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Piotr J. Mak

Marquette University, piotr.mak@marquette.edu

Warut Thammawichai

Marquette University

Dennis Wiedenhoeft

Marquette University

James R. Kincaid

Marquette University, james.kincaid@marquette.edu

# Resonance Raman Spectroscopy Reveals pH-Dependent Active Site Structural Changes of Lactoperoxidase Compound o and oIts Ferryl Heme O–O Bond Cleavage Products

Piotr J. Mak

*Department of Chemistry, Marquette University,  
Milwaukee, WI*

Warut Thammawichai

*Department of Chemistry, Marquette University,  
Milwaukee, WI*

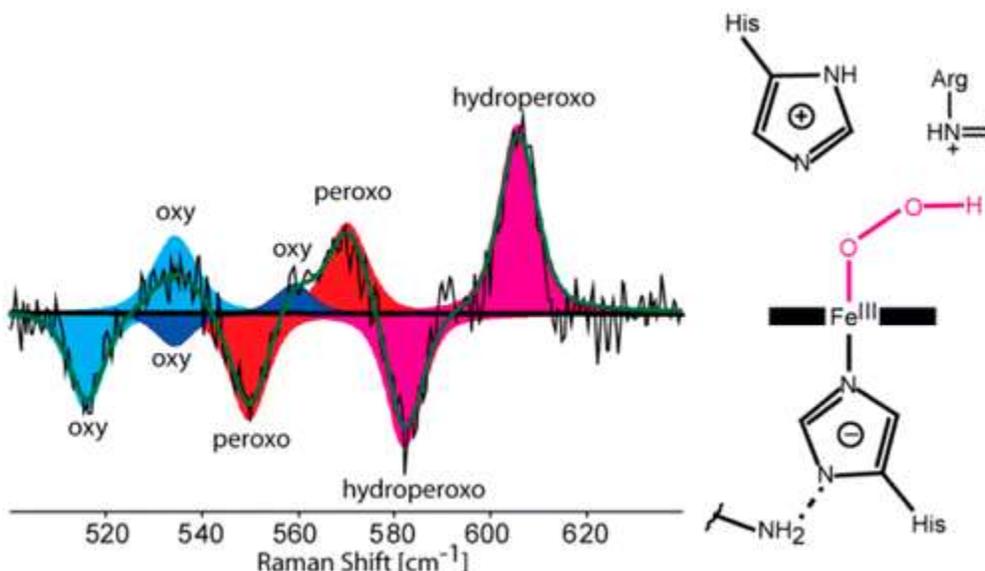
Dennis Wiedenhoeft

*Department of Chemistry, Marquette University,  
Milwaukee, WI*

James R. Kincaid

*Department of Chemistry, Marquette University,  
Milwaukee, WI*

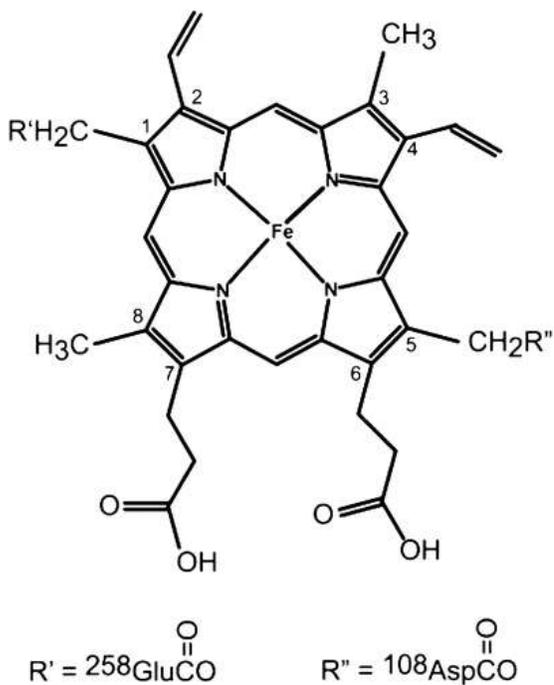
## Abstract



The first step in the enzymatic cycle of mammalian peroxidases, including lactoperoxidase (LPO), is binding of hydrogen peroxide to the ferric resting state to form a ferric-hydroperoxo intermediate designated as Compound 0, the residual proton temporarily associating with the distal pocket His109 residue. Upon delivery of this "stored" proton to the hydroperoxo fragment, it rapidly undergoes O–O bond cleavage, thereby thwarting efforts to trap it using rapid mixing methods. Fortunately, as shown herein, both the peroxy and the hydroperoxy (Compound 0) forms of LPO can be trapped by cryoradiolysis, with acquisition of their resonance Raman (rR) spectra now permitting structural characterization of their key Fe–O–O fragments. Studies were conducted under both acidic and alkaline conditions, revealing pH-dependent differences in relative populations of these intermediates. Furthermore, upon annealing, the low pH samples convert to two forms of a ferryl heme O–O bond-cleavage product, whose  $\nu(\text{Fe}=\text{O})$  frequencies reflect substantially different Fe=O bond strengths. In the process of conducting these studies, rR structural characterization of the dioxygen adduct of LPO, commonly called Compound III, has also been completed, demonstrating a substantial difference in the strengths of the Fe–O linkage of the Fe–O–O fragment under acidic and alkaline conditions, an effect most reasonably attributed to a corresponding weakening of the trans-axial histidyl imidazole linkage at lower pH. Collectively, these new results provide important insight into the impact of pH on the disposition of the key Fe–O–O and Fe=O fragments of intermediates that arise in the enzymatic cycles of LPO, other mammalian peroxidases, and related proteins.

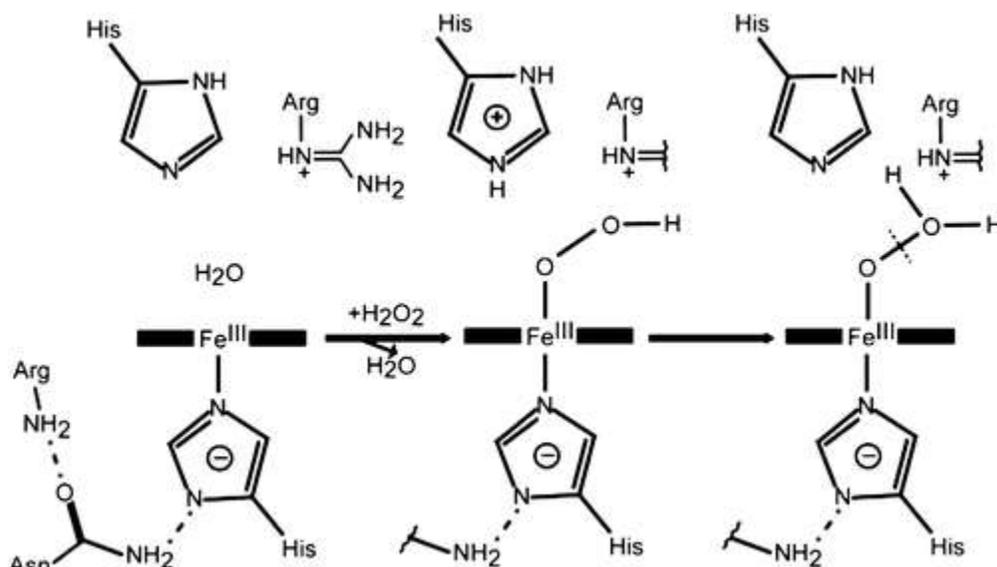
## Introduction

Lactoperoxidase (LPO), a secretory glycohemoprotein that belongs to the mammalian peroxidase superfamily, plays an important role in the innate immune defense system by generating the antimicrobial hypothiocyanate ion via oxidation of thiocyanate, employing endogenous hydrogen peroxide as the primary oxidant.<sup>1,2</sup> Similar to other mammalian peroxidases and some other heme proteins, including cytochrome c,<sup>3</sup> the heme group in this enzyme is covalently linked to the protein matrix. Recent crystallographic data of buffalo and bovine LPOs,<sup>4,5</sup> as well as previous magnetic circular dichroism<sup>6</sup> and NMR studies,<sup>7</sup> show that the prosthetic group of LPO is a modified derivative of protoheme, known as heme I, which bears ester linkages formed between two hydroxymethyl groups of heme I at positions 1 and 5 and the protein side chains of glutamate-258 (Glu258) and aspartate-108 (Asp108), respectively (Figure 1).<sup>4-7</sup> These covalent linkages, present in LPO and other mammalian peroxidases, serve to mediate protein-induced strain that results in an unusual degree of out-of-plane distortion of the heme prosthetic groups,<sup>4,5</sup> evidence for which had been obtained in earlier spectroscopic studies.<sup>9,10</sup>



**Figure 1.** Scheme of heme I in LPO.

The heme iron is coordinated to the imidazole fragment of the proximal histidine (His351) as the fifth ligand. Until recently, it had been accepted that the proximal histidyl imidazole residue of LPO and other mammalian peroxidases are H-bonded to a nearby, strictly conserved, Asn residue postulated to serve as an H-bond acceptor,<sup>11</sup> much as occurs for the plant proximal histidyl imidazoles that interact with a strictly conserved Asp residue, a strong H-bond acceptor.<sup>12,13</sup> However, inasmuch as the Asn of mammalian peroxidases is a much weaker H-bond acceptor, it was difficult to rationalize how such a configuration could give rise to the strong iron–nitrogen bonds documented for LPO and MPO, as measured by crystallographic distances and relatively high frequencies for the  $\nu(\text{Fe}-\text{N}_{\text{His}})$  stretching modes observed in the resonance Raman spectra.<sup>14</sup> Finally, acquisition of crucial experimental data and thoughtful analysis of related computational results led to a satisfying interpretation that invokes cooperative H-bonding interactions of a proximal side Arg-Asn-His ensemble that requires a heme-coordinated histidyl imidazolate residue, thereby reconciling the substantially elevated  $\nu(\text{Fe}-\text{N}_{\text{His}})$  frequencies (Figure 2).<sup>11</sup>



