Identification of the Fe−O−O Bending Mode in Oxycytochrome P450cam by Resonance Raman Spectroscopy

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Abstract: An oxygen-sensitive mode in oxygenated wild-type cytochrome P450cam (oxyP450cam) is observed at 401 cm^{-1} and assigned to the δ(Fe−O−O) bending mode, based upon $^{16}O_2$,$^{18}O_2$ isotopic shifts (19 cm^{-1}) and comparison with Co− and Fe−oxyporphyrin complexes. The detection of this Fe−O−O bending mode has structural implications for enzyme function since its frequency reflects the energies associated with Fe−O−O distortion in oxyhemeprotein active sites. Three body normal coordinate calculations adequately fit the experimental data set with a 125−130° bond angle for the Fe−O−O linkage in oxyP450cam. Observation of low-frequency isotope-sensitive vibrational patterns, some of which are hypothesized to be associated with out-of-plane porphyrin motions are also reported. These patterns, in conjunction with the high frequency of this bending mode and the abnormal isotopic shift of the Fe−O_2 stretching mode, suggest a "strained" Fe−O−O moiety in oxyP450cam, with comparable mobility to HbO_2 and MbO_2. Possible sources of this "strain" and implications for catalytic dioxygen activation in P450cam are discussed.

Introduction

Oxyhemeprotein complexes perform cellular functions as diverse as molecular oxygen carriers,[1] detoxifying catalysts,[2] biochemical energy production,[3] and the oxidative functionalization of many exogenous and endogenous substrates.[4] The cytochrome P450 hemeprotein family is believed to utilize the strong basicity of its proximal thiolate ligand to catalytically cleave the bound O−O moiety and facilitate highly stereospecific and regioselective monooxygenation of thousands of compounds.[4] Cytochrome P450cam, the most characterized P450 within this family, accepts reducing equivalents from β-nicotinamide adenine dinucleotide hydride (NADH) via putidaredoxin reductase (an FAD-containing flavoprotein) and putidaredoxin (an Fe_2S_2 iron−sulfur cluster protein) to reduce bound molecular oxygen for the hydroxylation of its substrate, camphor, and a variety of structural analogues.[5] The vibrational energies of the Fe−O−O linkage are important in delineating the ultimate function of the respective classes of hemeproteins. Resonance Raman (RR) spectroscopy directly probes the heme active-site structure and provides valuable vibrational information on iron−dioxygen bonds of oxygenated heme proteins as well as model porphyrin compounds.[6] In this paper we report the observation of a new oxygen-sensitive mode at 401 cm^{-1} for the thiolate-bound heme in oxycytochrome P450cam (oxyP450cam). The mode is assigned to the deformation motion of the iron−dioxygen ligand, δ(Fe−O−O). Such modes provide an excellent and sensitive structural probe of distal pocket influences on the bound dioxygen species for several oxyheme systems.[7] Three body normal coordinate calculations on the experimental data set provide satisfactory fitting levels using either 125° or 130° angles for the Fe−O−O linkage. The high frequency for this bending mode in oxyP450cam, abnormal isotopic shifts, and complicated low-frequency vibrational couplings, some of which are hypothesized to originate from out-of-plane porphyrin motions, suggest a degree of strain in the Fe−O−O moiety of oxyP450cam. Possible sources of these interactions on the oxygen-bound iron adduct are discussed and implications for the mechanism(s) of dioxygen activation by cytochromes P450 addressed. This first report of the bending mode should also enlighten structural studies of the large family of other thiolate-ligated heme proteins which include NO synthases and chloroperoxidases.

Experimental Procedures

Wild-type cytochrome P450cam was expressed and purified according to methodology described in a previous publication.[8] Ferrous P450cam was prepared by reduction anaerobically using 10 molar excess dithionite, which was subsequently removed using an equilibrated, 5-mL Sephadex G-25 column (Sigma, St. Louis, MO). Buffer conditions were 100 mM potassium phosphate, pH 8.0, 1.6 mM D-camphor (Sigma, St. Louis, MO). Protein concentation was adjusted

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to 200 μM. The sample was sealed anaerobically, flash-frozen in liquid nitrogen, and stored at −80 °C until required. Oxygenation was performed by the addition of 5 mL of the desired isotopic gas to the sealed 60 μL aliquots which were then transferred to the sample cell, whereupon data collection commenced. $^{18}O_2$ was purchased from ICON (Mt. Marion, NY). Raman instrumentation has been described previously.[9] Rayleigh scattering was removed by holographic filters (Kaiser Optics, Ann Arbor, MI). To minimize laser-induced autoxidation the sample was spun at ca. 1500 rpm. Spectra were analyzed and processed using Spectra-Calc (Galactic Industries, Salem, NH)) software which additionally removed cosmic spikes. Raman shifts were calibrated using indene as a spectral standard.

