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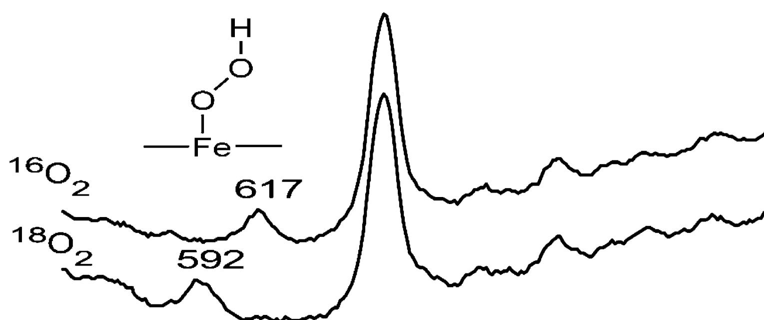

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Resonance Raman Spectroscopic Studies of Hydroperoxo-Myoglobin at Cryogenic Temperatures

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Abstract: In agreement with previous reports (Gasyna, Z. *FEBS Lett.* **1979**, *106*, 213−218 and Leibl, W.; Nitschke, W.; Huettermann, J. *Biochim. Biophys. Acta* **1986,** *870,* 20−30) radiolytically reduced samples of oxygenated myoglobin at cryogenic temperatures have been shown by optical absorption and EPR studies to produce directly the peroxo-bound myoglobin at 77 K. Annealing to temperatures near 185 K induces proton transfer, resulting in the formation of the hydroperoxo heme derivative. Resonance Raman studies of the annealed samples has permitted, for the first time, the direct observation of the key ν(Fe−O) stretching mode of the physiologically important Fe-OOH fragment of this ubiquitous intermediate. The assignment of this mode to a feature appearing at 617 cm^{-1} is strongly supported by documentation of a 25 cm^{-1} shift to lower energy upon substitution with $^{18}O_2$ and by a 5 cm^{-1} shift to lower energy for samples prepared in solutions of deuterated solvent.

Introduction

In the enzymatic cycles of many oxidative heme enzymes, including peroxidases,[1,2] catalases,[3] cytochrome P450,[4] nitric oxide synthases (NOS),[5] and some others,[6,7] high-valent transient heme-oxygen intermediates known as compound I, arise from peroxo- or hydroperoxo-ferric heme precursors via protonation of the distal oxygen atom and subsequent fast heterolytic scission of the oxygen−oxygen bond. Of the numerous spectroscopic techniques applied to the structural characterization of such enzymatic intermediates and model compounds,[8−11] resonance Raman (RR) spectroscopy is quite attractive inasmuch as, in principle, it provides a direct probe of both the heme macrocycle structure as well as the iron-oxo ligand fragments.[11]

While the RR technique has been effectively used to characterize the relatively stable compounds I and II of plant peroxidases[10a−e] and other enzymatic intermediates,[10f−h] all of which contain a ferryl fragment (Fe═O) that is directly manifested in the RR spectra as an oxygen-isotope sensitive feature, until now there have been no reports of the application of this powerful technique to the precursors of compound I; i.e., the fleeting peroxo/hydroperoxo heme complexes quickly disappear, and less is known about their spectral properties. The most direct method to isolate these species and to obtain their spectra is the cryogenic radiolytic reduction of corresponding oxy-ferrous heme proteins in aqueous glycerol buffers.[12−16] It was shown that the immediate products of radiolytic reduction

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of heme protein complexes are quite stable while kept immersed in liquid nitrogen at 100 K, well below T_g, the glass transition temperature of the solvent. Gradual thermal annealing at temperatures close to T_g and above induces conformational relaxation and product conversion; i.e., application of cryoradiolytic reduction to several enzymes showed that this path can mimic the physiological reaction cycle in several enzymes with stoichiometric substrate turnover.[4,17]

In the present work, the efficient cryoradiolytic reduction of oxy-Mb has been effectively exploited to isolate the peroxo-ferric heme complex at 77 K. After thermal annealing, the protonation of the distal oxygen atom occurred, and the hydroperoxo-ferric heme complex was formed. Using optical spectroscopy to monitor and control the yield of radiolytic reduction of oxy-Mb and EPR to identify transient intermediates, we were able, for the first time, to acquire high-quality RR spectra of an isolated hydroperoxo derivative of a heme protein.