**Figure 2.** Mechanism of Compound 0 formation in LPO.

The distal side of LPO contains a relatively constrained ligand binding site<sup>4,5,8</sup> and houses additional structural features that play crucial roles in the peroxidase catalytic cycle, including the imidazole of the distal His109 ( $pK_a$  5.8)<sup>15</sup> and Arg255, the former serving as a

general acid–base catalyst needed for the required deprotonation step involved in binding of H<sub>2</sub>O<sub>2</sub> and the latter mainly acting to stabilize developing negative charge on the terminal oxygen fragment during the O–O bond cleavage process (vide infra) (Figure 2). The first step of the LPO catalytic cycle is binding of hydrogen peroxide to the ferric state to form a fleeting ferric-hydroperoxo intermediate designated as Compound 0, temporarily storing the residual proton at the deprotonated (i.e., nonionized) His109 residue (Figure 2).<sup>16-18</sup> Subsequent delivery of this stored proton to the terminal oxygen of the bound hydroperoxo fragment of LPO Compound 0 triggers rapid heterolytic cleavage of the O–O bond, releasing a water molecule and forming a two-electron oxidized reactive intermediate, with the oxidizing equivalents presumably being initially localized on the heme as a ferryl heme  $\pi$ -cation radical, a species typically referred to as Compound I in the case of plant peroxidases and other oxidative heme enzymes.<sup>1-3,16-18</sup> However, in the case of LPO and other mammalian peroxidases, this Compound I species spontaneously undergoes an internal oxidation–reduction process where the Compound I transient oxidizes an aromatic protein residue, thereby producing a ferryl heme and a protein-based radical, the latter commonly being designated as aa<sup>\*</sup>; typically the radical site involves a tyrosine or tryptophan residue.<sup>16-19</sup>

In the case of LPO and other mammalian peroxidases, this overall process occurs with such efficiency that the primary intermediate, Compound 0, has eluded definitive structural characterization until now. The problem arising is that the primary oxidant, H<sub>2</sub>O<sub>2</sub>, carries with it into the active site the proton that ultimately facilitates the O–O bond cleavage reaction, making it quite difficult, using rapid mixing methods, to effectively isolate Compound 0.<sup>20-22</sup> To trap and structurally characterize this key intermediate, a method is required that can generate it while also preventing delivery of the catalytic (“stored”) proton, a seemingly difficult task. However, as is demonstrated in these current studies, the cryoradiolysis approach initiated by Symons and extended by others<sup>23-25</sup> does provide an effective method to generate and trap a hydroperoxo intermediate within the LPO active site in the absence of the catalytic proton. In this way, the peroxo and/or hydroperoxo derivatives of plant and nonmammalian hydroperoxidases,<sup>26,27</sup> as well as those of bacterial and

animal cytochromes P450 and related enzymes, have been trapped and characterized by EPR and ENDOR techniques,<sup>28-31</sup> with reports of a crystallographically characterized cryoreduced form of a fungal peroxidase,<sup>32</sup> CYP101,<sup>33</sup> myoglobin,<sup>34</sup> and a few others.<sup>35,36</sup>

In brief, the essential concept involved in cryoradiolysis is to freeze-trap the relatively stable dioxygen adduct, Fe-O<sub>2</sub> [formally Fe(III)-(O-O<sup>-</sup>)], of heme proteins within an aqueous buffer containing glycerol or certain other organic compounds; in the case of LPO and other peroxidases, this species is designated as Compound III.<sup>16-18,37</sup> Upon irradiation with  $\gamma$ -rays, free-electrons and organic radicals are produced, the electrons being mobile, while other movements, including proton transport, are more restricted.<sup>25</sup> The freeze-trapped [Fe(III)-(O-O<sup>2-</sup>)] peroxy fragment is generated and can be spectroscopically characterized.<sup>28-31</sup> Careful annealing to higher temperatures permits proton transfer that, depending upon the ability of a given protein to transport protons to its active site, can lead to formation and trapping of the hydroperoxy species (Fe(III)-O-O-H), while further annealing can lead to delivery of another proton to facilitate the O-O bond cleavage and product formation, as in the case of CYP101,<sup>28</sup> or possibly to the loss of the bound hydroperoxy fragment, an example of which is documented in the case of the T252A mutant of CYP101.<sup>28</sup> In fact, this cryoradiolysis method, combined with the powerful capability of resonance Raman (rR) spectroscopy, has been recently applied to generate, trap, and structurally characterize the key Fe-O-O and Fe-O-O-H fragments of ferric peroxy and hydroperoxy derivatives of several different proteins, including human globins as well as bacterial cytochromes P450.<sup>38-42</sup>

Inasmuch as mammalian peroxidases play critical roles in human physiology in both normal and diseased states,<sup>43</sup> drawing keen interest from the pharmaceutical industry as potential drug targets,<sup>43</sup> they are naturally attractive, but as yet neglected, candidates for study by the methods described above. Responding to this opportunity, this combination of methods, hereafter referred to as rR/cryoradiolysis, is used here to generate and structurally characterize, for the first time, the peroxy and hydroperoxy derivatives of LPO under varying pH conditions and to document the nature of their O-O bond cleavage ferryl heme products, which are seen to differ

between acidic and alkaline pH. In the process, earlier studies of the rR characterization of LPO Compound III, performed at pH 7.0,<sup>44</sup> have been extended to samples buffered at pH 5.6 and 8.2, where it is now revealed that pH-dependent changes in the linkage between the heme iron and the proximal N<sub>His</sub> donor group are sufficient to account for significant pH-induced alterations in the strength of the Fe–O linkage of LPO Compound III, as directly reflected in substantial shifts of the  $\nu(\text{Fe–O})$  stretching mode. This combination of newly acquired data for the Compound III, Compound 0, and ferryl heme derivatives of LPO at varying pH values, differing in the protonation status of the key distal histidine residue, as well as differences in the proximal side “push effect” associated with the strength of the Fe–N<sub>His</sub> linkage,<sup>12,45</sup> provides useful insight into the effect of pH on the disposition of the Fe–O–O and Fe=O fragments of these species and serves to demonstrate the power of this approach for future studies of the active site structures of the catalytic intermediates of mammalian peroxidases in the presence of natural substrates and potential drug candidates.

## Experimental Methods

### *Isolation and Purification of Lactoperoxidase*

Lactoperoxidase (LPO) was isolated from unpasteurized bovine milk obtained from a local dairy farm using Amberlite CG-50 weak cation exchanger resin according to previously published procedures.<sup>46,47</sup> Isolated raw material was further purified by cation exchange chromatography (CM-52), followed by two Bio-Gel P-100 (10 mM phosphate buffer, pH 6.8) column separations to obtain LPO fractions with Rz value ( $A_{412}/A_{280}$ ) greater than 0.90.<sup>18,48</sup> The final Rz value of the enzyme was 0.92, and approximately 0.420 g of pure LPO was obtained from 60 L of unpasteurized milk. The electronic absorption spectrum (Hewlett-Packard 8452A spectrophotometer) of the LPO resting state exhibits the Soret band at 412 nm and Q-bands at 500, 543, and 590 nm, as well as a ferric-heme charge transfer band at 630 nm, in agreement with the previously published data.<sup>18,48</sup>

### *Preparation of Lactoperoxidase Intermediates*

Ferric LPO samples were prepared by equilibrating the enzyme with 50 mM phosphate or acetate buffers at pH (pD) 8.2 and 5.6,

respectively; for example, the pD was adjusted employing  $\text{pD} = \text{pH}(\text{meter reading}) + 0.4$  formula. All samples contained 20% (v/v) purified glycerol or, when needed, glycerol-(OD)<sub>3</sub>; the glycerol was purified by vacuum distillation. The final concentration of protein was 300  $\mu\text{M}$  with 0.92 Rz value. The heme concentration was calculated according to the millimolar absorptivity at the Soret maximum at 412 nm,  $114 \text{ mM}^{-1} \text{ cm}^{-1}$ .<sup>18,48</sup> Ferrous LPO samples were obtained by anaerobic reduction of ferric LPO with approximately 3–4 mol equiv of dithionite solution at room temperature, and the reduction was confirmed by electronic absorption spectrophotometry (the Q-bands of the ferrous form being at 562 and 597 nm).<sup>18</sup> The dithionite solution was prepared anaerobically and was used fresh, and its concentration was determined spectrophotometrically using  $\epsilon_{316} = 8500 \text{ mM}^{-1} \text{ cm}^{-1}$ .<sup>49</sup> The LPO compound III was prepared in NMR tubes connected to the Schlenck line by sequential application of vacuum, high purity argon, vacuum, and then gaseous oxygen (1 atm of <sup>16</sup>O<sub>2</sub> or <sup>18</sup>O<sub>2</sub>) to ferrous LPO at  $-10 \text{ }^\circ\text{C}$ , followed by vigorous mixing with a Vortex apparatus for 10 s and then rapid freezing by submersion in liquid nitrogen (77 K). Dithionite reduction is known to exhibit time-dependent spectral changes; for example, the prolonged incubation of ferrous samples results in formation of additional ferrous states.<sup>14</sup> Thus, the rR spectra of ferrous samples (pH 8.5) measured immediately after reduction exhibit a dominant  $\nu(\text{Fe}-\text{N}_{\text{His}})$  mode that decreases with time (within 60 min), and a new  $\nu(\text{Fe}-\text{N}_{\text{His}})$  band at  $221 \text{ cm}^{-1}$  appears.<sup>14</sup> In the present work, care was taken to introduce the O<sub>2</sub> and complete mixing within a few minutes, to facilitate formation of the single dioxygen adduct associated with the form exhibiting the  $\nu(\text{Fe}-\text{N}_{\text{His}})$  mode at  $254 \text{ cm}^{-1}$ ; that is, the observation of a single  $\nu(\text{Fe}-\text{O})$  mode confirms the presence of a single Compound III. The formation of LPO Compound III was confirmed by acquiring rR spectra at 77 K with the laser power of 1 mW, using the 415 nm excitation line from a Kr ion laser, that is, by inspection of the intensity of the  $\nu(\text{Fe}-\text{O})$  mode at  $534 \text{ cm}^{-1}$ .