The difference spectra were fitted to positive and negative Lorentzian peaks, one isotopically sensitive peak for the Fe−O_2 stretch and one isotopically sensitive peak for the Fe−O−O bend. These peaks were then incorporated into the original spectra, together with other nonisotopically sensitive peaks, and gave a reasonable reconstruction of the original spectra. Both the $^{18}O_2$ and $^{16}O_2$ spectra were simultaneously fit using a commercially available least-squares peak-fitting package (Origin 4.1 PFM). The positions of the nonisotopically sensitive peaks were constrained to the same degree in both spectra ($^{18}O_2$ and $^{16}O_2$). The widths and areas of these peaks were constrained to be within 10% of each other. All variables for the isotopically sensitive peaks, the Fe−O_2 stretch and Fe−O−O bend, were unconstrained and free to vary in the Levenberg−Marquardt minimization of χ-squared.

The accuracy of Raman shifts are ±1 cm^{-1} for absolute shifts and 0.25 cm^{-1} for relative shifts. The GF-matrix method was utilized for three body normal-mode analyses. One program, which was kindly supplied by Dr. Satoshi Takahashi and Professor Denis L. Rousseau, utilized the Urey−Bradley-type potential function described below

$$2V = K_1(\Delta r_{\mathrm{Fe-O_2}})^2 + K_2(\Delta r_{\mathrm{oo}})^2 + H(\Delta\delta_{\mathrm{FeOO}})^2 + F(\Delta q_{\mathrm{Fe^{OO}O}})^2 \quad (1)$$

where $\Delta r_{\mathrm{Fe-O^2}}$, Δr_{OO}, $\Delta\delta_{\mathrm{FeOO}}$ and $\Delta q_{\mathrm{Fe^{OO}O}}$ correspond to alterations in the Fe−O and O−O bond distances, the Fe−O−O bending angle, and the distance from the terminal oxygen to Fe center, respectively. K_1, K_2, H, and F are the respective stretching, bending, or nonbonding interaction force constants for the atoms in question.

Results and Discussion

Figure 1 displays the low frequency (50−800 cm^{-1}) RR data for $^{16}O_2$ [1A], $^{18}O_2$ [1B], and the normalized difference spectra ($^{16}O_2$−$^{18}O_2$) for wild-type oxyP450cam at 356.4-nm excitation. Numerical results are summarized in Table 1. Inspection of the $^{16}O_2$−$^{18}O_2$ difference spectrum between 300 and 800 cm^{-1} clearly reveals the presence of two clean derivative signals. The band positioned at 540 cm^{-1}[$^{18}O_2$ → 510 cm^{-1}] has been assigned to the ν(Fe−O2) stretching mode which was originally identified by Hu et al.[10] A weak second derivative signal for oxyP450cam is observed at 401 cm^{-1}. On the basis of the recorded frequency and isotopic downshift of 19 cm^{-1} for $^{18}O_2$ oxyP450cam we attribute it to originating from the δ(Fe−O−O) bending mode. This bending mode has not previously been reported for any thiolate-based hemeprotein, and this observation should have significance for other enzymes in this class, in addition to cytochromes P450. These assignments are further supported by measurements with an ^{16}O−^{18}O isotopic mixture of dioxygen [data not shown] and their frequencies are consistent with previous observations.[10,11] A third strong derivative signal was observed at 1139 cm^{-1} [data not shown]. This band is assigned to the ν(O−O) vibration and has been reported previously.[10,11] The 66 cm^{-1} isotopic shift upon $^{18}O_2$ substitution matches that expected from an isolated two-body oscillator calculation.

