EPR data places some 10 Å or more from the heme iron.

Mechanism of Compound I Formation in Arginine 38 Mutants. Kinetic data for the R38L and R38G variants are consistent with a two-step mechanism where H_2O_2 binding to the heme iron is followed by O−O cleavage with Arg38 modulating the pK_a of the Nϵ proton of His42. In the absence of Arg38, the distal histidine has a pK_a around 8.0. Below this value, His42 cannot act as a proton acceptor and compound I formation is slower. At acid pH, the lower limiting value for the rate of H_2O_2 binding is $\sim 10^2$ M^{-1} s^{-1}. A similar value was determined for the reactions between metmyoglobin or methemoglobin and H_2O_2.[39] Lack of arginine in the distal pocket of these oxygen storage/carrier proteins explains their poor peroxidase activity. When His42 is ionized at more alkaline pH, the rates of H_2O_2 binding to R38L and R38G variants increase by a factor of 10^3 but are still 2 orders of magnitude lower than for wild-type HRPC. These results strongly support our previous observation that Arg38 plays an important role in facilitating the rapid binding of H_2O_2 to HRPC. The effect of pH on k_2^{app} indicates that peroxide anion complex formation is a prerequisite for rapid O−O cleavage. Arg38 clearly modulates the pK_a of the initial enzyme−substrate complex.

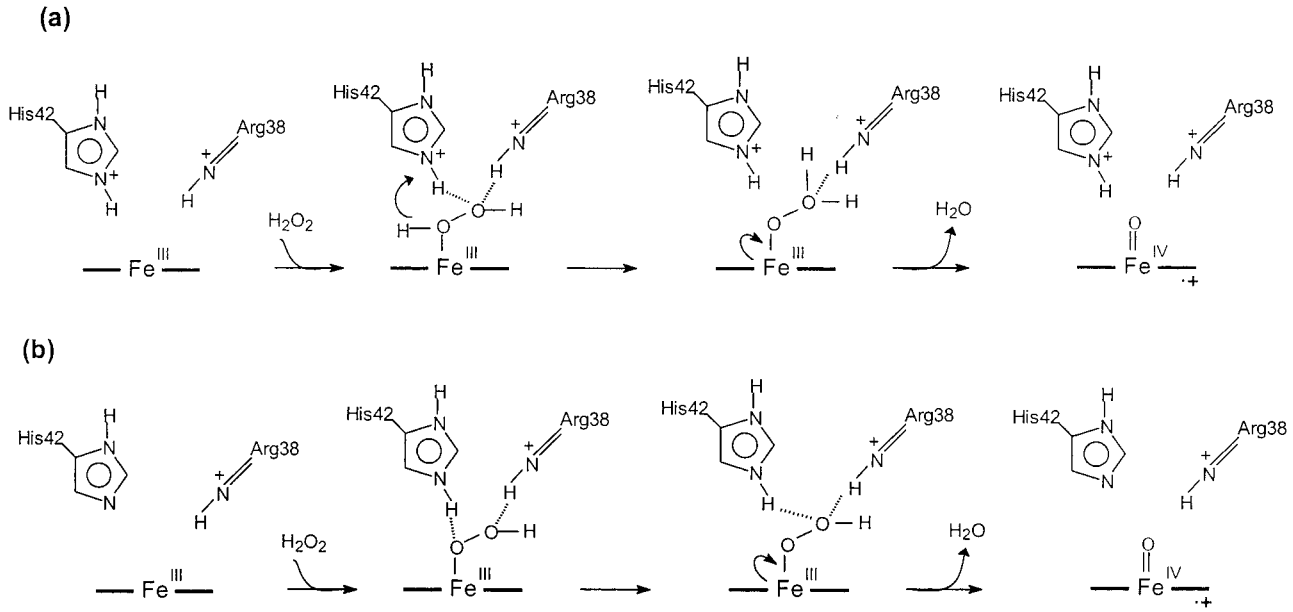
The effect of different buffer systems on the rate constants is more difficult to rationalize. Differential effects of phosphate and nitrate buffers on C*c*P have previously been observed.[4b,c] Phosphate has been proposed to bind in the distal heme pocket of C*c*P and function as a general-base catalyst.[4b] Phosphate, borate, and Tris-HCl buffers also show effects on the kinetics of Arg38 mutants of HRPC. The reaction of R38L and R38G variants with H_2O_2 (k_1^{app}) is slower in the presence of these buffers. Also, the rate of cleavage of the peroxide O−O bond increases when the concentration of these buffers is raised. An explanation for these observations is that these low molecular weight buffers can bind in the distal pocket of HRPC and

(37) Khan, K. K.; Mondal, M. S.; Padhy, L.; Mitra, S. *Eur. J. Biochem.* **1998**, *257*, 547−555.