### *The Cryoradiolytic Reduction*

The cryoradiolytic reduction of LPO Compound III samples was done by exposing the samples to 3.5 Mrad of  $\gamma$ -irradiation from a <sup>60</sup>Co source at the Notre Dame Radiation Laboratory (University of Notre

Dame, South Bend, IN). During irradiation, the samples were contained in a modified Dewar vessel and continuously immersed in the liquid nitrogen. Annealing was done by immersing a given irradiated sample into *n*-pentane/liquid nitrogen solution at a desired temperature for 60 s and then very quickly (<1 s) transferring back into the liquid nitrogen vessel. Temperatures of 170 and 180 K were chosen as appropriate for conversion of peroxo to hydroperoxo forms, based on earlier work with HRP,<sup>26</sup> while a temperature of 200 K was selected to generate the O–O bond cleavage ferryl heme product.<sup>26</sup>

## The Resonance Raman Measurements

The resonance Raman spectra of LPO Compound III and Compound 0 were acquired using a Spex 1269 spectrometer equipped with a Spec-10 LN liquid nitrogen-cooled detector (Princeton Instruments, NJ). The data were measured with the 415.4 nm excitation line from a Kr<sup>+</sup> laser (Coherent Innova Sabre Ion Laser). The spectra of irradiated samples annealed at 180 and 200 K, where the O–O bond cleavage product (ferryl heme) is formed, were measured with a 441.6 nm line provided by a He–Cd laser (IK Series He–Cd laser, Kimmon Koha CO., Ltd.), an excitation line that is appropriate for effective enhancement of this species. The rR spectra were collected using back scattering (180°) geometry with the laser beam being focused by a cylindrical lens to form a line image on the sample.<sup>50</sup> The laser power was adjusted to 1 mW or less. All measurements were done at 77 K, and the total collection time was 2 h in the high-frequency region and 2–3 h in the low-frequency region. The slit width was set at 150 μm, and the 1200 g/mm grating was used. The NMR tubes were positioned into a double-walled quartz low temperature cell filled with liquid nitrogen. The sample tubes were spun to avoid local heating. Spectra were calibrated with fenchone (Sigma-Aldrich, WI) and processed with Grams/32 AI software (Galactic Industries, Salem, NH). The difference traces in Figure 7 were deconvoluted using 50/50% Gaussian/Lorentzian functions according to the procedure described in the Supporting Information.

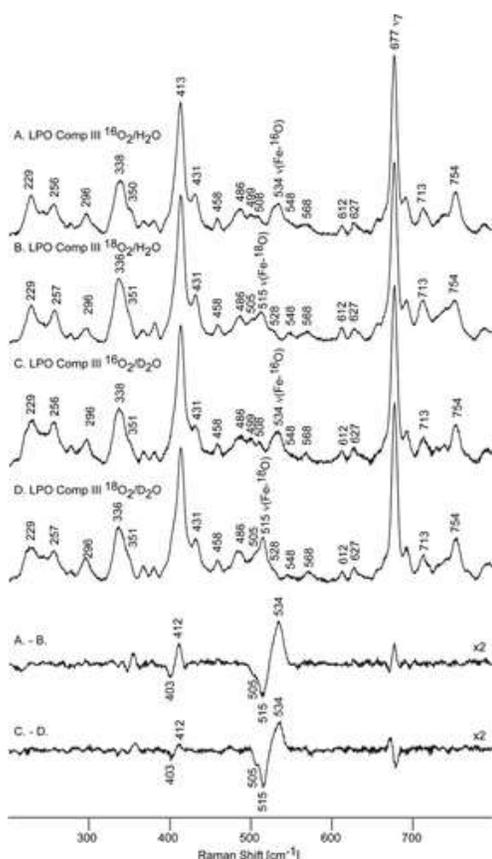
## Results

### *1LPO Compound III*

The rR spectra of the  $^{16}\text{O}_2$  and  $^{18}\text{O}_2$  adducts (Compound III) in  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  buffers, at pH 8.2 and 5.6 in the high-frequency region, where heme macrocycle modes occur, are illustrated in Figures S1 and S2 of the Supporting Information. There it is shown that no significant differences are seen in the rR spectra for these two pH values; that is, as expected for a  $\text{Fe(III)}-(\text{O}-\text{O}^-)$  formulation, in both cases the LPO Compound III is in a 6-coordinate low-spin (6cLS) ferric state with identical heme macrocycle structures. These rR spectra are in agreement with previously published data collected at  $-45\text{ }^\circ\text{C}$  with solutions buffered at pH 7.0.<sup>44</sup> It is noted that there were no  $^{16}\text{O}_2/^{18}\text{O}_2$  or  $\text{H}_2\text{O}/\text{D}_2\text{O}$ -sensitive modes detected in the high-frequency region (bottom two difference traces in Supporting Information Figures S1 and S2). This observation is consistent with the fact that the  $\nu(\text{O}-\text{O})$  stretching mode is commonly not enhanced for histidyl ligated heme protein dioxygen adducts,<sup>51</sup> although some precedent exists for unusual enhancement of these modes in cases where the  $\text{Fe}-\text{O}-\text{O}$  fragment is distorted by steric restrictions within the distal pocket (vide infra).<sup>51-53</sup>

On the other hand, the  $\nu(\text{Fe}-\text{O})$  stretching modes of the  $\text{Fe}-\text{O}-\text{O}$  fragment of oxygenated heme proteins are typically enhanced, and this behavior is confirmed for these Compound III samples as seen in Figure 3, which presents the low-frequency rR spectra of LPO Compound III at pH 8.2 and their  $^{16}\text{O}/^{18}\text{O}$  difference spectra in both  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  buffers. The most intense band at  $677\text{ cm}^{-1}$  is assigned to the  $\nu_7$  heme macrocycle mode, and the modes associated with heme peripheral groups are expected in the region of  $330\text{--}460\text{ cm}^{-1}$ .<sup>54-57</sup> Most importantly, the oxygen-sensitive modes are observed at  $534$  and  $412\text{ cm}^{-1}$  in the spectra of  $^{16}\text{O}_2$  in  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  buffers (traces A and C in Figure 3). These modes shift by  $19$  and  $9\text{ cm}^{-1}$  upon  $^{18}\text{O}_2$  substitution (traces B and D and the difference traces in Figure 3) and are assigned to the  $\nu(\text{Fe}-\text{O})$  stretching and  $\delta(\text{Fe}-\text{O}-\text{O})$  bending modes, respectively, noting that (in the absolute spectra) the latter is hidden under the intense  $413\text{ cm}^{-1}$  out-of-plane heme mode.<sup>9</sup> On the basis of the two-body harmonic oscillator approximation, the  $\nu(\text{Fe}-\text{O})$

stretching mode should exhibit a  $24\text{ cm}^{-1}$  downshift upon  $^{18}\text{O}$  substitution. The unusually small apparent  $^{16}\text{O}_2\text{-}^{18}\text{O}_2$  shift ( $19\text{ cm}^{-1}$ ) observed here has been previously explained by invoking a "Fermi-resonance type interaction"<sup>44,58-60</sup> between the  $\nu(\text{Fe-}^{18}\text{O})$  stretching mode, whose inherent frequency is at  $512\text{ cm}^{-1}$ , and the nearby  $508\text{ cm}^{-1}$  heme mode, whose inherent frequency is verified by inspection of traces A and C. The vibrational interaction between these two modes results in the upshift of the  $\nu(\text{Fe-}^{18}\text{O})$  mode by  $3\text{ cm}^{-1}$  to  $515\text{ cm}^{-1}$  and a  $3\text{ cm}^{-1}$  downshift of the  $508\text{ cm}^{-1}$  mode to  $505\text{ cm}^{-1}$ , as can be seen in traces B and D and the difference traces in Figure 3, the derived  $^{16}\text{O}_2\text{-}^{18}\text{O}_2$  shift ( $22\text{ cm}^{-1}$ ) of the  $\nu(\text{Fe-O})$  mode being satisfyingly closer to the  $24\text{ cm}^{-1}$  calculated shift.



**Figure 3.** Low-frequency rR spectra of LPO Compound III at pH 8.2,  $^{16}\text{O}_2/\text{H}_2\text{O}$  (A),  $^{18}\text{O}_2/\text{H}_2\text{O}$  (B),  $^{16}\text{O}_2/\text{D}_2\text{O}$  (C),  $^{18}\text{O}_2/\text{D}_2\text{O}$  (D), and their difference traces. Spectra measured with  $415\text{ nm}$  excitation line at  $77\text{ K}$  and normalized to the  $\nu_7$  mode at  $677\text{ cm}^{-1}$ .

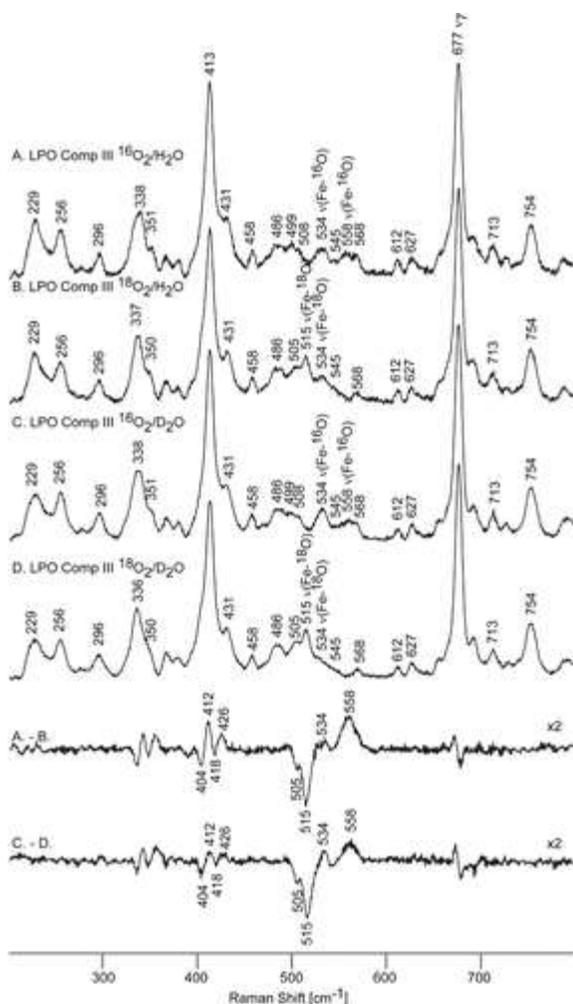
The corresponding spectra of Compound III acquired for solutions at pH 5.6 are presented in Figure 4. Although complicated,