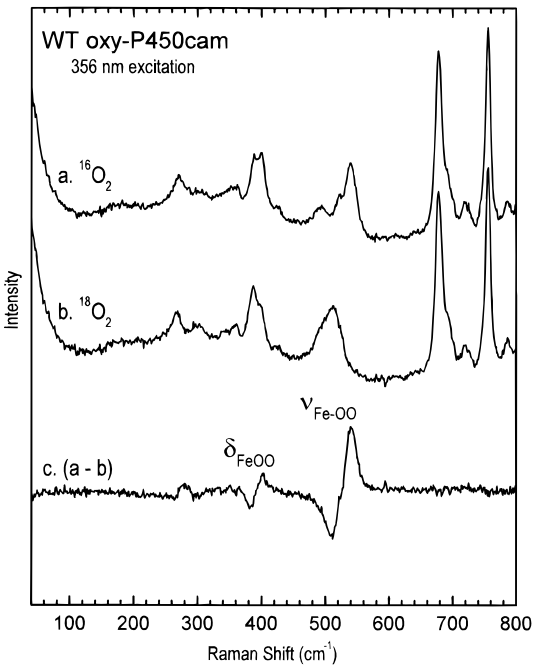

Figure 1. RR spectra of wild-type oxyP450cam for the 50−800 cm^{-1} range (a) $^{16}O_2$ and (b) $^{18}O_2$ and the difference spectrum (c) $^{16}O_2$−$^{18}O_2$ at an excitation line of 356.4 nm. P450cam produced according to the methodology of Gerber[8] and the sample prepared anaerobically.[40] Spectra were recorded as previously described.[9] Data collected with an accumulation of 44 scans, each of 30s duration. Sample retained at 4 °C. The broad $^{18}O_2$ difference band at ∼510 cm^{-1} possibly has contributions from spectral congestion and vibrational coupling. The ferric autoxidation product and background fluorescence were subtracted from the presented spectra. A water peak (∼140 cm^{-1}) was also subtracted from each spectrum.

Table 1. Observed Frequencies for Metal−Dioxygen Modes for oxyP450cam, CoHbCTTO$_2$, HbAO$_2$, CcO−O_2, and HO−O_2. All Values are in Wavenumbers (cm^{-1})

modes	oxyP450cam	CoHbO$_2$	HbAO$_2$	CcO−O_2	HO−O_2
δ(Fe−O−O)	402 (381)[a]	390[b]	425 (405)[d]	435 (415)[d]	414 (403)[f]
ν(Fe−O_2)	540 (510)[a]	537 (519)[b]	568 (544)[d]	571 (544)[d]	565 (538)[f]
ν(OO)	1139 (1073)[a]	1136, 1153[b,c]	∼1130[d,e]		

[a] This work (the frequencies were obtained by simultaneous fitting analysis of the Raman spectra and the isotopic difference spectra). [b] See ref 17. [c] Split nature is due to vibronic effects probably with internal imidazole stretches. See refs 18, 26, and 27 for full explanation. [d] Data from ref 7. [e] Estimate based upon vibronic coupling effects. See ref 7. [f] Data from ref 26.

Fe−O−O bending modes have been observed for iron/imidazole hemeproteins such as oxygenated hemoglobin (HbO$_2$) (425 cm^{-1}),[7] oxygenated cytochrome *c* oxidase (CcO−O_2) (435 cm^{-1}),[7] and compound III of lactoperoxidase (490 cm^{-1})[12] as well as some five-coordinate model compounds including FeO$_2$-TPP (349 cm^{-1})[6,13] (TPP = tetraphenyl porphyrin) and FeO$_2$-TMP (343 cm^{-1}) (TMP = tetramesitylporphyrin).[6,13] Curiously no such δ(Fe−O−O) mode has been observed for oxygenated myoglobin (MbO$_2$). Distortion from equilibrium of a triatomic linkage, such as Fe−O−O, leads to an increase in the frequency for the associated bending mode.[14−16] The high frequency for CcO−O_2 is consistent with a strong distortion of the proposed

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oxygen binding cleft by the proximity of a Cu_B center and the surrounding polar environment.[3] The downshift observed in the bending frequency upon $^{18}O_2$ labeling for oxyP450cam (19 cm^{-1}) is similar to those reported for HbO_2 (20 cm^{-1})[7] and $CcO-O_2$ (20 cm^{-1}),[7] as listed in Table 1. Moreover, the frequency of δ(Fe−O−O) for oxyP450cam is detected 20−30 cm^{-1} lower than in HbO_2, $CcO-O_2$ and other imidazole ligated dioxygen iron porphyrin complexes.[6] This reflects the strong basicity of the thiolate ligand, increasing the antibonding character of the π-interaction with the diatomic orbitals[17,18] which, consequently, lowers the frequencies of the ν, δ modes of the Fe−O−O linkage in oxyP450cam.

