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# Resonance Raman Spectroscopic Studies of Hydroperoxo Derivatives of Cobalt-substituted Myoglobin

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# Resonance Raman Spectroscopic Studies of Hydroperoxo Derivatives of Cobalt-substituted Myoglobin

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**Abstract:** Recent progress in generating and stabilizing reactive heme protein enzymatic intermediates by cryoradiolytic reduction has prompted application of a range of spectroscopic approaches to effectively interrogate these species. The impressive potential of Resonance Raman spectroscopy for characterizing such samples has been recently demonstrated in a number of studies of peroxo- and hydroperoxo-intermediates. While it is anticipated that this approach can be productively applied to the wide range of heme proteins whose reaction cycles naturally involve these peroxo- and hydroperoxo-intermediates, one limitation that sometimes arises is the lack of enhancement of the key intraligand v(O-O) stretching mode in the native systems. The present work was undertaken to explore the utility of cobalt substitution to enhance both the v(Co-O) and v(O-O) modes of the CoOOH fragments of hydroperoxo forms of heme proteins bearing a trans-axial histidine linkage. Thus, having recently completed RR studies of hydroperoxo myoglobin, attention is now turned to its cobalt-substituted analogue. Spectra are acquired for samples prepared with $^{16}O_2$ and $^{18}O_2$ to reveal the v(M-O)

*Journal of Inorganic Biochemistry*, Vol. 102, No. 10 (October 2008): pg. 1952-1957. DOI. 

and ν(O-O) modes, the latter indeed being observed only for the cobalt-substituted proteins. In addition, spectra of samples prepared in deuterated solvents were also acquired, providing definitive evidence for the presence of the hydroperoxo- species.

# Introduction

Structural characterization of reactive heme-oxygen species, including peroxo-and hydroperoxo- ferric forms, as well as the ferryl (Compound II) and ferryl π-cationic (compound I) intermediates, is an important step in gaining useful insight into the precise catalytic mechanisms of various heme-containing enzymes [1–7]. While the resonance Raman (RR) technique has proven itself to be of immense value for probing many of these species [8–16], it has been only recently that this method has been successfully applied to interrogate hydroperoxo myoglobin as well as key intermediates in the enzymatic cycle of Cytochrome P450, the peroxo- and hydroperoxo- forms [17–19]. These recent studies were inspired by pioneering work of several research groups showing that such heme protein intermediates can be efficiently generated and stabilized by radiolytic cryoreduction techniques [20–34].

Given these initial successes in acquiring RR spectra of these important species, it is anticipated that this approach can be productively applied to the wide range of heme proteins whose reaction cycles naturally involve these peroxo-hydroperoxo intermediates. However, while the studies of the cytochrome P450 intermediates, bearing a cysteine-thiolate as the trans-axial ligand, revealed effective enhancement of both the ν(Fe-O) and ν(O-O) modes [35–37], the acquired spectra of hydroperoxo-Mb exhibited a distinctive peak attributable to the ν(Fe-O) stretch of the FeOOH fragment, but no evidence for enhancement of the structurally sensitive intraligand ν(O-O) mode, a result that is consistent with the lack of enhancement generally seen for the *dioxygen* adducts of histidyl ligated heme proteins; to date there is only one known exception to this general trend, with both the ν(Fe-O) and ν(O-O) modes being seen only for a *Chlamydomonas* and *Synechocystis* hemoglobins [38].

*Journal of Inorganic Biochemistry*, Vol. 102, No. 10 (October 2008): pg. 1952-1957. DOI. 

The lack of enhancement of the v(O-O) modes of dioxygen adducts of the native (iron-containing) heme proteins was overcome in many early RR studies by employing cobalt-substituted analogues, wherein the v(O-O) of the CoOO fragments are quite efficiently enhanced via resonance with underlying charge-transfer transitions [39]. The present work was undertaken to explore the utility of cobalt substitution to enhance both the v(Co-O) and v(O-O) modes of the CoOOH fragments of hydroperoxo forms of heme proteins bearing a trans-axial histidine linkage. Specifically, having completed RR studies of hydroperoxo myoglobin [17,18], attention is now turned to its cobalt-substituted analogue. In order to secure assignments, spectra are acquired for samples prepared with $^{16}O_2$ and $^{18}O_2$, comparison of which reveals modes associated with the v(M-O) and v(O-O) modes, the latter indeed now being observed here only for the cobalt-substituted proteins. In addition, in seeking to distinguish between peroxo- and hydroperoxy- species, spectra of samples prepared in deuterated solvents were also acquired and compared to those obtained for samples prepared with natural abundance solvents, any shifts to lower frequencies observed for the deuterated samples being taken as evidence for the presence of the hydroperoxo- species. It is emphasized that while these cobalt-substituted proteins may reasonably be considered to adopt active site structures similar to those of the native proteins, comparable reactivity should not be assumed.