these new spectral data can be readily understood by first noting that the presence of the 515/505 pair in trace B, along with the 534  $\text{cm}^{-1}$  peak in trace A, shows that the species present at pH 8.2 is retained at pH 5.6. However, it is noted that a second form is present, as evidenced by the new 558  $\text{cm}^{-1}$  feature seen in trace A, which disappears and apparently is observed at 534  $\text{cm}^{-1}$  for the sample prepared with  $^{18}\text{O}_2$ , as shown in trace B; that is, the 534  $\text{cm}^{-1}$  feature seen in trace A, assigned to  $\nu(\text{Fe}-^{16}\text{O})$  of the form present at pH 8.2, cannot contribute intensity near 534  $\text{cm}^{-1}$  for the samples prepared with  $^{18}\text{O}_2$ . Thus, the new form appearing at pH 5.6 exhibits a  $\nu(\text{Fe}-^{16}\text{O})$  mode at 558  $\text{cm}^{-1}$  and a  $\nu(\text{Fe}-^{18}\text{O})$  mode at 534  $\text{cm}^{-1}$ , a shift that is in good agreement with that calculated for a diatomic oscillator. It is noted that the difference patterns (two bottom traces in Figure 4) at first glance show a confusing  $\nu(\text{Fe}-\text{O})$  difference trace, where there is only one positive  $\nu(\text{Fe}-^{16}\text{O})$  stretching mode at 558  $\text{cm}^{-1}$  that is seemingly shifted to the negative 515  $\text{cm}^{-1}$  mode, giving an apparent, but quite unreasonable, 43  $\text{cm}^{-1}$  downshift. However, this apparent anomaly is easily explained by the assignments described above. Thus, the  $\nu(\text{Fe}-^{16}\text{O})$  mode of the lower frequency Fe-O-O conformer (at 534  $\text{cm}^{-1}$ ) overlaps with the  $\nu(\text{Fe}-^{18}\text{O})$  mode of the higher frequency Fe-O-O conformer (also at 534  $\text{cm}^{-1}$ ), resulting in their near cancellation; that is, the intensity of the higher frequency conformer is weaker than that of the lower frequency conformer, so that there is residual positive intensity near 534  $\text{cm}^{-1}$ . Finally, it is also noted that the difference patterns (two traces at the bottom of Figure 4) show two sets of the  $\delta(\text{Fe}-\text{O}-\text{O})$  bending modes: the 412  $\text{cm}^{-1}$  feature that shifts to 404  $\text{cm}^{-1}$  in the  $^{18}\text{O}_2$  sample is identical to that seen previously at pH 8.2 and is associated with the lower frequency Fe-O-O conformer, and a new  $\delta(\text{Fe}-\text{O}-\text{O})$  bending mode at 426  $\text{cm}^{-1}$ , with a comparable 8  $\text{cm}^{-1}$  downshift, that is associated with the higher frequency Fe-O-O conformer.

The frequency of the  $\nu(\text{Fe}-\text{O})$  mode strongly depends on the character of the heme proximal ligand, and for histidine-ligated heme proteins, like LPO and globins, there is an inverse correlation between  $\nu(\text{Fe}-\text{N}_{\text{His}})$  and  $\nu(\text{Fe}-\text{O}_2)$  modes; that is, the greater is the Fe-N<sub>His</sub> bond strength (i.e., higher  $\nu(\text{Fe}-\text{N}_{\text{His}})$  frequency), the weaker is the Fe-O bond strength (i.e., lower  $\nu(\text{Fe}-\text{O}_2)$  frequency).<sup>51,61</sup> Previous rR studies of ferrous LPO revealed that at basic pH (8.5) there are two  $\nu(\text{Fe}-\text{N}_{\text{His}})$

modes observed; that is, the dominant one is seen at  $254\text{ cm}^{-1}$  and the lower intensity one at  $221\text{ cm}^{-1}$ .<sup>14</sup> At pH 5.0, these modes had reversed intensities; the higher frequency  $254\text{ cm}^{-1}$  mode has smaller intensity, and the  $221\text{ cm}^{-1}$  mode becomes dominant.<sup>14</sup> The  $\nu(\text{Fe-O})$  stretching mode of Compound III, seen in this present work at basic pH at  $534\text{ cm}^{-1}$ , is relatively low as compared to other heme proteins that contain histidine as a proximal ligand, such as myoglobin and hemoglobin ( $\sim 572\text{ cm}^{-1}$ ); this lowering of the  $\nu(\text{Fe-O})$  frequency is entirely consistent with the more imidazolate-like character of the proximal histidine, as reflected in the observed high  $\nu(\text{Fe-N}_{\text{His}})$  frequency of  $254\text{ cm}^{-1}$ .<sup>62</sup>

In summary of this section, earlier rR studies of LPO had shown that solutions with pH values near 5 contain two forms of ferrous LPO, a dominant form having a relatively weak linkage between the heme iron and the proximal histidine, exhibiting a  $\nu(\text{Fe-N}_{\text{His}})$  frequency of  $221\text{ cm}^{-1}$ , and a less populated form possessing a relatively strong linkage, associated with a  $\nu(\text{Fe-N}_{\text{His}})$  frequency of  $254\text{ cm}^{-1}$ . At pH 8.5, the same two frequencies were observed, but having opposite relative intensities; that is, now the higher frequency mode was much stronger than that of the  $221\text{ cm}^{-1}$  feature. Consistent with those results, in the present work dealing with Compound III derivatives at these widely varying pH values, it is seen that for a low pH value of 5.6, two forms of the dioxygen adduct exist, with a dominant one exhibiting a  $\nu(\text{Fe-O})$  mode at  $558\text{ cm}^{-1}$  and a lower population of a species exhibiting its  $\nu(\text{Fe-O})$  mode at  $534\text{ cm}^{-1}$ . As in the case of the corresponding ferrous derivatives, raising the pH of the Compound III form leads to a reversal of populations, with the  $534\text{ cm}^{-1}$  species becoming dominant.



**Figure 4.** Low-frequency rR spectra of LPO Compound III at pH 5.6,  $^{16}\text{O}_2/\text{H}_2\text{O}$  (A),  $^{18}\text{O}_2/\text{H}_2\text{O}$  (B),  $^{16}\text{O}_2/\text{D}_2\text{O}$  (C),  $^{18}\text{O}_2/\text{D}_2\text{O}$  (D), and their difference traces. Spectra measured with 415 nm excitation line at 77 K and normalized to the  $\nu_7$  mode at 677  $\text{cm}^{-1}$ .

## 2LPO Compound 0

On the basis of the results of the relatively few previous rR studies of hydroperoxo derivatives of heme proteins,<sup>38,39,42</sup> the  $\nu(\text{Fe}-\text{O})$  stretching mode of these species is usually observed at  $\sim 25\text{--}40\text{ cm}^{-1}$  higher frequency than the corresponding mode of the dioxygen adduct (Compound III) [Table 1]. It is noted that, for the peroxo and hydroperoxo derivatives of globins and other histidyl-ligated heme proteins, the  $\nu(\text{O}-\text{O})$  modes are not generally enhanced. However, in the cases of the peroxo and hydroperoxo derivatives of cytochromes P450, where the trans-axial proximal ligand is thiolate and the

associated  $\nu(\text{O}-\text{O})$  modes are resonance enhanced, this mode is observed between 750 and 800  $\text{cm}^{-1}$ , a region consistent with a bound peroxy/hydroperoxy fragment. It is further noted that in recent studies of the hydroperoxy derivatives of cobalt-substituted myoglobins,<sup>41</sup> the  $\nu(\text{O}-\text{O})$  modes were also enhanced, an observation consistent with the fact that these modes are strongly enhanced in the corresponding dioxygen adducts of cobalt-substituted myoglobin and hemoglobin.<sup>59,62</sup> Upon careful inspection of the data given in Table 1 for the cytochrome P450 samples, it can be noticed that there exists an inverse correlation between the  $\nu(\text{Fe}-\text{O})$  and  $\nu(\text{O}-\text{O})$  stretching modes; that is, there is a strengthening of the Fe-O bond and attendant weakening of the O-O bond as one proceeds from the dioxygen adduct to the peroxy and then hydroperoxy forms.

**Table 1.** Summary of Frequencies and  $^{16}\text{O}_2/^{18}\text{O}_2$  and  $\text{H}_2\text{O}/\text{D}_2\text{O}$  Isotopic Shifts for the Oxy, Peroxy, and Hydroperoxy Species of Native Mb and Its Co Substituted Analogues, LPO at Basic and Acidic pH, and Cytochrome P450 and Its D251N Mutant<sup>a</sup>

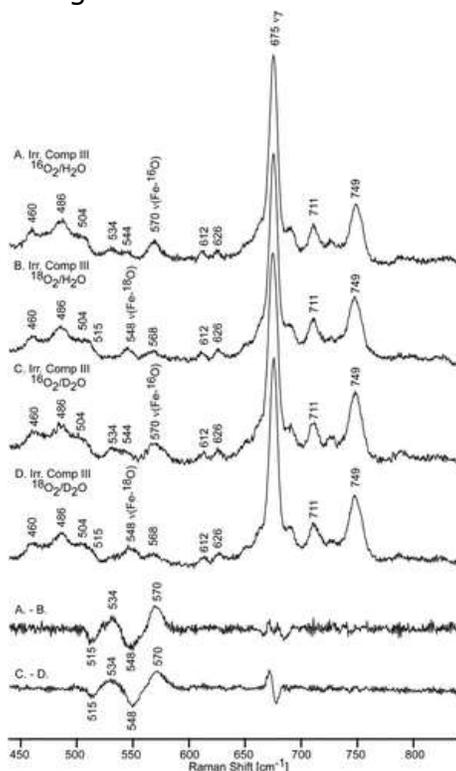
	frequency and isotopic shift [ $\text{cm}^{-1}$ ]		ref
	$\nu(\text{M}-\text{O})$	$\nu(\text{O}-\text{O})$	
	$\Delta[^{16}\text{O}_2/^{18}\text{O}_2; \text{H}_2\text{O}/\text{D}_2\text{O}]$	$\Delta[^{16}\text{O}_2/^{18}\text{O}_2; \text{H}_2\text{O}/\text{D}_2\text{O}]$	
FeMb			
oxy	578 [-29; 0]		39
peroxy	n.o.		39
hydroperoxy	617 [-25; -5]		39
CoMb			
oxy	549 [-24; 0]	1136 [-68; +2]	41
peroxy	n.o.	n.o.	41
hydroperoxy	583 [-28; -5]	851 [-45; -6]	41
LPO pH 8.2			
oxy	534 [-22; 0]		44, t.w.
peroxy	570 [-22; 0]		t.w.
hydroperoxy	n.o.		t.w.
LPO pH 5.6			
oxy	558 [-24; 0]		t.w.
peroxy	n.o.		t.w.
hydroperoxy	604 [-24; -5]		t.w.
CYP101 WT			
oxy	546 [-31; 0]	1139 [-66; 0]	42
peroxy	n.o.	n.o.	42
hydroperoxy	559 [-27; -3]	799 [-40; -3]	42
CYP101 D251N			

	frequency and isotopic shift [cm <sup>-1</sup> ]		ref
	$\nu(\text{M-O})$	$\nu(\text{O-O})$	
	$\Delta[^{16}\text{O}_2/^{18}\text{O}_2; \text{H}_2\text{O}/\text{D}_2\text{O}]$	$\Delta[^{16}\text{O}_2/^{18}\text{O}_2; \text{H}_2\text{O}/\text{D}_2\text{O}]$	
oxy	537 [-30; 0]	1136 [-66; 0] 1125 [-65; +2]	38
peroxo	553 [-27; 0]	792 [-38; 0]	38
hydroperoxo	564 [-28; -2]	774 [-37; -4]	38

<sup>a</sup>n.o., not observed. t.w., this work.