The observation by Thomson et al.[17] of an oxygen-sensitive mode at ∼390 cm^{-1} in Co-substituted monomeric hemoglobin (HbO_2 CTT III) is particularly pertinent to the interpretation of our results. The addition of an extra d-electron [Co relative to iron] into the porphyrin system mimics the effect of the strongly electron-donating thiolate axial ligand in the iron porphyrins found in the P450s.[10,11,17−20] Such effects are reflected in the similar frequencies for the oxygen-stretching frequency, ν(O−O), the oxygen−metal (Fe or Co) stretching frequency, ν(M−O_2), and the bending frequency δ(M−O−O) for oxyP450s and Co-substituted oxygenated imidazole/porphyrin systems (see Table 1).

As noted earlier, the frequency of the δ(Fe−O−O) mode can reflect geometric and/or electronic distortions caused by pocket influences on the dioxygen moiety. Thomson et al.[17] reported the δ(Co−O−O) at ∼390 cm^{-1} for Co-reconstituted HbO_2 (see Table 1). Several studies suggest that the native protein structure for hemoglobin is maintained upon Co-reconstitution.[21] Hydrogen bonding from a distal polar residue (histidine) is implicated in stabilizing the bound dioxygen moiety in Hb and Mb.[3,22] The δ(Fe−O−O) mode for oxyP450cam is 12 cm^{-1} higher than in $CoHbO_2$. Assuming that Co/imidazole systems effectively mimic the basicity of the thiolate ligand in this heme system, the 12 cm^{-1} upshift suggests a higher degree of perturbation of the Fe−O−O linkage in oxyP450cam than in HbO_2. Plausible origins of this effect may involve a combination of steric interactions from the proximity of the camphor substrate to this linkage[23] and distal pocket polarity/hydrogen-bonding influences from the active site. Proximal effects, such as trans ligand vibrational coupling,[24] are another possibility. Steric and distal pocket interactions have been invoked to explain the highly bent Fe−O−O linkage in oxygenated heme oxygenase ($HO-O_2$).[25] These interactions on the Fe−O−O moiety in P450cam may be involved in catalytic dioxygen activation by this enzyme, as discussed below.

To relate these observations to a structurally based system, we utilized the experimentally obtained frequencies for the ν(O−O), ν(Fe−O_2), and δ(Fe−O−O) modes in a three body normal coordinate calculation, assuming a Urey−Bradley force field, eq 1. Satisfactory fitting of the observed frequencies and isotopic shift patterns was obtained by applying the following force constants: $K_1 = 2.01$ mdyn/Å, $K_2 = 5.67$ mdyn/Å, $H = 0.65$ mdyn/Å, and $F = 0.99$ myn/Å with θ(Fe−O−O) = 130°. It should be noted that an adequate fitting level was achieved for an angle of 125° using $H = 0.74$ mdyn/Å, $F = 0.85$ mdyn/Å, and adjusting K_1 and K_2 accordingly. The size of F determines the magnitude of coupling between the stretching and bending coordinates. In this case the value of F is moderately high but within the range quoted for oxyhemeprotein complexes.[7] Lower values of F result in a requirement for a larger K_1 to achieve the correct frequency for the ν(Fe−O_2) mode but fail to reproduce the observed O_2-isotopic frequency shifts for the δ(Fe−O−O) bending mode.

Similarly, a moderately high value for H was used in a failed attempt to account for the large (30 cm^{-1}) isotopic shift of the ν(Fe−O_2) mode. The downshift upon $^{18}O_2$ exchange, 540 to 510 cm^{-1}, is in good agreement with values reported in earlier work,[10] suggesting that these large shifts are not spurious. Isotopic shifts for heme/imidazole systems are between ∼22 and 26 cm^{-1}, whereas that calculated for a simple diatomic oscillator is 24 cm^{-1}. $CoHbO_2$ has an isotopic shift of only 18 cm^{-1}.[17] To investigate whether the 30 cm^{-1} isotopic shift reported for the ν(Fe−O_2) mode can be explained using a three body harmonic oscillator, we performed a general and unrestrained GF-matrix calculation to fit the experimental data. This calculation can explain the 30 cm^{-1} isotopic shift for the ν(Fe−O_2) mode and can also accurately reproduce the experimental frequencies and isotopic shifts, simply by altering the stretch−bend coupling force constants which occur as off-diagonal elements in the F matrix.