# Experimental

## *Materials*

The Co(III)-protoporphyrin IX was purchased from Frontier Scientific (Logan, UT) and used for preparation of cobalt analogues of horse heart myoglobin (Mb) (Sigma-Aldrich, Milwaukee, WI). The proteins purification and buffer exchanges were performed using Bio-Gel P-6, AG 501-X8 (D) Resin (Bio-Rad Laboratories, Hercules, CA), Sephadex G-25 (Sigma-Aldrich, Milwaukee, WI) and CM-52 cellulose (Whatman, Fairfield, NJ).

*Journal of Inorganic Biochemistry*, Vol. 102, No. 10 (October 2008): pg. 1952-1957. DOI. 

## *Protein Preparation*

The apoMb was prepared using the acid-acetone method [40] and insertion of reduced Co(II) was performed anaerobically according to previously published procedures [41]. Briefly, the 1.2 molar excess of reduced Co(II) protoheme (30 % aqueous pyridine solution, 10% of apoMb volume) was added anaerobically to apoMb dissolved in 100 mM PB pH 7.0. The mixture was stirred under Ar gas for 5 minutes and then applied on Sephadex G-25 column equilibrated with degassed 10 mM PB pH 6.0. The mixture of deoxy/oxy MbCo(II) protein was collected first and then purified on CM-52 column equilibrated with 10 mM PB pH 6.0. The cobalt Mb was adsorbed on the ion exchanger and washed with the same buffer until the eluent was clear. The pure protein was eluted using 100 mM PB pH 7.0. The protein was then saturated with oxygen and gave UV-VIS spectrum characteristic for Co(II)-oxy myoglobin (426 nm, 539 nm, 577 nm). In order to make sure that protein didn't contain any traces of oxidized Co(III) the sample was deoxygenated using argon gas and the UV-Vis spectrum was collected. The absorption spectrum showed only deoxy form of Co(II) myoglobin (406 nm, 558 nm) without any traces of oxidized Co(III) Mb [41]. The $^{16}O_2$ to $^{18}O_2$ substitution, as well as $H_2O$ to $D_2O$ exchange, were performed as previously reported [17–19]. The formation of oxy samples was confirmed using UV-Vis and resonance Raman spectroscopies [39,41]. The samples were in 100 mM phosphate buffer, pH 8.3 with 25 % purified glycerol (by volume) (or glycerol-$d_3$, where applicable). The final concentration of protein was 0.30 mM in heme. The samples were stored in NMR tubes (WG-5-ECONOMY, Wilmad Glass) at 77 K.

## *Irradiation and Annealing Procedures*

The oxygenated samples immersed in liquid nitrogen were irradiated for 5 hours using the $^{60}Co$ source at Notre Dame Radiation Laboratory (Notre Dame University, IN) and were exposed to 0.97 × 10.1 krad/min. The irradiated samples were stored in liquid nitrogen. The cryoreduced samples were annealed at higher temperatures by quickly (around 1 second) transferring the irradiated sample into a solvent/liquid nitrogen bath at desired temperatures and immersing it for 1.5 min, then quickly transferring back into liquid nitrogen. The

outside walls of NMR tubes after annealing were usually covered by ice that formed during freezing the sample back in liquid nitrogen (the samples were not wiped with paper or cloth since that could warm up the surface of the tube from which the scattered light is collected); instead, the ice was gently scraped away from the tube under liquid nitrogen using a pre-cooled (in liquid nitrogen) spatula.