Experimental Section

Horse-heart met-myoglobin (Sigma) was purified according to a previously reported procedure.[18] The purified met-Mb was converted to Mb-CO by applying sodium dithionite under an Ar atmosphere followed by passing the sample through a Biogel P-6 (Biorad) column equilibrated with a 100 mM phosphate buffer, pH 7.0, saturated with CO. The oxy-myoglobin samples were obtained by photolyzing the Mb-CO samples (in an ice bath) by light and simultaneously applying $^{16}O_2$. The integrities of the samples were checked with optical absorption spectroscopy.

The H_2O/D_2O buffer exchange was carried out by concentrating 2 mL of Mb-$^{16}O_2$ to 0.5 mL at the centrifuge using Amicon Microconcentration membranes (YM-10 membrane). The sample was then diluted to 2 mL with 100 mM phosphate (D_2O) buffer, pH 7.0, and again concentrated to 0.5 mL. This process was repeated three times (25 h).

The $^{16}O_2$ oxy-myoglobin samples with 50% (v/v) glycerol[19] were prepared by adding either the degassed glycerol (99.5+%, Aldrich) to the Mb-$^{16}O_2$ samples in 100 mM phosphate buffer, pH 7.0, or adding the glycer(ol-d_3) (D 98%, Aldrich) to the Mb-$^{16}O_2$ samples in 100 mM phosphate (D_2O) buffer, pH 7.0.

The $^{18}O_2$ exchange was carried out on a vacuum line as follows: the Mb-$^{16}O_2$ samples sealed with rubber septa in the NMR tubes (WG-5M-ECONOMY, Wilmad Glass Co., Beuna, NJ) were first degassed until all $^{16}O_2$ had been removed. The complete removal of $^{16}O_2$ was monitored by UV−vis and RR spectroscopies. Then $^{18}O_2$ gas (^{18}O 99%, Icon Isotopes) was carefully passed into the sample on the vacuum line. The formation of the Mb-$^{18}O_2$ was confirmed by UV−vis and RR spectroscopies. Concentrations of the oxy-Mb samples in 50% glycerol were around 0.5 mM.

We note that some attempts were made to avoid high concentrations of glycerol as a sample medium for these irradiation experiments because a relatively high degree of fluorescence was observed in some early RR measurements; i.e., in the present study, trial experiments were conducted with solutions of 1% (v/v) *tert*-butyl alcohol being employed as solvent. However, the RR spectra acquired for the irradiated samples of oxy-Mb showed no changes from the nonirradiated samples (data not shown), indicating that this solvent is not effective in mediating radiolytic reduction of the protein samples studied here. Consequently, all further experiments were conducted in 50% (v/v) glycerol solutions.

Cryoradiolytic reduction of oxy-Mb samples was achieved by exposing to γ-irradiation from a ^{60}Co source (dose rate 9.5 kilograys/h, 4 h) at the Notre Dame Radiation Laboratory (University of Notre Dame, South Bend, IN). The samples were fully immersed in liquid nitrogen during the irradiation and during the transport to Marquette University. Annealing was carried out by immersing the irradiated Mb samples into solvent/liquid nitrogen mixtures at desired temperatures (142 and 185 K) for 1 min.[20] Samples were then quickly (less than 2 s) placed back into liquid nitrogen.

RR spectra were obtained by a Spex 1269 spectrometer equipped with a Princeton Instruments ICCD-576 UV-enhanced detector. The 413 and 568 nm notch filters (Kaiser Optical Systems, Inc., Ann Arbor, MI) were used for the respective lines from a Coherent Innova 100 Kr laser; the 442 nm notch filter was used for the 442 nm line from a Liconix Helium−Cadmium laser. The spectra were calibrated with fenchone and processed with GRAMS/32 AI software (Thermo Galactic, Salem, NH). Power at the sample was ∼4−5 mW unless otherwise noted. The samples were kept frozen in sealed NMR tubes (WG-5M-ECONOMY, Wilmad Glass) that were positioned in a backscattering geometry and spun during the measurement to avoid local heating effects. The sample NMR tubes were kept cooled at 77 K in a double-walled low-temperature glass cell of in-house design by periodically refilling with liquid nitrogen.