Moreover, additional plausible explanations have been reported in the literature which can address the abnormally large isotopic shifts. Takahashi et al.[26] have reported an O_2-isotopic substitution frequency shift of 27 cm^{-1} for the ν(Fe−O_2) mode of $HO-O_2$ (see Table 1). In this paper, higher isotopic shifts are suggested to arise from increasing angular distortion of the Fe−O−O linkage. Such an effect would make the oscillator more "Fe-O like", lowering the reduced mass of the system and increasing isotopic shifts. However, it should be indicated that the theoretical assumptions in the Urey−Bradley description are fraught with difficulty in that it is not known to what extent force constants are affected by changes in the bending angle. The authors tentatively suggested an angle of 110° for the Fe−O−O linkage in $HO-O_2$.[26] Another reasonable explanation involves vibrational mode mixing. Such mixing is fairly common within the class of oxyhemeproteins and is associated with either porphyrin modes or proximal ligands, especially imidazolate heme ligands, interacting with the dioxygen moiety of these systems.[24,27,28] Such mode-mixing effects can easily alter apparent isotopic shifts and often lend themselves to complicated and misleading isotopically derived vibrational patterns. Much of the pioneering work into the identification and investigation of factors associated with this coupling phenomenon in oxyheme systems can be attributed to Kincaid

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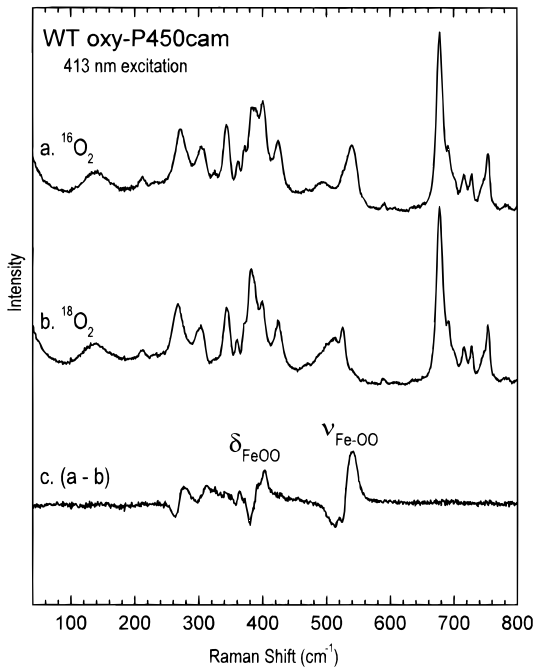

Figure 2. RR spectra of wild-type oxyP450cam for the 50−800 cm^{-1} range (a) $^{16}O_2$ and (b) $^{18}O_2$ and the difference spectrum (c) $^{16}O_2-^{18}O_2$, using 413.1-nm excitation. Experimental conditions identical to those described in Figure 1.

and Nakamoto.[24,27] At this stage we are unable to precisely identify the origin of the 30 cm^{-1} isotopic shift, but we can reasonably argue that a combination of Fe−O−O distortion along with stretch−bend coupling and/or trans-ligand mode mixing offers a plausible explanation for the large isotopic shift.

Closer examination of the difference spectrum in Figure 1 reveals a weak band between 250 cm^{-1} and 300 cm^{-1}. Further investigation of this phenomenon was performed by repeating these experiments using 413.1-nm excitation. The respective isotopically labeled oxyP450cam spectra and their difference spectrum at 413.1-nm, for the frequency range 50−800 cm^{-1}, are presented in Figure 2. A complicated spectral pattern emerges in the region of 250−400 cm^{-1}, as shown in the difference spectrum. To suggest possible origins for these observations it is necessary to identify whether these shifts are large movements of weak lines or small differences in strong porphyrin lines. We have adopted the methodology of Takahashi et al.[26] to distinguish between these possibilities. The authors observed weak and random shifts of porphyrin bands due to spectral drift and therefore introduced a "screening" technique to eliminate experimental artifacts. This involved creating a "simulated" spectrum by deliberately shifting an experimental trace and then subtracting the "shifted" spectrum from the original. This screening methodology eliminated several artifacts which occurred in the isotopically labeled difference spectrum in HO−O_2.[26] In the data we present in this paper no spectral drift is detected, and therefore, no experimental artifacts are present in our observed shifts.