## *Resonance Raman Measurements*

Resonance Raman spectra were obtained using a Spex 1269 spectrometer equipped with an Andor Newton EMCCD detector (Model DU971, Andor Technologies). Excitation at 415.4 nm from $Kr^+$ laser (Coherent Innova 100-K3) was used to measure RR spectra of the samples before and after irradiation and annealing. The RR spectra were collected using back scattering (180°) geometry, a cylindrical lens was used to form a line image [42] with 1.0 mW (or less) laser power on the sample. The frozen sample, contained in a 5 mm O.D. NMR tube, was positioned into a double-walled quartz low temperature cell filled with liquid nitrogen and fitted with an NMR spinner. The sample tubes were spun to avoid local heating. Resonance Raman spectra of the oxygenated forms of MbCo were taken at 77 K before irradiation to document complete oxygenation. The spectra were calibrated with fenchone and processed with Grams/32 AI software.

## *Results*

In this current effort to extend the earlier study of cryoreduced oxy Mb [17,18], samples of oxy CoMb and its cryoreduced product have been prepared and studied. For completeness, corresponding spectra of native oxyMb are also included here (Figure 1), noting that the earlier work was carried out at pH 7.0, while the data obtained here for oxyCoMb and oxyMb are acquired at pH 8.3, a value matching that used in an earlier work on oxyCoMb [39]. In Figure 1 it is seen that a feature attributable to the v(Fe-O) of the oxyMb sample is observed at 578 $cm^{-1}$, a value that is slightly higher than the 572 $cm^{-1}$ frequency observed at room temperature. As shown previously [17,18], upon cryoreduction the 578 $cm^{-1}$ feature virtually disappears and is replaced by a new feature seen at 617 $cm^{-1}$ in trace C that exhibits the ~25 $cm^{-1}$ $^{16}O/^{18}O$ isotopic shift expected for the v(Fe-O)

*Journal of Inorganic Biochemistry*, Vol. 102, No. 10 (October 2008): pg. 1952-1957. DOI. 

mode of an Fe-O-O fragment. Furthermore, the observed ~5 $cm^{-1}$ downshifts observed for both the $^{16}O_2$ and $^{18}O_2$ samples in $D_2O$ solution as shown in Figure 1, trace D, is most consistent with formulation of this species as a hydroperoxo derivative; i.e., Fe-O-OH(D).

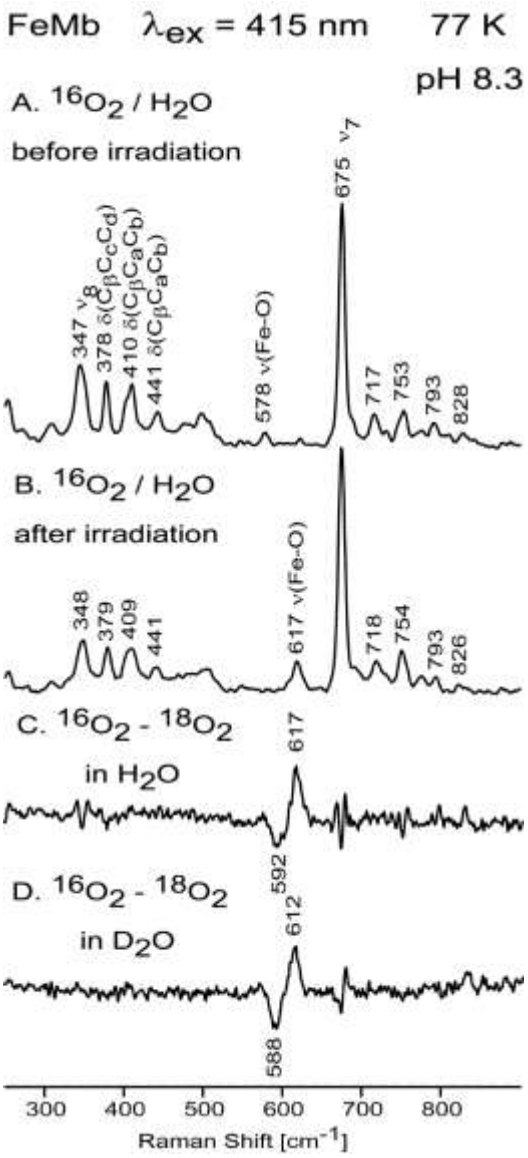


**Figure 1** Low frequency resonance Raman spectra of native oxy myoglobin ($^{16}O_2/H_2O$) before (A) and after (B) irradiation. The two bottom traces show the difference spectra of $^{16}O_2$-$^{18}O_2$ in $H_2O$ (C) and $^{16}O_2$-$^{18}O_2$ in $D_2O$ buffer (D) of irradiated native oxyMb.