The EPR spectra were obtained on a Varian E-122 X-band (9.08 GHz) spectrometer at the University of Illinois at Urbana-Champaign EPR Resource Center. A liquid helium flow system (Air Products, Allentown, PA) was used for measurements at 10−20 K. A microwave power of 0.5 mW was used with a modulation amplitude of 10 G at 100 kHz. The samples were fully immersed in liquid nitrogen before and after the EPR experiment and during the transport.

Inasmuch as the measurements reported herein were conducted on temperature-sensitive samples at different locations, it seems warranted to provide further details on experimental protocol, as follows:

1. A set of three oxy-Mb samples were transported under liquid nitrogen to the Radiation Laboratory and irradiated with γ radiation at 77 K to form peroxo species.

2. Samples were transported under liquid nitrogen to Marquette University, where RR spectra were acquired for two samples of the set.

3. All three samples were then transported under liquid nitrogen to the University of Illinois where EPR spectra were acquired, the spectral results showing no differences among the three samples; i.e., acquisition of RR spectra caused no apparent changes to the bulk samples (vide infra).

4. The samples were then annealed at 142 K (to remove organic radicals) and then at 185 K (to facilitate conversion to the hydroperoxo species), EPR spectra being recorded from samples annealed at both temperatures.

5. The annealed samples were then transported under liquid nitrogen to Marquette University, where RR spectra were acquired for all three annealed (i.e., hydroperoxo) samples.

In addition to this, UV−visible spectra of radiolytically reduced oxy-Mb (after annealing to 142 K) were also measured as previously described.[14,21] These spectra are especially useful to monitor the state

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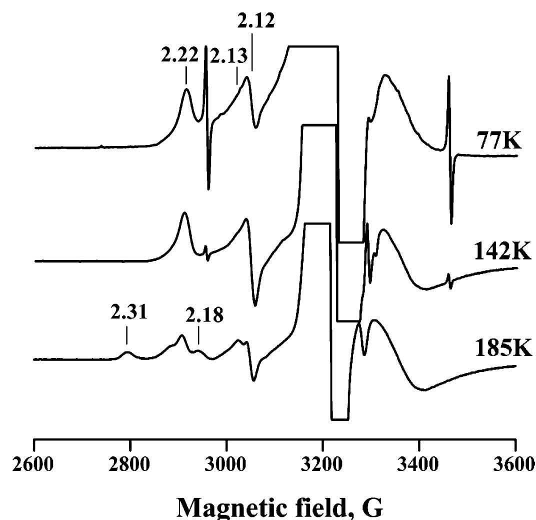

Figure 1. EPR spectra of the irradiated and annealed irradiated oxy-Mb.

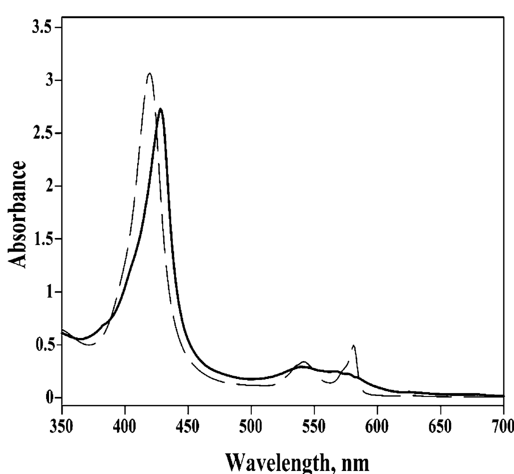

Figure 2. UV−vis spectra of oxy- (dashed line) and peroxo-myoglobin (solid line) measured at 100 K.