(38) From the observed value of k_{-2}^{app} at pH 4.0 (3.6×10^{-3} s^{-1}) the value of the absolute second-order rate constant for the reaction of compound 0 with protons (k_{-2}) can be calculated ($k_{-2}^{app} = k_{-2}[H^+]$; Table 1).

(39) (a) Dalziel, K.; O'Brien, J. R. P. *Biochem. J.* **1954**, *56*, 648−659. (b) Fox, J. B., Jr.; Nicholas, R. A.; Ackerman, S. A.; Swift, C. E. *Biochemistry* **1974**, *13*, 5178−5186.

Scheme 5. Role of Distal Heme Pocket Residues of HRPC in Compound I Formation[a]



[a] Mechanism of formation of HRPC compound I at (a) acid pH (cationic His42) and (b) neutral or basic pH (neutral His42).

differentially modulate these reactions. We can only speculate that the decrease in k_1^{app} is due to an increase in the pK_a of His42 when the anionic forms of the buffers bind close to the active center or that they cause a steric/electrostatic impediment to the access of H_2O_2 to the heme. Negative charges adjacent to His42 could stabilize the protonated form of this residue. The effect on k_2^{app} can be explained if the anionic forms of these buffers act as base catalysts accepting the departing proton from oxygen α of H_2O_2. These buffer effects were not detected with wild-type HRPC*.

Factors Controlling the pK_a of His42 in Ferric HRPC. The reactions of native ferric peroxidases with peroxides are so rapid that it has only been possible to observe an ES complex directly at low temperature.[11] The pH dependence of k_1^{app} in HRPC* (Figure 5) has been explained by Scheme 2, resulting in the same rate expression as given in eq 1. With the H42L variant, k_1^{app} was found to have only a weak pH dependence in contrast to the clear pH dependence for the reaction of HRPC* or Arg38 variants with H_2O_2. These data clearly indicate that His42 is the acid−base group that, when protonated, inhibits the reaction between HRPC and H_2O_2. Several residues in the distal pocket of HRPC play an important role in maintaining the basicity of His42. Thus, Asn70 modulates acid−base catalysis by His42 through the hydrogen bond between its side-chain oxygen and the Nδ nitrogen atom of His42.[10a] We have also shown that Arg38 modulates the pK_a of His42 in the carbon monoxide complex of HRPC.[40] A large change of pK_a was observed in an HRPC* mutant in which His42 had been changed to arginine.[9] The formation of a heme−hydroxyl complex at pH 7.0 with this variant suggested a lowering of the pK_a of the introduced Arg residue (Arg42), from its intrinsic value of 12, due to the adjacent Arg38. By analogy, it is reasonable to propose that Arg38 controls the pK_a of His42 in HRPC. The decrease in the pK_a of His42 in native HRPC to a value of 3.8 in acetate buffer, or 2.5 taking into account buffer effects, is attributed to electrostatic destabilization of the histidine imidazole group by the adjacent positive charge on Arg38. In the mutants in which Arg38 is changed, the pK_a value of His42 was increased by up to four pH units (Figure 8).

Mechanism of Formation of HRPC Compound I at Acid pH (Cationic His42). Recently, a more important role has been suggested for Arg38 in HRPC compound I formation at low pH than for His42 in it cationic form.[12] Thus, at acid pH, with the distal His protonated in its cationic form, two possible mechanisms of formation of HRPC compound I have been suggested. The first involves a single peroxide-bound complex. Either His42 or Arg38 can act as the proton donor to the distal peroxide oxygen atom (Oβ), while a stable bound water H_2O_{400} can act as the proton acceptor from the heme ligand peroxide oxygen (Oα). The second possible mechanism involves a dynamically interchanging end-on peroxide ligand with Arg38 acting as the proton donor and the same water H_2O_{400} as the proton acceptor. The construction of HRPC distal heme pocket mutants in which the reaction with H_2O_2 is much slower has permitted the detection of new intermediates and a better understanding of the mechanism of compound I formation. The data presented here are not consistent with Arg38 being a proton donor to H_2O_2 at any pH value examined. Thus, HRPC Arg38 mutants showed behavior similar to the wild-type enzyme at alkaline pH with respect to H_2O_2 binding and transformation. The drastic effect on activity in Arg38 mutants at neutral or acid pH can be explained by the protonation of the distal His42 at these pH values (pK_a = 8.0). In fact, the reaction mechanism of Arg38 mutants resembles that of wild-type HRPC at acid pH (cationic His42). If Arg38 was the proton donor in the mechanism, an increase would be expected in the relative amount of homolytic cleavage of bound peroxide. Arg38 mutants gave normal compound I at all pHs studied, and no homolytic cleavage was detected. Therefore, we favor a mechanism similar to that proposed by Filizola and Loew[14] for HRPC compound I formation at acid pH but in which His42 is both proton donor and acceptor. The mechanism is described in Scheme 5a and involves the donation of a proton from the cationic His42 (acid catalyst) to the peroxide β-oxygen and the further abstraction of a proton from the α-oxygen by the same His residue (base catalyst). This process ultimately leads to the formation of the oxywater complex and facile O−O bond