The RR spectra for oxy CoMb in both the low frequency and high frequency regions, obtained at 77 K, are given in Figure 2, where it is

*Journal of Inorganic Biochemistry*, Vol. 102, No. 10 (October 2008): pg. 1952-1957. DOI. 

seen that distinctive features are observed at 1136 and 1069 $cm^{-1}$ for the $\nu(^{16}O\text{-}^{16}O)$ and $\nu(^{18}O\text{-}^{18}O)$ modes, the observed frequencies being slightly perturbed from their inherent values by a thoroughly documented and well understood vibrational coupling interaction between the v(O-O) and internal modes of the trans-axial histidyl-imidazole ligand [39, 43, 44]. As has been documented in many previous studies [12, 45], no feature attributable to an enhanced v(O-O) mode is observed for the oxyMb sample (data not shown). In the low frequency region a distinctive v(Co-O) mode is observed at 549 $cm^{-1}$, a value that (like the frozen oxyMb sample mentioned above) is also somewhat higher ( ~ 10 $cm^{-1}$) than that seen at room temperature in previous studies [17–19].This feature exhibits a nearly ideal $^{16}O/^{18}O$ isotopic shift and, as expected for a simple dioxygen adduct, no shift in buffers prepared with $D_2O$; there are no interfering coupled modes near the v(Co-O) mode.

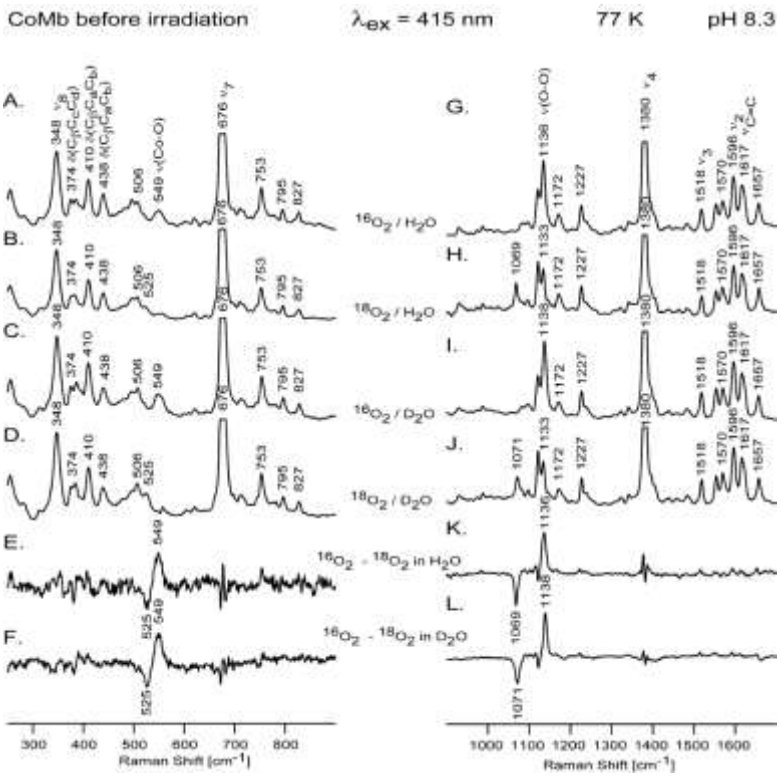


**Figure 2** Low and high frequency resonance Raman spectra of $^{16}O_2/H_2O$, $^{18}O_2/H_2O$, $^{16}O_2/D_2O$ and $^{18}O_2/D_2O$ samples of Co(II) myoglobin (MbCo) before irradiation and their difference spectra.

*Journal of Inorganic Biochemistry*, Vol. 102, No. 10 (October 2008): pg. 1952-1957. DOI. 