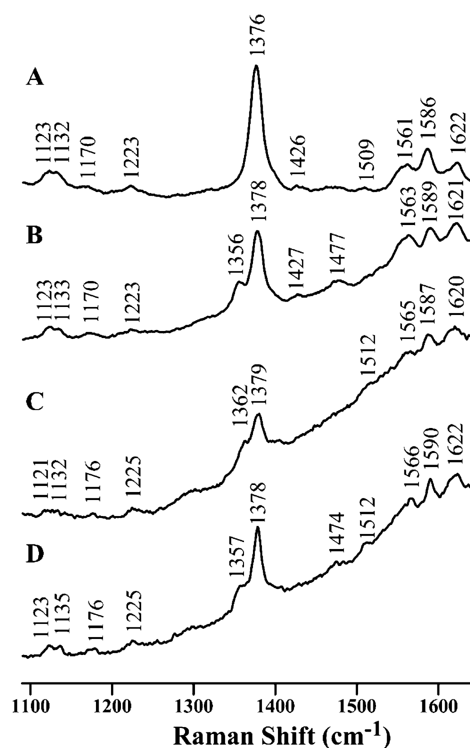

Figure 3. High-frequency RR spectra of oxy-Mb (50% glycerol) at 413 nm excitation. (a) At room temperature. (b) At 77 K. (c) Irradiated, at 77 K. (d) Irradiated and annealed to 185 K, at 77 K.

of the cryoreduced oxy-Mb, showing clear differences upon conversion of the oxy form to the reduced, peroxo form.

Results and Discussion

EPR and UV−Vis Studies of the Irradiated and Annealed Oxy-Mb. Previous EPR studies of the ^{60}Co γ-irradiated samples of oxy-Mb[15,16] and oxy-Hb[12a,22] in aqueous ethylene glycol medium at 77 K suggested the species formed after the irradiation is the low-spin ferric-peroxo (Fe(III)-O-O^{2-}) intermediate, with $g \approx$ [2.22, 2.11, 1.96]. EPR spectra of the initial cryoreduced oxy-myoglobin in 50% glycerol studied here yield a similar low-spin rhombic signal (g_x = 2.22, g_y = 2.12), indicative of the one-electron reduced form of the ferrous oxy form $[Fe^{3+}$-$O_2]^{2-}$ (i.e., peroxo form) with end-on geometry[15,23] (Figure 1) (note: annealing at 142 K eliminates interference signals from the trapped organic radicals, while not altering the g values). These results were confirmed by the absorption spectra of radiolytically reduced oxy-Mb (Figure 2). The Soret maximum of the unprotonated peroxo-ferric heme complex is observed at 428 nm, which is 7 nm red-shifted as compared to the data obtained for protonated hydroperoxo-ferric complexes in HRP[21] and heme oxygenase.[24] This shift can be compared to the similar 3-nm shift of the Soret band observed as a result of protonation of peroxo-ferric intermediate in the distal mutant D251N of cytochrome P450cam.[25] In general, the absorption spectrum of peroxo-ferric Mb is typical for the low-spin complexes of His-ligated heme proteins with anionic ligands.

Upon annealing of the irradiated oxy-Mb samples in 50% glycerol at 185 K, a loss of the main signal at g_x = 2.22 is seen (Figure 1), with the appearance of a new signal (g_x = 2.31, g_y = 2.18) also indicative of rhombic symmetry. This characteristic change in g-tensor upon annealing, as reported in conjunction with EPR studies of irradiated oxy-Mb[16] and oxy-Hb[22] and ^{1}H and ^{14}N ENDOR work on Mb[15] and cytochrome P450cam,[4] is indicative of a change in the protonation state of the distal oxygen atom upon first proton transfer to form a hydroperoxo-type low-spin ferric heme (Fe(III)-O-OH^-). In addition, it is noted that no difference in EPR signals is seen between samples which were not exposed to the laser beam and samples for which Raman spectra were acquired, indicating that the electronic integrity of the peroxo complexes and their characteristic annealing patterns has been retained, at least for the bulk samples; however, the RR results suggest that the laser beam may cause local heating sufficient to induce proton transfer (vide infra).