## Compound 0 at pH 8.2

Returning attention to the present work, first it is noted that, as expected, all irradiated samples are in the ferric low spin state, as judged by positions of oxidation and spin state marker bands seen in the high-frequency region (Supporting Information Figure S3). The low-frequency region of the rR spectra of the samples obtained after  $\gamma$ -ray irradiation of the Compound III samples of LPO at pH 8.2 is shown in Figure 5.



**Figure 5.** Low-frequency rR spectra of LPO irradiated Compound III at pH 8.2, <sup>16</sup>O<sub>2</sub>/H<sub>2</sub>O (A), <sup>18</sup>O<sub>2</sub>/H<sub>2</sub>O (B), <sup>16</sup>O<sub>2</sub>/D<sub>2</sub>O (C), <sup>18</sup>O<sub>2</sub>/D<sub>2</sub>O (D), and their difference traces. Spectra measured with 415 nm excitation line at 77 K and normalized to the  $\nu_7$  mode at 675 cm<sup>-1</sup>.

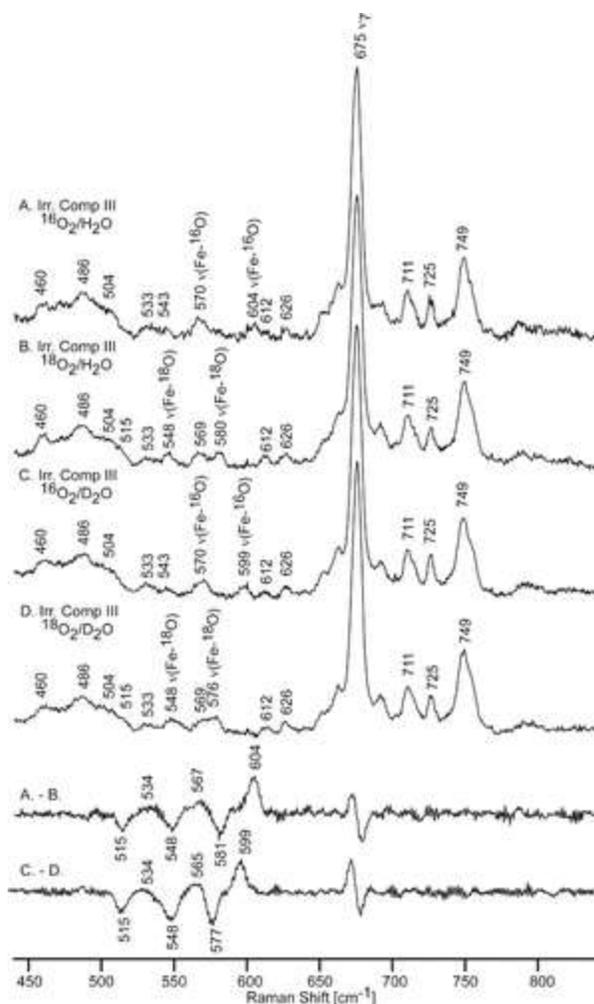
In the spectrum of the  $^{16}\text{O}_2/\text{H}_2\text{O}$  sample, a new mode is observed at  $570\text{ cm}^{-1}$  that shifts to  $548\text{ cm}^{-1}$  for the sample prepared with  $^{18}\text{O}_2$  (spectrum B), yielding a slightly small isotope shift of only  $22\text{ cm}^{-1}$ . (The experimental isotopic shifts of the  $\nu(\text{Fe}-\text{O})$  mode, being small as compared to its calculated shifts, are usually associated with coupling of the  $\nu(\text{Fe}-\text{O})$  mode with underlying heme or proximal trans axial ligand modes (as explained above for Comp III). Another possible explanation of the smaller  $^{16}\text{O}_2/^{18}\text{O}_2$  shift observed here is that it arises from a larger (i.e., more linear) bending angle of the  $\text{Fe}-\text{O}-\text{O}$  fragment; e.g., significantly larger isotopic shifts were observed for the dioxygen complex of heme oxygenase (HO) and were attributed to its rather small (heavily bent)  $\text{Fe}-\text{O}-\text{O}$  angle.<sup>63</sup>) This  $570\text{ cm}^{-1}$  value is  $36\text{ cm}^{-1}$  higher than the corresponding  $\text{Fe}-^{16}\text{O}$  stretching mode of Compound III, seen at  $534\text{ cm}^{-1}$ , a shift to higher frequency that is consistent with the behavior observed for other peroxo/hydroperoxo heme proteins listed in Table 1, thereby strongly supporting the generation and trapping of a cryoreduced  $\text{Fe}-\text{O}-\text{O}$  fragment. The samples in  $\text{D}_2\text{O}$  buffers (traces C and D) exhibit the same spectral pattern as the corresponding samples in  $\text{H}_2\text{O}$  buffer, the lack of an H/D shift prompting the assignment of this species to the ferric peroxo form. It is important to again note that, as in the case of the Compound III species discussed above, there are no  $^{16}\text{O}_2/^{18}\text{O}_2$ -sensitive modes observed in the region of  $750\text{--}800\text{ cm}^{-1}$ , showing that the  $\nu(\text{O}-\text{O})$  modes of the peroxo/hydroperoxo species are not enhanced for this protein.

The additional sets of  $^{16}\text{O}_2/^{18}\text{O}_2$ -sensitive modes at  $534\text{ cm}^{-1}$  ( $^{16}\text{O}_2$ ) and  $515\text{ cm}^{-1}$  ( $^{18}\text{O}_2$ ) in both  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  buffers (difference traces at the bottom of Figure 5) are associated with residual Compound III precursor (Figure 3), indicating incomplete conversion of Compound III to peroxo/hydroperoxo species in the cryoreduction process. If it is assumed that the relative cross sections for the  $\nu(\text{Fe}-\text{O})$  stretching modes of both Compound III and the peroxo form are the same, the analysis of the difference traces in Figure 3 would indicate that the intensities of positive and negative bands associated with peroxo form have roughly twice the intensity of the corresponding positive and negative bands associated with Compound III, implying that the cryoreduction was  $\sim 60\text{--}70\%$  effective. While this estimate is admittedly based on an unexplored assumption, it is also worth

making the important point that the precise degree of conversion does not impact the validity of the  $\nu(\text{Fe-O})$  mode assignment to the peroxy species. Attempts were made to generate the hydroperoxy species by annealing the trapped peroxy species at 170 and 180 K. At 170 K, the intensity of the band assigned to the  $\nu(\text{Fe-O})$  of the peroxy form had diminished significantly with respect to the  $534\text{ cm}^{-1}$  mode of the residual Compound III precursor, with no other  $^{18}\text{O}$ -sensitive peaks being observed. In fact, after annealing at 180 K, the mode associated with peroxy species at  $570\text{ cm}^{-1}$  had disappeared completely, and only the  $534\text{ cm}^{-1}$   $^{18}\text{O}$ -sensitive band of the residual (more stable) Compound III remained; that is, there was no conversion to the hydroperoxy form upon annealing to 180 K, the eventual fate of this ferric peroxy species being discussed later.

### *Compound 0 at pH 5.6*

Having completed an rR spectral characterization of the active site structure of peroxy LPO at elevated pH (8.2), attention was focused on samples prepared under acidic conditions. Before proceeding to discussion of the key features in the low-frequency region, it is first noted that the high-frequency spectral region indicates that all species present in the irradiated and subsequently annealed samples are, as expected, in the ferric low spin state (Supporting Information Figures S3 and S4). Now, focusing on the spectral region wherein the vibrational modes of the Fe-O-O fragment appear, Figure 6 displays rR spectra of irradiated LPO Compound III samples prepared at pH 5.6. Only the stronger intensity bands sensitive to the  $^{16}\text{O}_2/^{18}\text{O}_2$  substitution can be observed in the absolute spectra. Spectrum A shows two  $^{16}\text{O}_2/^{18}\text{O}_2$ -sensitive features appearing at  $570$  and  $604\text{ cm}^{-1}$  in the spectra of the  $^{16}\text{O}_2$  adducts that shift upon employing  $^{18}\text{O}_2$  to  $548$  and  $580\text{ cm}^{-1}$ , respectively (spectrum B). It is noted that only the  $604\text{ cm}^{-1}/580\text{ cm}^{-1}$  pair of bands is sensitive to H/D exchange (spectra C and D as well as the difference traces A-B vs C-D). However, in addition to these two more obvious pairs of features, the two difference traces of Figure 6 (bottom) show evidence for the presence of other components in the difference patterns, occurring near  $534\text{ cm}^{-1}/515\text{ cm}^{-1}$ .