The observation of complex low frequency isotope-dependent vibrational patterns in oxyhemeprotein systems has been reported only once in the literature by Takahashi et al.[26] for HO−O_2. In this system the authors attribute these observations to a coupling of several porphyrin modes with the δ(Fe−O−O) mode. This suggestion is reasonable in light of data consistent with a highly bent structure. Such distortion can induce vibrational coupling between the Fe−O−O bending mode and out-of-plane porphyrin modes as previously discussed.[26] The oxyP450cam vibrational patterns reported here are similar to those for heme oxygenase. However, it is emphasized that the precise molecular factors responsible for such coupling effects in oxyP450cam are unknown and may differ from those responsible for similar experimental observations in HO−O_2. Indeed, we wish to stress that the isotopic evidence for oxyP450cam suggests that the Fe−O−O linkage is less bent than that in HO−O_2. The three body calculation adequately supports an angle of 125−130° for the iron dioxygen linkage in oxyP450cam, although it is tentatively suggested that an 110° angle exists in HO−O_2.[26] Nevertheless, the complicated low energy vibrational patterns suggest that there is unusual "strain" or vibrational mode mixing associated with the Fe−O−O linkage in oxyP450cam.

The presence of several isotopically sensitive bands, in addition to the three identified Fe−O−O modes, suggests that the three body normal-mode calculation, often used in the literature, is an oversimplification for oxyP450cam. Application of a four body normal coordinate calculation to this system is precluded by the absence of a full data set. While the iron−thiolate stretch has been explicitly detailed in the ferric high-spin state of P450cam,[29] no report in the literature has identified this mode in low-spin protein forms, including the oxy complex. Moreover, the Raman intensity of low frequency isotopically sensitive bands are enhanced as the excitation line is changed from 356.4 to 413.1 nm (more in "resonance" with the Soret band) which is in stark contrast to the ν,δ Fe−O−O modes. Since we do not know the precise electronic transitions from which these isotopically sensitive bands originate we cannot rule out Fermi resonance couplings involving the proximal thiolate ligand. However, the Soret band Raman intensity enhancement of the features near 260−320 cm^{-1} suggests that at least some of these modes occur from out-of-plane porphyrin motions which are affected by the O_2 isotopic substitution through vibrational coupling with the dioxygen linkage of oxyP450cam.

The unique intensity relationships for modes arising from the Fe−O−O moiety for thiolate-ligated hemes are deserving of discussion in relation to imidazolate-bound hemes. OxyCo/imidazole heme systems and model compounds display a relationship whereby the intensity of the ν(O−O) vibration upon Soret band excitation is significantly greater than that displayed by the M−O−O modes.[10,11,17,18] This behavior is contrary to that reported for iron/imidazole porphyrin systems where strong M−O−O mode scattering is observed. In fact, the ν(O−O) mode has never been directly observed in these systems.[7,27] The ν(Fe−O_2) of several heme/imidazolate systems has been reported in the literature.[6] This extensive list includes HbO_2 (568 cm^{-1}),[7] MbO_2 (571 cm^{-1}),[7] CcO−O_2 (571 cm^{-1}),[7] FeO_2TPP (575 cm^{-1}),[28,30] FeO_2OEP (572 cm^{-1}),[31] and several FeO_2-TpivPP analogues (558−571 cm^{-1}).[6,32−34] To date only one paper has tentatively assigned the ν(Fe−O_2) for P450cam and a thiolate-ligated model compound.[10] The high quality data presented in this manuscript supports this initial assignment. The selective enhancement of M−O−O modes in oxyCo

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systems has been attributed by Mackin and co-workers[18] to a charge-transfer band, underlying the intense Soret, involving the π^* on the dioxygen moiety and the Co d_{z^2}. Imidazolate-bound oxyhemes utilize a porphyrin $\pi \rightarrow \pi^*$ transition for enhancement of ν, δ (Fe−O−O) modes. Thiolate-ligated porphyrins display strong ν(O−O) intensity but weak scattering from M−O−O modes, which is similar to that displayed in the analogous oxyCo/imidazole porphyrins.[10,11] Charge-transfer conditions are suggested to be responsible for selective mode enhancement. Upon thiolate ligation a mercaptide orbital may overlap with the π^* orbital and donate electron density into the antibonding σ^* orbital, comprising Fe d_{z^2} and $\pi^*(O_2)$, thereby lowering the energies of the Fe−O−O modes, with respect to histidyl-ligated oxyhemes, and providing an alternative path of resonant enhancement.