RR Study of the Irradiated and Annealed Oxy-Mb. The high-frequency RR spectra of the oxygenated, irradiated oxy and annealed irradiated oxy-Mb samples in 50% glycerol are shown in Figure 3. The spectrum of the oxygenated form

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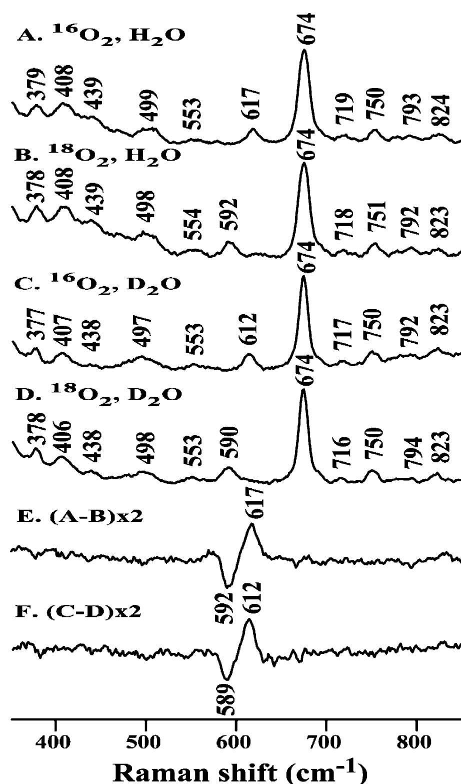

Figure 4. Low-frequency RR spectra of irradiated oxy-Mb (50% glycerol) at 413 nm excitation at 77 K.

acquired for the frozen sample (trace B) exhibits bands at 1356 and 1477 cm^{-1} that are absent in the spectrum of the room-temperature sample (trace A) because of a greater degree of photolysis observed for the frozen sample; i.e., prolonged laser irradiation of a fixed sample spot vs irradiation of a fluid solution. In the spectrum of the γ-irradiated sample (trace C), the ν_3 and ν_4 modes were observed at 1512 and 1379 cm^{-1}, respectively, which are indicative of a low-spin ferric heme. A shoulder at 1362 cm^{-1} arises from a small amount of Fe(II) contribution (trace C), which moves to 1357 cm^{-1} when the sample was annealed to 185 K (trace D). This feature does not arise from photolysis of any traces of the dioxygenated species remaining in the sample after γ-irradiation; in that case, the frequency would be closer to 1356 cm^{-1} (see trace B). In fact, the observed 1362 cm^{-1} frequency is close to that observed for a reduced sample of myoglobin prepared by γ irradiation of frozen buffer solutions of met-myoglobin (spectrum not shown); in that case also, the band gradually shifts from 1362 to 1357 cm^{-1} upon warming to 185 K. This behavior has been reported in a previous work,[26] wherein the 1362 cm^{-1} feature was assigned to a low-spin Fe(II)-H_2O myoglobin species. Furthermore, it is noted that no evidence is found in the low-frequency region for the presence of residual oxy-Mb; i.e., there is no evidence of a ν(Fe$-O_2$) mode, expected to occur as a difference feature near 580/555 cm^{-1}, in Figure 4, traces E and F (vide infra).

Thus, as indicated above, the high-frequency RR spectrum of the γ-irradiated sample is consistent with formation of a peroxo myoglobin, and it is therefore expected that the

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Table 1. RR Spectroscopic Features of Low-Spin Ferric-Hydroperoxo [Fe(III)-OOH] Complexes

complexes	ν(Fe–O) $^{16}O^{16}O$ ($\Delta H/D$)	$^{18}O^{18}O$ ($\Delta ^{16}O/^{18}O$)	ref
[(N4Py)Fe(OOH)]$^{2+}$	632 (−5)	616 (−16)	27, 31−33
[(TPA)Fe(OOH)]$^{2+}$	626		27
[(trispicMeen)Fe(OOH)]$^{2+}$	617 (−4)	600 (−17)	28c
[(TPEN)Fe(OOH)]$^{2+}$	617 (−4)		28c
[(trispicen)Fe(OOH)]$^{2+}$	625 (−7)	602 (−23)	28c
[(Py5)Fe(OOH)]$^{2+}$	627		33
[(TACNPy2)Fe(OOH)]$^{2+}$	639		33
hydroperoxo-Mb	617 (−5)	592 (−25)	this work