Following irradiation of the oxygenated CoMb sample with the $^{60}Co$ source, the RR spectrum (Figure 3) shows a dramatic decrease in modes associated with the bound Co-O-O fragment, an observation that is made most evident by noting the decrease of the 1069 ($H_2O$) and 1071 ($D_2O$) $cm^{-1}$ features associated with the $\nu(^{18}O\text{-}^{18}O)$ modes in the high frequency region; e.g., comparing traces K and L in Figure 3 with those shown in Figure 2. This decrease in the intensities of the features associated with the bound dioxygen fragment is obviously consistent with cryoreduction of the dioxygen adduct to the peroxo- or hydroperoxo- form and this expectation is confirmed by the appearance of *new* modes in the low frequency region displayed in Figure 3. There, distinct difference patterns are seen with components being evident at 851/806 $cm^{-1}$ and between 583 and 525 $cm^{-1}$. The higher frequency (851/806 $cm^{-1}$) pair, reasonably associated with the $\nu$(O-O) mode of a peroxo- or hydroperoxo- fragment, exhibits a clear difference pattern yielding an extracted isotopic shift of 45 $cm^{-1}$, in good agreement with theory for such a $\nu$(O-O) mode. Furthermore, the 6 $cm^{-1}$downshifts observed in buffers prepared in $D_2O$ support the formulation of these derivatives as hydroperoxo species, as was the case with the native (iron-containing) myoglobin samples [17,18].

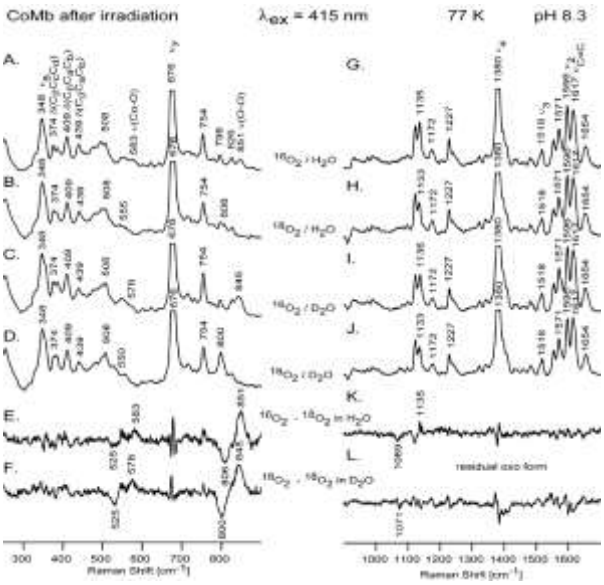


**Figure 3** Low and high frequency resonance Raman spectra of $^{16}O_2/H_2O$, $^{18}O_2/H_2O$, $^{16}O_2/D_2O$ and $^{18}O_2/D_2O$ samples of Co(II) myoglobin (MbCo) after irradiation and their difference spectra.

*Journal of Inorganic Biochemistry*, Vol. 102, No. 10 (October 2008): pg. 1952-1957. DOI. 

While the ν(O-O) modes yield the expected isotopic shifts, the difference pattern observed between 583 and 525 $cm^{-1}$ is complicated by overlap of modes associated with unreduced Co-O-O fragments, the modes for the latter appearing at 549 and 525 $cm^{-1}$, as documented in Figure 2. Thus, given a ν(Co-$^{16}$O) appearing at 583 $cm^{-1}$ for the new hydroperoxo- form, one expects a corresponding ν(Co-$^{18}$O) at ~555 $cm^{-1}$, which is closely overlapped with the ν(Co-$^{16}$O) mode of the residual non-reduced parent that is seen in Figure 2 at 549 $cm^{-1}$. This partial cancellation of the 555 and 549 $cm^{-1}$ features leaves the 583 and 525 $cm^{-1}$ peaks; i.e., the *apparently* non-ideal (large) isotopic shift is actually an expected consequence of the presence of residual non-reduced form. This expected cancellation effect indeed also occurs for the samples in deuterated solvent, noting that the observed 578 $cm^{-1}$ component in the E–F difference trace of Figure 3 is shifted down by 5 $cm^{-1}$ compared to its value in protonated solvents, whereas its $^{18}$O counterpart is again cancelled leaving only the 525 $cm^{-1}$ remnant from residual $^{18}O_2$-CoMb precursor, which is naturally insensitive to H/D effects.