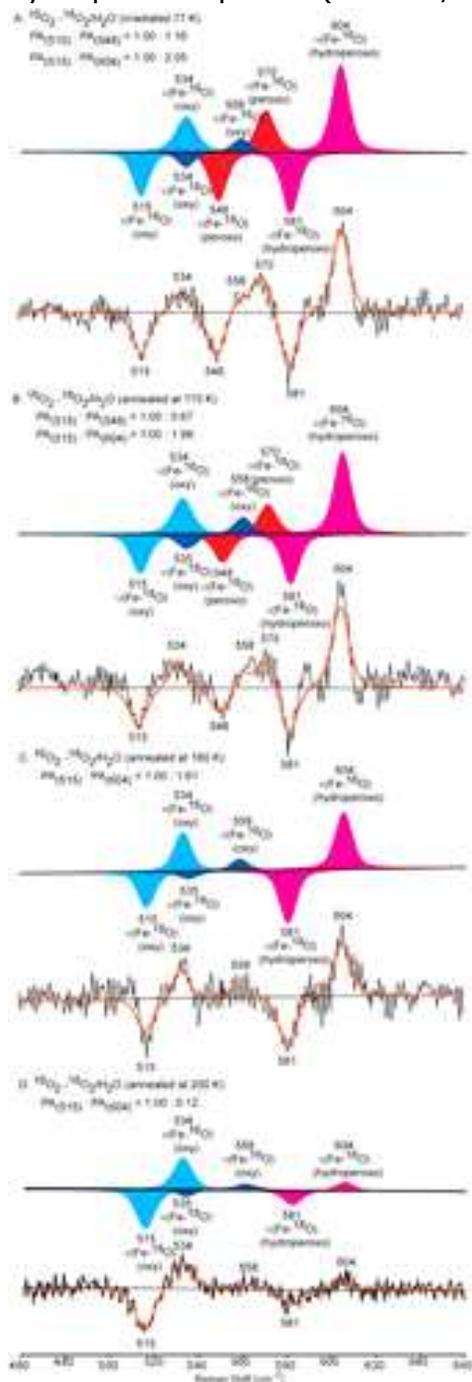
**Figure 6.** Low-frequency rR spectra of LPO irradiated Compound III at pH 5.6,  $^{16}\text{O}_2/\text{H}_2\text{O}$  (A),  $^{18}\text{O}_2/\text{H}_2\text{O}$  (B),  $^{16}\text{O}_2/\text{D}_2\text{O}$  (C),  $^{18}\text{O}_2/\text{D}_2\text{O}$  (D), and their difference traces. Spectra measured with 415 nm excitation line at 77 K and normalized to the  $\nu_7$  mode at  $675\text{ cm}^{-1}$ .

These important new, but complex, difference traces are most effectively analyzed with the aid of simulated spectra, as shown in the upper (A) part of Figure 7. The top pattern in the figure exhibits the sets of ( $^{16}\text{O}_2/^{18}\text{O}_2$ ) paired bands that were needed to produce an overall difference pattern that closely matched the experimental; the experimental and simulated (orange) differences traces are overlaid immediately below the difference trace of simulated components (top trace). While three pairs of bands (red, cyan, and magenta) are obviously present, it is noted that a fourth pair of weak bands (dark blue pair) was required to maximize the fit between simulated and observed difference patterns. All of the derived frequencies are listed

in Table 1. Referring to the top set of the derived difference patterns, the highest energy pair exhibits  $^{16}\text{O}_2/^{18}\text{O}_2$  components at 604/581  $\text{cm}^{-1}$  (magenta). As seen in Figure 6, this pair exhibits an H/D shift of 4–5  $\text{cm}^{-1}$  (i.e., shifting to 599/577  $\text{cm}^{-1}$ ), consistent with its assignment to hydroperoxo-LPO. The pair of bands seen at 570/548  $\text{cm}^{-1}$  (red) is consistent with assignment to peroxo-LPO, exhibiting the same frequencies seen in the cryoreduced samples measured at pH 8.2 with no H/D sensitivity (vide supra). The other two pairs of bands detected, both associated with LPO Compound III, include the weak difference pattern with derived frequencies of 558/534  $\text{cm}^{-1}$  (dark blue) and the lowest energy pair having frequencies of 534/515  $\text{cm}^{-1}$  (cyan), the last pair corresponding precisely to that seen for LPO Compound III at pH 8.2; it is again noted that the derived  $\nu(\text{Fe}-^{18}\text{O})$  mode is the result of the fitting procedure matching the observed 515  $\text{cm}^{-1}$  peak, but it must be recalled that the inherent frequency of this mode is  $\sim 512 \text{ cm}^{-1}$ , its observed frequency being 515  $\text{cm}^{-1}$  due to the interaction with a heme mode whose inherent frequency is close to 508  $\text{cm}^{-1}$ .<sup>44,59</sup>

The relative stability of the various forms can be effectively determined by conducting controlled annealing experiments whereby the temperature is raised from 77 K to higher temperatures, noting that the relatively stable Compound III is unlikely to be significantly affected throughout the temperature range spanned here (i.e., 77–200 K). Thus, in Figure 7, the isolated 515  $\text{cm}^{-1}$  feature of LPO- $^{18}\text{O}_2$  serves as a convenient internal intensity standard throughout the annealing excursion. Referring to the middle two traces (B) in Figure 7, focusing on the upper difference trace of the simulated components, it can be seen that upon raising the temperature from 77 to 170 K, the intensity of the peroxo-LPO mode at 570  $\text{cm}^{-1}$  diminishes substantially as compared to that of the hydroperoxo-species (at 604  $\text{cm}^{-1}$ ), the latter possessing virtually constant intensity at 77 and 170 K, relative to the 515  $\text{cm}^{-1}$  internal standard (i.e., 1.00/2.05 vs 1.00/1.98). Furthermore, upon warming to 180 K (Figure 7C), the peroxo-LPO (570  $\text{cm}^{-1}$ ) disappears completely, while the hydroperoxo-LPO form (at 604  $\text{cm}^{-1}$ ) has diminished only slightly. The important point to emphasize is that the loss of intensity of the 570  $\text{cm}^{-1}$  feature, while maintaining constant relative intensity of the 604  $\text{cm}^{-1}$  band until about 180 K, implies that the peroxo species does not convert to the hydroperoxo (Compound 0) species, but independently converts to a

different form, the nature of which is discussed below. Further annealing to 200 K results in almost complete disappearance of the hydroperoxy species (trace D, Figure 7).



**Figure 7.** Deconvoluted difference traces ( $^{16}O_2$ - $^{18}O_2$  in  $H_2O$ ) of irradiated Compound III at pH 5.6 (A), annealed at 170 K (B), annealed at 180 K (C), and annealed at 200 K

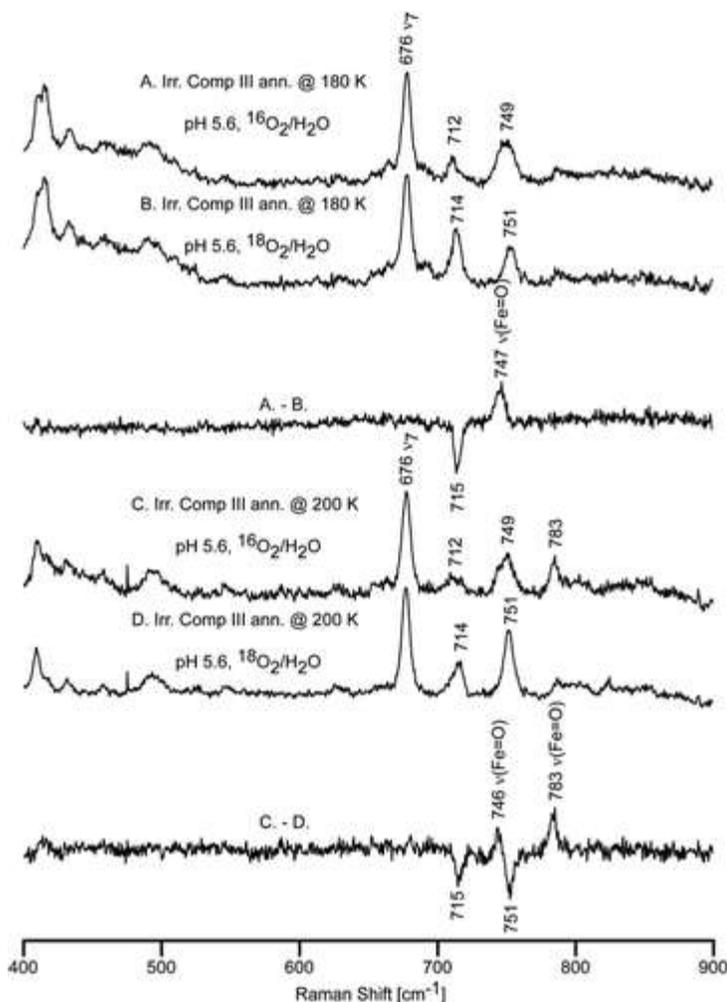
(D). The upper parts show corresponding extracted Fe-<sup>18</sup>O and Fe-<sup>16</sup>O modes associated with lower frequency Compound III form (cyan), higher frequency Compound III form (dark blue), peroxy form (red), and hydroperoxy form (magenta). The bottom traces show overlapped experimental difference traces (black) with corresponding fitted curve derived from the components shown in the upper part (orange). The PA<sub>(515)</sub> stands for peak area of mode at 515 cm<sup>-1</sup> that was used as internal standard used to calculate the changes in peak areas of modes at 548 cm<sup>-1</sup> (representing peroxy species; PA<sub>(548)</sub>) and 604 cm<sup>-1</sup> (representing hydroperoxy form; PA<sub>(604)</sub>).

Summarizing these results at pH 5.6, a lower frequency mode, seen at 570 cm<sup>-1</sup> and shifting to 548 cm<sup>-1</sup> with <sup>18</sup>O<sub>2</sub>, corresponds to the ν(Fe–O) stretching mode of the same peroxy form that arises for the sample prepared at pH 8.2, as shown earlier. The 604 cm<sup>-1</sup> mode is 46 cm<sup>-1</sup> higher than the new 558 cm<sup>-1</sup> mode of the Compound III species seen in spectrum A of Figure 4, and in D<sub>2</sub>O buffer shifts by 5 cm<sup>-1</sup> to lower frequency (599 cm<sup>-1</sup>). This significant H/D sensitivity supports assignment of this 604 cm<sup>-1</sup> feature to the ν(Fe–O) mode of a new hydroperoxy (Compound 0) species appearing at pH 5.6. As in the cases of the irradiated (pH 8.2) samples discussed earlier, although carefully checked, there are no (<sup>16</sup>O<sub>2</sub>–<sup>18</sup>O<sub>2</sub>) difference bands observed in the frequency region between ~750–800 cm<sup>-1</sup>, where the ν(O–O) stretching modes of peroxy or hydroperoxy fragments would be expected to occur. The rR spectra of the carefully annealed cryotrapped mixture of peroxy-LPO and hydroperoxy-LPO forms demonstrate that the peroxy-LPO does not convert to hydroperoxy-LPO, but decomposes independently throughout the temperature range of 170–180 K, at which point only a small percentage of the hydroperoxy-species has been lost.