(40) (a) Rodriguez-Lopez, J. N.; George, S. J.; Thorneley, R. N. F. *J. Biol. Inorg. Chem.* **1998**, *3*, 44−52. (b) Feis, A.; Rodriguez-Lopez, J. N.; Thorneley, R. N. F.; Smulevich, G. *Biochemistry* **1998**, *37*, 13575−13581.

cleavage. Although crystallographic water molecules could be involved in the process, the alkaline pK_a of the Fe(III)−H_2O_2 complex in Arg38 mutants of HRPC (similar to the pK_a of His42 in these mutants) suggests that His42 acts as an acid−base catalyst in this reaction.

Mechanism of Formation of HRPC Compound I at Neutral or Basic pH (Neutral His42). While there is evidence for a peroxide-bound peroxidase intermediate, the mechanism by which it forms an oxywater species, leading to compound I formation, and the roles of nearby amino acids in facilitating this transformation are not fully understood. In the most widely accepted mechanism, it is hypothesized that the conserved distal histidine plays the principal role as both acceptor of the proton from one oxygen and donor to the other, with the distal arginine facilitating the cleavage of the O−O bond in the resulting oxywater species. Although this mechanism has been supported by hybrid density functional theory results,[41] molecular dynamics simulations of the HRP−H_2O_2 complex do not support it.[14] A mechanism has therefore been proposed in which neutral His42 acts as a base accepting a proton from the peroxide Oα while Arg38 is the acid catalyst donating a proton to Oβ. We do not favor this mechanism for the following reasons: (i) if Arg38 was the critical residue for rapid heterolytic cleavage, it should be possible to detect the hyperporphyrin spectrum of Fe−OOH in the reaction of Arg38 mutants with H_2O_2; (ii) the increase in homolytic cleavage of the O−O bond in H42L supports His42, and not Arg38, being the proton donor in the reaction. Therefore, we favor the classical mechanism of compound I formation at neutral or basic pH in which His42 is the acid−base catalyst in the reaction. The mechanism is shown in Scheme 5b and is consistent with the spectroscopic and kinetic evidence and with the recent X-ray structure of HRPC*.[6] We have shown that compound I formation involves the binding of the neutral form of H_2O_2 to the ferric enzyme. Thus, HRPC, like C*c*P, binds neutral ligands in preference to their anionic forms.[42] Clearly His42 and Arg38 have critical roles in the binding/orientation/activation of H_2O_2 in the distal pocket of ferric HRPC. Arg38 has multiple functions: (i) it modulates the ionization state of His42 by decreasing the pK_a of the imidazole Nδ2 to a value close to 4; (ii) through electrostatic and hydrogen-bonding interactions involving His42 and the incoming H_2O_2, the positively charged guanidinium side chain of Arg38 induces polarization of the oxygen−oxygen bond to promote nucleophilic attack at the heme and subsequent heterolytic cleavage. The pronounced decrease in the rate of H_2O_2 binding to H42L is explained by a stringent requirement for hydrogen bond formation between His42 and the Oα proton of H_2O_2. Proton transfer from bound H_2O_2 to the His42 is proposed to be the second step in the reaction sequence. The pK_a of H_2O_2 is .∼12, and binding of peroxide to the heme iron promotes ionization of peroxide, while synergistically, interactions with the positively charged Arg38 and His42 decrease the pK_a to a value close to 4.0. An apparent pK_a of 4.0 has been estimated for the ionization of heme-bound H_2O_2 in C*c*P.[4b] This pK_a is similar to that calculated for the E−H_2O_2 complex in the H42L mutant of HRPC (pK_a = 3.6; Table 1) but is lower than that of the predicted for the ES complex of the Arg38 mutants. These pK_a shifts demonstrate the role of Arg38 in modulating the pK_a of the heme-bound H_2O_2. Proton abstraction that yields the anionic form of the peroxide complex, designated compound 0, promotes the charge separation necessary for heterolytic cleavage of the O−O bond and proton transfer from His42 to Oβ. It is likely that other distal and proximal residues in HRPC such as Asn70, His143, or Asp147 also contribute to facile compound I formation. The present studies clearly show that His42 and Arg38 are critical for rapid formation of this key intermediate in HRPC, as originally suggested by Poulos and Kraut.[4a]

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