## Discussion

Relatively recent work on γ-irradiated samples of oxygenated globins that employed both EPR and Mossbauer techniques, as well as carefully conducted annealing procedures, have helped define the composition of such cryoreduced samples [32, 33]. There it was found that γ-irradiation of oxyMb yields a low-spin ferric peroxo species, which persists up to about 195 K, but then converts to a mixture of mainly peroxo-Mb and hydroperoxoMb; however, the authors of that work did note that the annealing procedure also generates another product considered to be a ferryl heme (i.e., Compound II) [33,34]. In our previous RR studies of cryoreduced oxyMb [17,18], even at lower temperatures (77 K and 145 K) we were able to observe only one ν(Fe-O) mode, which was assigned to the hydroperoxo-form, based on an observed 5 $cm^{-1}$ down shift in deuterated solvents. This somewhat surprising lack of evidence for the non-protonated peroxo-species was attributed to unintentional annealing of the *surface* of the sample with the laser beam used to excite the RR spectrum; as was duly noted there [17], subsequent EPR studies of the *same tube* showed that the *bulk* of the sample was indeed still in the non-protonated peroxo-

*Journal of Inorganic Biochemistry*, Vol. 102, No. 10 (October 2008): pg. 1952-1957. DOI. 

form. The data acquired here for the cryoreduced OxyCoMb samples, exhibiting ~ 5cm$^{-1}$ H/D shifts, also confirm the hydroperoxo-formulation for these cobalt-substituted myoglobins.

The most important finding in the present work is the confirmation that, while the v(O-O) mode of the hydroperoxo-derivative of the native iron-containing globin is not resonance enhanced with excitation near the Soret transition, this internal v(O-O) mode is indeed effectively enhanced in the case of the cobalt-substituted analogues. While the dioxygen adducts of nearly all histidyl-ligated heme proteins fail to exhibit efficient enhancement of these key modes, it is noted that dioxygen adducts of cytochromes P450 do exhibit strongly enhanced v(O-O) modes; i.e., the native (iron-containing) thioloate-ligated proteins show enhancements similar to the cobalt-substituted, histidyl-ligated heme proteins. These similarities extend to the peroxo- and hydroperoxo derivatives of these proteins. Thus, as was recently shown in our laboratory, in a manner analogous to the cobalt globins studied here, Cytochrome P450 camphor (P450cam) forms a hydroperoxo-derivative whose v(O-O) mode is readily observed at 799 cm$^{-1}$, exhibiting expected isotopic shifts upon both $^{18}O_2$ and $D_2O$ substitutions [19].

Finally, inasmuch as this is apparently the first report of the preparation and characterization of a hydroperoxo- derivative of a cobalt-substituted heme protein, it is of interest to compare the vibrational behavior of these with the corresponding native myoglobin samples. As has been well documented in many earlier studies comparing the M-O-O fragments of the parent *dioxygen* adducts of heme proteins and model compounds, the v(Co-O) is typically observed to occur at frequencies that are 30–50 cm$^{-1}$ lower than the corresponding v(Fe-O) modes [12,39,45–48]. The newly acquired data presented here for the hydroperoxo derivative of CoMb are entirely consistent with this trend, documenting a 34 cm$^{-1}$ lower frequency for the v(Co-O) mode compared with the 617 cm$^{-1}$ value observed for the native protein; it is also noted that similar differences in v(M-O) have been observed [46] and calculated [49, 50] in comparing the hydroperoxo forms of cobalt (545 cm$^{-1}$) and iron (575 cm$^{-1}$, calculated) bleomycins. A comparison of corresponding v(O-O) modes is strictly not possible, given the fact that these are not resonance enhanced for the native systems; the relatively scarce data available

for the parent dioxygen adducts, obtained mainly from IR difference spectroscopy, generally show that this mode is reflective of a bound superoxide formulation and is about 7–10 $cm^{-1}$ lower for the native systems than for the cobalt-substituted analogues [47,48]. Given this difference observed for the parent dioxygen adducts, a reasonable expected value for the v(O-O) of the hydroperoxoMb would be near 840 $cm^{-1}$. While this species has yet to be studied by IR difference spectroscopy, data has been published for several end-on hydroperoxo complexes of ferric model compounds [51], which suggests that for complexes having v(Fe-O) modes near 630 $cm^{-1}$, the corresponding v(O-O) modes are seen near 790 $cm^{-1}$. Inasmuch as these modes are expected to be inversely correlated [52–54], the 617 $cm^{-1}$ frequency observed for hydroperoxo Mb would be expected to yield a v(O-O) frequency between these 790 and ~850 $cm^{-1}$ values; i.e., the 617 $cm^{-1}$ value observed for v(Fe-O) is reasonably consistent with an expected value of ~840 $cm^{-1}$ for the *unobserved* v(O-O).

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## Footnotes



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