*3Ferry Derivatives Arising from Controlled Annealing To Induce O–O Bond Cleavage Reactions.* Having fully documented the appearance and spectral properties of the peroxy and hydroperoxy species in the samples at both pH values, studies were then undertaken to clarify the nature of the products formed upon controlled annealing of these samples. The first sample investigated was the same (pH 5.6) sample annealed at 180 K, whose rR spectrum is shown in Figure 7C. That spectrum showed that the peroxy species (appearing at 570 cm<sup>-1</sup> at 77 K) was totally depleted at 180 K, while the (Compound 0) hydroperoxy form (604 cm<sup>-1</sup>) was almost completely retained. As can be seen in Figure 8, top trace, using the 441.6 nm excitation line, which is known to efficiently enhance Raman

lines of the ferryl species, the lost peroxy form has been converted to a ferryl heme species, whose  $747\text{ cm}^{-1}$  observed frequency (shifting to  $715\text{ cm}^{-1}$  upon  $^{18}\text{O}_2$  substitution) is entirely consistent with the previously assigned  $\nu(\text{Fe}=\text{O})$  stretching frequency of the ferryl derivative of LPO in aqueous buffer.<sup>64</sup> It is noted that this frequency is also consistent with a strong trans-axial Fe–N<sub>His</sub> linkage in its ferrous form. Thus, the peroxy form that had disappeared by 180 K and the ferryl form that had formed by 180 K are both associated with a strong trans-axial histidine linkage, that is,  $\sim 254\text{ cm}^{-1}$ .<sup>14</sup>

As is seen in the traces in the bottom half of Figure 8, upon annealing to 200 K, a new set of  $^{16}\text{O}/^{18}\text{O}$ -sensitive modes with components at  $783$  and  $\sim 748\text{ cm}^{-1}$  appears in addition to the initially formed  $747/715\text{ cm}^{-1}$  component. The difference trace shown at the bottom reveals a pattern that is consistent with partial cancellation of the  $\nu(\text{Fe}=\text{}^{18}\text{O})$  mode of the higher frequency set with the  $\nu(\text{Fe}=\text{}^{16}\text{O})$  mode of the lower frequency component. This higher frequency set of modes, with its  $\nu(\text{Fe}=\text{}^{16}\text{O})$  frequency of  $783\text{ cm}^{-1}$ , is quite consistent with that expected for a ferryl heme bearing a relatively weak trans-axial Fe–N<sub>His</sub> linkage, the species with a  $\nu(\text{Fe}=\text{N}_{\text{His}})$  frequency of  $221\text{ cm}^{-1}$  dominating the spectrum for ferrous LPO at pH = 5; that is,  $\nu(\text{Fe}=\text{}^{16}\text{O})$  values of between  $780$  and  $800\text{ cm}^{-1}$  are typically observed for heme proteins whose  $\nu(\text{Fe}=\text{N}_{\text{His}})$  varies from  $\sim 240$  to  $220\text{ cm}^{-1}$ .<sup>14</sup> Finally, it is noted that for comparable studies conducted for the isolated peroxy species trapped at pH 8.2, where there exists a paucity of mobile protons, no evidence is obtained for a ferryl species, the expected product of O–O bond cleavage. Apparently, in the absence of an adequate supply of protons, the ferric peroxy species decomposes by an as yet undetermined mechanism; further studies, employing a wider range of conditions and a larger number of resonance excitation lines, are planned for the future.



**Figure 8.** Low-frequency rR spectra of irradiated LPO Compound III (pH 5.6) annealed at 180 K  $^{16}\text{O}_2/\text{H}_2\text{O}$  (A) and  $^{18}\text{O}_2/\text{H}_2\text{O}$  (B) and annealed at 200 K  $^{16}\text{O}_2/\text{H}_2\text{O}$  (C) and  $^{18}\text{O}_2/\text{H}_2\text{O}$  (D). The  $^{16}\text{O}_2$ - $^{18}\text{O}_2$  difference traces are shown directly under absolute spectra. Spectra were measured with 441.6 nm excitation line and normalized to the  $\nu_7$  mode at  $676\text{ cm}^{-1}$ . The spectra shown here were acquired for the same samples used to generate the difference traces in Figure 7C; that is, the samples contain both the ferryl and the hydroperoxo species, but only the ferryl form is effectively in resonance with the 441.6 nm excitation line, noting that relative enhancement of heme modes may vary from derivative to derivative and with different excitation lines.

## Discussion

Faced with the difficulties encountered with rapid mixing methods to trap the elusive Compound 0 enzymatic intermediate of mammalian peroxidases for definitive structural characterization, here cryoradiolytic reduction of the trapped ferrous dioxygen adduct, Compound III, is employed to generate the peroxo- and hydroperoxo-

LPO (Compound 0) species, the relative populations of which depend on the pH. Controlled annealing procedures are used to isolate peroxo and hydroperoxo species and to induce O–O bond cleavage, producing the ferryl heme intermediates. Resonance Raman spectroscopy is used to identify and characterize the Fe–O–O and Fe–O–O–H fragments of the Compound III, peroxo, and Compound 0 intermediates, as well as the  $\nu(\text{Fe}=\text{O})$  of the ferryl heme O–O bond cleavage products. Comparison of acquired data reveals quite dramatic effects of pH on the relative strengths of the Fe–O and Fe=O bonds of these species, thereby permitting a more rational interpretation of the corresponding effects on the reactivity of these species.

### *Alternate Forms of Compound III*

As described earlier in discussing the reaction sequence depicted in Figure 2, the distal histidine His109 is typically assigned a  $\text{pK}_a$  value of 5.8–6.4,<sup>15,65</sup> implying that for the studies conducted here at pH 8.2, one would expect that the population of LPO molecules bearing a nonprotonated His109 would strongly dominate. Indeed, the rR spectral data provide clear evidence that only one Compound III form, exhibiting its  $\nu(\text{Fe}-^{16}\text{O})$  mode at  $534\text{ cm}^{-1}$ , is observed. The spectra of the samples prepared at pH 5.6, on the other hand, demonstrate that a second form of Compound III is present, exhibiting its  $\nu(\text{Fe}-^{16}\text{O})$  mode at  $558\text{ cm}^{-1}$ , signaling a significant increase in the strength of the Fe–O bond relative to the species seen at alkaline pH. While it is tempting to ascribe this behavior to H-bond donation by the protonated distal His109 residue to the Fe–O–O fragment of Compound III based on results from previously published work for dioxygen adducts of other heme proteins,<sup>38,51,66–68</sup> it is difficult to accept a suggested H-bond donor effect of this magnitude on the  $\nu(\text{Fe}-\text{O})$  mode. For example, in the case of dioxygen adducts of myoglobin, where the distal histidine was removed, only slight ( $<5\text{ cm}^{-1}$ ) shifts of the  $\nu(\text{Fe}-\text{O})$  mode were seen relative to WT Mb.<sup>51,66</sup> Similarly, comparison of a series of cytochrome P450 dioxygen adducts with varying degrees of distal side H-bonding configurations, including some with H-bond donating substrates, shows that shifts of the  $\nu(\text{Fe}-\text{O})$  mode attributable to the H-bonding interaction are typically in the neighborhood of only  $5\text{--}10\text{ cm}^{-1}$ , with an interesting and very informative detail being that the shift for H-bonding to the terminal

oxygen in the Fe–O–O fragment induces upshifts in  $\nu(\text{Fe–O})$ , while H-bonding to the proximal oxygen caused downshifts of similar magnitude.<sup>38,51,67,68</sup>

Given these reservations about ascribing the unexpectedly large positive shifts of the  $\nu(\text{Fe–O})$  mode solely to distal side interactions, further consideration was given to possible pH-induced alterations of proximal side architecture that might account for these vibrational data. Fortunately, a directly relevant and carefully conducted study on the impact of pH changes on the heme-proximal histidine linkage of LPO offers some useful insight.<sup>14</sup> In that work, Smulevich, Obinger, and co-workers show that two  $\nu(\text{Fe–N}_{\text{His}})$  stretching modes are observed, appearing at 221 and 254  $\text{cm}^{-1}$ , whose relative intensities are pH dependent, with the 254  $\text{cm}^{-1}$  feature dominating at high pH (8.5) and the 221  $\text{cm}^{-1}$  band dominating at low pH (5.0). Those authors point out that, while the protonation status of the proximal histidine undoubtedly plays a crucial role in modulating the donor strength, with an “imidazolate” formulation being applicable at alkaline pH, other factors, including reorientation of the imidazole plane with respect to the four Fe–N bonds of the heme macrocycle, brought about by structural rearrangements within the heme pocket, could also alter the strength of the Fe–N<sub>His</sub> linkage. In any case, the observed variation of the  $\nu(\text{Fe–N}_{\text{His}})$  as a function of pH is an important finding, because there exists a well-documented inverse correlation between the frequency (bond strength) of the  $\nu(\text{Fe–N}_{\text{His}})$  mode of the ferrous form and the  $\nu(\text{Fe–O})$  frequencies of various oxy forms of heme proteins.<sup>51,61</sup>



protonated His109, the influence of which on its  $\nu(\text{Fe-O})$  frequency would depend on whether the H-bond forms between the proximal or terminal oxygen atom of the Fe-O-O fragment.<sup>51 67</sup> To the extent that deoxyMb and this low-pH form of ferrous LPO both possess similar Fe-N<sub>His</sub> bond strengths, as evidenced by similar  $\nu(\text{Fe-N}_{\text{His}})$  stretching frequencies of  $\sim 221 \text{ cm}^{-1}$ , it would be expected that both would exhibit similar  $\nu(\text{Fe-O})$  frequencies in the corresponding dioxygen adducts, yet these differ by  $\sim 16 \text{ cm}^{-1}$  ( $558 \text{ cm}^{-1}$  for frozen LPO Compound III and  $574 \text{ cm}^{-1}$  for frozen oxyMb).<sup>39,41,56</sup> Given the fact that the Fe-O-O fragment of oxyMb is known to be stabilized by an H-bonding interaction between the terminal oxygen and a distal pocket histidyl imidazole group that would lead to an increase in the inherent  $\nu(\text{Fe-O})$  frequency, the  $558 \text{ cm}^{-1}$  frequency observed here for the low-pH form of LPO Compound III could suggest that the H-bonding interaction of the protonated distal His109 is either not properly oriented to interact with the terminal oxygen or may even be positioned to interact with the proximal oxygen atom of the Fe-O-O fragment, the latter situation being known to cause negative shifts of the  $\nu(\text{Fe-O})$  mode, as was clearly seen for the dioxygen adduct CYP17 in the presence of hydroxy pregnenolone.<sup>67</sup> However, although possible alignment of the His109 H-bond with the proximal oxygen of the Fe-O-O fragment is consistent with the data for this Compound III form, the data for the corresponding Compound 0 derivative formed upon cryoreduction are entirely consistent with the expected hydroperoxo LPO formulation (vide infra).