vibrational modes of the Fe$-O_2$ fragment may be manifested in the RR spectrum. Indeed, as shown in Figure 4 (traces A, B, and E), the low-frequency RR spectra of the sample that had been annealed only to 142 K showed a distinct new band at 617 cm^{-1} for Mb-$^{16}O_2$, which shifted to 592 cm^{-1} in the case of Mb-$^{18}O_2$. The observed isotopic shift, $\Delta\nu = -25$ cm^{-1}, is very close to the calculated shift for a pure Fe-O vibration (−27 cm^{-1}).[27] This isotopic-sensitive band is most reasonably assigned as the ν(Fe−O) stretching mode of a peroxo or hydroperoxo species; i.e., this new feature cannot be ascribed to the ν(Fe−O) of the familiar dioxygen adduct, which occurs near 570 cm^{-1} at room temperature and near 580 cm^{-1} at the low temperatures used here (spectrum not shown). This 617 cm^{-1} mode observed here is significantly higher in frequency compared to those reported for side-on iron-peroxo species, which are typically observed between ∼438−468 cm^{-1},[28] but quite close to those reported for model compounds containing a bound hydroperoxy fragment (Table 1).

To examine possible deuterium exchange effects on the vibrational modes of the FeO_2/FeO_2H fragment, measurements on the irradiated oxy-Mb samples prepared with 50% glycer(ol-d_3) in D_2O buffer were conducted. As can be seen from traces C, D, and F of Figure 4, a 5 cm^{-1} downshift in D_2O was observed for the irradiated oxy-Mb. This shift is similar to the deuterium isotopic shifts of model ferric hydroperoxo complexes (Table 1). The ∼4 cm^{-1} ^{2}H isotopic shift strongly supports the formulation of this species as a hydroperoxo heme complex.

The low-frequency RR spectra of the irradiated oxy-Mb after being annealed at 185 K and then cooled to 77 K (Supporting Information, Figure S1) exhibit the same set of modes as those shown in Figure 4; i.e., the isotope sensitive modes are again observed at 617 and 593 cm^{-1} for Mb-$^{16}O_2$ and Mb-$^{18}O_2$, respectively (and at 613 and 590 cm^{-1} in D_2O buffer). Inasmuch as it is considered unlikely that the vibrational modes of both a bound peroxo and a bound hydroperoxo-Fe fragment would exhibit precisely the same frequencies, the most reasonable explanation for observing the same frequencies for the ν(Fe−O) modes of both the nonannealed and annealed irradiated oxy-Mb samples is as follows: as the RR spectrum of the nonannealed irradiated oxy-Mb is being acquired, the laser beam

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causes local heating, resulting in an unintentional annealing the sample. However, this local heating apparently is restricted to the surface, since the subsequent EPR measurements demonstrated that the bulk sample (probed by EPR) was unchanged by the laser beam exposure. Since the scattered light comes from the surface of the frozen sample, the Raman spectra obtained corresponds to that of the (laser) annealed sample; i.e., the hydroperoxo-Mb species.

Although careful efforts were made, using excitation lines both at 442 and 568 nm (the former still in resonance with the measured Soret band of peroxo-ferric Mb, as noted above, see Figure 2), no evidence was found here for enhancement of the ν(O−O) of the bound hydroperoxo fragment. This is not surprising, given the fact that, with the exception of thiolate-ligated heme proteins (cytochrome P450, etc.), the ν(O−O) mode of dioxygen-bound heme proteins is not usually observable in RR spectra.[10] (In a most recent exception, Das et al. reported simultaneous observation of the ν(O−O) and $\nu(Fe-O_2)$ stretching modes in the oxy-Hb of *Chlamydomonas eugametos* and *Synechocystis PCC6803*[29]).

Conclusions

The ν(Fe−O) mode of the hydroperoxo-Mb species, produced by the radiolytic reduction of the oxy-Mb, has been observed to occur at 617 cm^{-1}, exhibiting a 25 cm^{-1} shift for the ^{18}O analogue and a 4−5 cm^{-1} shift to lower frequencies in D_2O. This is the first reported observation of the important ν(Fe−O) mode for a hydroperoxo complex of any heme protein. The results of the annealing experiment were consistent with the previous EPR studies mentioned above; namely, the hydroperoxo-Mb species was formed at 185 K. Similar experiments are in progress for a number of other heme proteins, including cytochrome P450cam, where it is hoped that, by analogy with the dioxygen adducts,[30] both the ν(Fe−O) and ν(O−O) modes for the hydroperoxo complex of P450cam will be observed.

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Supporting Information Available: RR spectra of the annealed irradiated oxy-Mb samples (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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