The observation of several coupled modes, an abnormally high isotopic shift in the ν(Fe−O2) mode, normal coordinate calculations, and a high frequency for the δ(Fe−O−O) bending mode at 402 cm^{-1}, compared with the analogous deformation in $CoHbO_2$ (390 cm^{-1}), are all consistent with the assertion that the Fe−O−O moiety in oxyP450cam appears to be strained in some manner. The highly bent structure in HO−O2 was attributed to unspecified distal pocket interactions[26] which the authors hypothesized had enabled an α-meso carbon of the porphyrin ring to contact the terminal oxygen atom. It has also been suggested in the literature that the ν(Fe−O_2) bandwidths reflect the relative mobility of the Fe−O−O moiety in the active site of hemeproteins.[7] The width of the ν(Fe−O2) for oxyP450cam, at 21 cm^{-1}, is comparable to that reported for HbO_2 (21 cm^{-1}) and MbO_2 (20 cm^{-1}).[7] In both of the latter cases, it is known that specific hydrogen-bonding contact exists between a distal pocket histidine residue and the dioxygen moiety which consequentially restricts the mobility of the Fe−O−O linkage. A similar degree of restraint to that exhibited by HbO_2 and MbO_2 is indicated for oxyP450cam by the width of the ν(Fe−O_2) mode.

The sources of these "restraining forces" in oxyP450cam are possibly a combination of steric camphor substrate contacts (there is no such substrate in HbO_2 or MbO_2) and polarity/hydrogen bonding influences on the Fe−O−O linkage. Evidence does exist for nonbonded contact between camphor substrate and a diatomic adduct of P450cam. Steric contact between camphor and the carbon monoxide adduct of wild-type P450cam was suggested by X-ray crystallography.[25] Moreover, distortion of the Fe−CO linkage in P450cam in the presence of substrate was strongly suggested by solution resonance Raman studies wherein appreciable δ(Fe−CO) intensity was observed.[35] Substrate-free CO-bound P450cam did not display this behavior, and further studies of many CO-bound heme systems indicated activation of this deformation mode required strain being applied.[36] It is not unreasonable to expect stress or strain on this linkage when one considers the functionality of this class of enzyme. Cleavage of the O−O bond in this system is believed to proceed via injection of a second electron and protonation of this species.[4,37,38] This produces 1 equiv of water and generates an, as yet, unidentified intermediate which is hypothesized to be a highly reactive ferryl oxene species, analogous to the semistable compound I -type species formed by the peroxidase class of enzymes. Strain is, therefore, a reasonable requirement to assist bond breakage in P450 chemistry. Moreover, it is known in the literature[4] that different substrates alter the efficiency of hydroxylating reactant and utilize electron equivalents in side reactions such as peroxide production and water formation from the hypothesized compound I branch point. These reaction pathways may be substrate-mediated and the δ(Fe−O−O) mode could prove an excellent probe of active site strain on the linkage, aiding our understanding of the mechanism(s) involved.

The role of hydrogen bonding in proton delivery within this catalytic system has been addressed by extensive use of kinetic solvent isotope effects.[37,38] At least two protons in flight are involved in the rate determining step of substrate hydroxylation for wild-type enzyme[37] while five protons in flight, involving a water wire to bulk solvent, are suggested by removal of the carboxylate switch in the active site.[38] It should be noted that preliminary data using deuterium oxide suggests no exchangeable protons affect the Fe−O−O linkage in oxyP450cam. Such an observation (may) indicate a requirement for conformational changes to occur in the active site, upon injection of the second electron, to assist in formation of these proton wires for bond breakage. Threonine, suggested to play a crucial role in controlled O−O bond cleavage in P450s,[4] is too distant to be a strong hydrogen-bond donor to the dioxygen species but perhaps plays a pivotal role in mediating a transient hydrogen-bonding water network, stabilizing the bound dioxygen before catalysis.[38,39]

Our data set suggests a strain in the Fe−O−O linkage in camphor-bound oxyP450cam which could be important for catalytic coupling. We are currently investigating the nature of substrate structure on active site strain by utilizing the δ(Fe−O−O) mode as an informative and sensitive probe of distal pocket distortions in oxyP450cam.

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