### *Characterization and Fate of Compound 0 and Peroxo LPO*

Cryoreduction of Compound III under alkaline conditions produced only peroxo LPO, with an  $\nu(\text{Fe-}^{16}\text{O})$  frequency at  $570 \text{ cm}^{-1}$  that, as expected, is not sensitive to exchange of H<sub>2</sub>O with D<sub>2</sub>O (Figure 9). This frequency shift of  $36 \text{ cm}^{-1}$  to higher frequency upon conversion of LPO Compound III to its peroxo derivative is in reasonable agreement with that expected from consideration of previously acquired rR data for globins,<sup>69</sup> where the  $\nu(\text{Fe-O})$  mode for the dioxygen adduct occurs near  $578 \text{ cm}^{-1}$  while that for the corresponding peroxo derivative occurs near  $606 \text{ cm}^{-1}$ . Interestingly, annealing of this frozen solution of trapped peroxo LPO at 180 K leads

only to loss of the  $570\text{ cm}^{-1}$  feature without producing any new  $^{16}\text{O}_2/^{18}\text{O}_2$ -sensitive features with excitation at 415 nm, thereby demonstrating that conversion to the hydroperoxo form, compound 0, does not occur under alkaline conditions; that is, in this situation, proton delivery to the heme active site is apparently quite inefficient due to the very low concentration of protons. In line with this observation is the fact that interrogation of the annealed sample using 441.6 nm excitation, where any ferryl heme would be detected, failed to yield any  $^{16}\text{O}/^{18}\text{O}$ -sensitive features. A rather extensive amount of further work will be required to clarify the fate of the lost peroxo form.

As shown in Figure 6 and described above, cryoreduction of Compound III of LPO in frozen solutions under acidic conditions at pH 5.6 produces a new feature observed at  $604\text{ cm}^{-1}$ , which shifts by  $5\text{ cm}^{-1}$  to lower frequency in solutions prepared in  $\text{D}_2\text{O}$ , a result that is consistent with its assignment to Compound 0. With the precursor (Compound III form) possessing a protonated distal His109 at pH 5.6 and a  $\nu(\text{Fe}-^{16}\text{O})$  mode at  $558\text{ cm}^{-1}$ , the resultant shift of  $46\text{ cm}^{-1}$  upon converting from Compound III to Compound 0 is reasonably consistent with the corresponding shift seen for globins, where the  $\nu(\text{Fe}-\text{O})$  mode of oxyMb shifted from  $578$  to  $617\text{ cm}^{-1}$  for hydroperoxo Mb.<sup>39-41</sup> Thus, it is seen that at pH 5.6, two distinct populations of cryoreduced species exist, peroxo and hydroperoxo, an observation consistent with two populations of each precursor; that is, ferrous LPO exhibited a dominant  $\nu(\text{Fe}-\text{N}_{\text{His}})$  mode at  $221\text{ cm}^{-1}$  and a weaker one at  $254\text{ cm}^{-1}$ , while the two populations of LPO Compound III were confirmed by a strong  $\nu(\text{Fe}-\text{O})$  mode at  $558\text{ cm}^{-1}$  and a weak one observed at  $534\text{ cm}^{-1}$ , the latter being associated with the (less populated) form having an imidazolate-like proximal histidine with a  $\nu(\text{Fe}-\text{N}_{\text{His}})$  mode of  $254\text{ cm}^{-1}$ .

Before turning attention to the fate of these peroxo and hydroperoxo forms upon further annealing, it is interesting to consider the implications of these findings under acidic conditions with respect to the issue of proton delivery to the bound peroxo-fragment. The rR spectra of the Compound III sample (Figure 4) exhibited evidence for a non-H-bonded form ( $\nu(\text{Fe}-^{16}\text{O}) = 534\text{ cm}^{-1}$ ) and an H-bonded form ( $\nu(\text{Fe}-^{16}\text{O}) = 558\text{ cm}^{-1}$ ), the source of the H-bond being most reasonably ascribed to protonated His109. Upon cryoreduction,

without further annealing above 77 K, two reduced forms are detected, a peroxo species and an H/D-sensitive hydroperoxo species. Now, on the basis of consideration of shifts typically observed in conversion from dioxy to peroxo/hydroperoxo forms, as well as the observation of the telltale shift observed upon deuteration of hydroperoxo forms, the most reasonable interpretation of the rR spectral data acquired for irradiated samples is that the resulting Compound 0 form, with a  $\nu(\text{Fe}-^{16}\text{O}) = 604 \text{ cm}^{-1}$ , is derived by direct and quite efficient proton transfer from the protonated His109 to an initially formed transient peroxo-species. This conclusion receives support from consideration of many previous cryoradiolysis studies, wherein it is generally possible to trap the peroxo forms at 77 K before accessing the hydroperoxo form by annealing at higher temperatures, the implication being that facile proton transfer at 77 K is likely to originate only from an H-bond donor group within the distal pocket. Clearly, this argument is further supported by the fact that the trapped peroxo-form in this same sample, which arose from reduction of the non-H-bonded Compound III, remained unprotonated at 77 K due to the lack of an effective active site proton donor. Although the above considerations effectively argue for protonation of the terminal oxygen atom of the Fe–O–O fragment by protonated His109, it is noted that a recently published study of a different peroxidase, cytochrome c peroxidase (ccp), employing neutron diffraction methods for the ferric and ferryl species, presents effective arguments that (in that enzyme) the protonation of the hydroperoxo species (Compound 0) involves a different distal pocket residue, probably a protonated arginine (Arg48), with the distal histidine remaining protonated following Compound I formation.<sup>70</sup> Assuming those results and arguments are essentially correct, the difference from the present work on LPO and those studies on ccp may arise from the fact that in the latter work the proton transfer being dealt with is that of the key “catalytic” proton to the actual Compound 0, while that being addressed in the present work is a proton transfer step to form a trapped Compound 0 species for structural characterization.

Having defined the nature and relative populations of the peroxo and hydroperoxo forms trapped at 77 K under acidic conditions, it remains only to consider their relative stability as reflected by their behavior observed upon controlled annealing. As seen in Figure 7,

annealing of this sample resulted in loss of the peroxo-LPO content of the sample before any substantial loss of the Compound 0. Inspection of Figure 8 provides definitive evidence that the peroxo fragment converted to a ferryl species with a  $\nu(\text{Fe}-^{16}\text{O})$  at  $747\text{ cm}^{-1}$ ; that is, both the peroxo form and the resultant ferryl form are associated with the population of LPO having a strong  $\text{Fe}-\text{N}_{\text{His}}$  bond, with a  $\nu(\text{Fe}-\text{N}_{\text{His}})$  of  $254\text{ cm}^{-1}$ . During this process, the Compound 0 form, with its weaker trans-axial histidyl ligand, does not undergo O–O bond cleavage. Indeed, the O–O bond cleavage for the hydroperoxide form associated with the weaker  $\text{Fe}-\text{N}_{\text{His}}$  bond occurs only at 200 K. The most reasonable interpretation of this behavior is that upon annealing to higher temperatures, where protons become more mobile and enter the active site, the stronger “push effect” of the imidazolate-like form substantially increases the efficiency of the O–O bond cleavage process. While the population expressing a strong push effect is depleted by 180 K, the population possessing the weaker  $\text{Fe}-\text{N}_{\text{His}}$  linkage was seen to undergo substantial O–O bond cleavage only at 200 K, the difference in O–O bond cleavage efficiency, as documented spectroscopically herein for the first time, directly reflecting the dominant influence of the “push” effect relative to the “pull” effect.<sup>71,72</sup>

Early work by Kimura and Yamazaki<sup>73</sup> confirming even earlier work by Dunford and co-workers<sup>74</sup> shows the rate of the reaction is pH independent between about 5 and 11, the rate decreasing relatively sharply below pH 5, results that are most reasonably attributed to protonation of the distal histidine, which would in turn hamper the required deprotonation of the incoming  $\text{H}_2\text{O}_2$ , presumably the rate-limiting step. While at pH values between 5 and 9, there are apparently different populations of LPO having weak and stronger  $\text{Fe}-\text{N}_{\text{His}}$  linkages (based on the rR data in ref 14), the corresponding effects on the rate of Compound I formation are small relative to the effect of distal side protonation. In the present work, to capture the peroxo/hydroperoxo intermediates, the reactive  $\text{Fe(III)}-(\text{O}-\text{O}^{2-})$  and  $\text{Fe(III)}-(\text{O}-\text{OH}^-)$  fragments are generated without permitting delivery of a “catalytic” proton. This approach permits the determination of the effects of proximal side differences on the efficiencies of O–O bond cleavage, such differences being masked in solution phase work by the slowness of the primary  $\text{H}_2\text{O}_2$  binding step. Further definition of these processes will require more highly resolved pH variations and

additional annealing temperatures (i.e., between 77 and 180 K), whereupon it is anticipated that a new hydroperoxo species will emerge, exhibiting a  $\nu(\text{Fe-O})$  frequency between 580 and 590  $\text{cm}^{-1}$ .

Finally, one of the key findings of the present work is the demonstration of an essential difference between mammalian peroxidases from their counterparts in plants. In the latter case, it is known that the strength of the Fe-N<sub>His</sub> bond is affected only slightly by variations in pH between ~5–11, as reflected in small ( $<5 \text{ cm}^{-1}$ ) changes in the  $\nu(\text{Fe-N}_{\text{His}})$  frequencies.<sup>75</sup> On the other hand, as is seen here, the pH-induced changes in the  $\nu(\text{Fe-N}_{\text{His}})$  lead to substantial changes in the strengths of the Fe-O bonds of the Compound III and all subsequent Fe-O-O and Fe=O intermediates, effects that clearly hold implications for differences in the complex reactivity patterns of these mammalian enzymes.<sup>11,12,17,18</sup>

The authors declare no competing financial interest.

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## Supporting Information

Description of the deconvolution procedure, and high-frequency spectra of Compound III and its irradiated form at basic and acidic pH. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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