RESONANCE RAMAN STUDIES OF Co—O_2 AND O—O STRETCHING VIBRATIONS IN OXY-COBALT HEMES

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ABSTRACT Strong evidence suggests that the stretching vibration of the bound oxygen can be perturbed by an accidentally degenerate porphyrin ring mode, resulting in two split frequencies. In the Co(II)(TpivPP) (pyridine) $^{18}O_2$ complex, we demonstrate that the $\nu(^{18}O—^{18}O)$ mode, after being shifted from its $\nu(^{16}O—^{16}O)$ value at 1,156 cm^{-1}, undergoes a resonance interaction with the 1,080 cm^{-1} porphyrin mode, giving rise to two lines at 1,067 and 1,089 cm^{-1}. In the O_2 complex of Co(II) mesoporphyrin IX–substituted sperm whale myoglobin, we observed a dramatic intensity increase at 1,132 cm^{-1} upon $^{16}O_2 \rightarrow {}^{18}O_2$ substitution, which is due to the reappearance of the 1,132-cm^{-1} porphyrin mode after the removal of resonance conditions. A decrease in O_2 binding affinity, caused by the proximal base tension, corresponds to an increase in the Co—O_2 stretching frequency. The ν(Co—O_2) at 527 cm^{-1} for the low affinity Co(II)(TpivPP)(1,2-Me_2Im) O_2 complex is 11 cm^{-1} higher than the 516-cm^{-1} value for the high affinity complex (with *N*-MeIm replacing 1,2-Me_2Im). However, in the corresponding iron complexes the reverse behavior is observed, i.e., the ν(Fe—O_2) decreases for the (1,2-Me_2Im) complex. There is a 24-cm^{-1} difference in the Co—O_2 stretching frequencies between Co(II)(TpivPP)(*N*-MeIm)O_2 (at 516 cm^{-1}) and oxy meso CoMb (at 540 cm^{-1}), suggesting a protein induced distortion of the Co—O—O linkage. However, the values for ν(Fe—O_2) are nearly identical between Fe(II)(TpivPP)(*N*-MeIm)O_2 (at 571 cm^{-1}) and oxy Mb (at 573 cm^{-1}), indicating that O_2 binds to myoglobin in the same manner as in the sterically unhindered "picket fence" complex. Evidence is presented that suggests the presence of two dioxygen stretching frequencies due to two different conformers in each of the *N*-MeIm and 1,2-Me_2Im complex of oxy Co(II)(TpivPP).

INTRODUCTION

The determination of the exact nature of dioxygen binding to hemoglobin/myoglobin has been the subject of a considerable amount of research over the past few years (1–13). Various compounds that serve as model systems for the native hemoproteins have proven quite useful. Among these are the cobalt-substituted hemoproteins (4) and cobalt "picket fence" porphyrins (1). Both of these systems are capable of reversible dioxygen binding, with affinities comparable to native hemoglobin/myoglobin.

Some of the most valuable information concerning dioxygen binding may be derived from the vibrational characteristics of the iron (or cobalt)—O_2 moiety. Unfortunately the frequency of bound O—O stretching vibration in oxy hemoproteins has been a puzzle. Infrared studies by Caughey and co-workers (9) reported the first ν(O—O) frequencies at 1,107 (oxy HbA) and 1,103 cm^{-1} (oxy Mb). Collman *et al.* (1) have located the ν(O—O) frequency at 1,160 cm^{-1} in oxy iron picket fence porphyrin, which is quite different from the values for oxy HbA/Mb. The interesting development was the detection (13) of an additional IR (infrared) band at 1,156 cm^{-1} in oxy HbA. Alben et al. (13) interpreted the splitting of the ν(O—O) vibration into two IR bands at 1,107 and 1,156 cm^{-1} in oxy HbA as due to Fermi resonance involving the first overtone of the ν(Fe—O_2) stretch (14) at ~570 cm^{-1}.

Recently, Tsubaki and Yu (15) detected three isotope-sensitive Raman lines at 1,103 (1,107), 1,137 (1,137), and 1,153 (1,152) cm^{-1} in the spectra of oxy CoMb (oxy CoHbA) excited at 406.7 nm. They concluded that the kind of Fermi resonance suggested by Alben et al. (13) is not operative in Co hemoproteins. It has been proposed (15) that the first two frequencies at ~1,105 and 1,137 cm^{-1} arise from resonance interaction between a ν(O—O) mode at ~1,122 cm^{-1} and an accidentally degenerate porphyrin ring mode at 1,123 (1,121) cm^{-1}, whereas the third one represents an unperturbed ν(O—O) vibration from a second conformer. Although the ν(O—O) in Fe hemoproteins and model hemes cannot be resonance enhanced with excitation in the Soret and Q-band regions, available IR data do suggest the possibility of a close correspondence between Fe and Co systems. For example, the IR ν(O—O) in cobalt-substituted oxyhemoglobin A (containing Co-deuteroporphyrin IX) was reported (3) at 1,105 cm^{-1}, which is similar to the 1,107 cm^{-1} value in oxy FeHbA. Also, the ν(O—O) at 1,155 cm^{-1} in oxy Co picket

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fence porphyrin is close to the 1,160 cm^{-1} value in the corresponding Fe complex (1).

The evidence of the aforementioned resonance interaction in oxy CoMb/HbA may not be considered as unequivocal because of the presence of a second conformer and the complex nature of the porphyrin ring vibrations near 1,130 cm^{-1}. Furthermore, there are still a number of unanswered questions related to Raman intensities. Thus, it is desirable to have a model system that exhibits resonance interaction involving a single $\nu(\text{O—O})$ and a porphyrin ring mode.

In the present study, we present $\nu(\text{Co—O}_2)$ and $\nu(\text{O—O})$ data from the resonance Raman study of oxy Co picket fence porphyrin and oxy meso porphyrin IX–substituted CoMb. Clear evidence is found for resonance interaction involving a single $\nu(\text{O—O})$ vibration and a porphyrin ring mode, which generates two split Raman lines with an intensity ratio similar to that found in Co hemoproteins. We also have studied the effects of proximal base tension on the $\nu(\text{Co—O}_2)$ and $\nu(\text{O—O})$ frequencies because of its importance in understanding the phenomenon of cooperativity and the mechanisms of protein control of heme reactivity. The variations of the Co—O_2 and Fe—O_2 bond strengths with respect to binding affinity will be compared and discussed. In addition, we suggest the existence of two conformers in oxy Co hemes, which are induced by proximal bases.

EXPERIMENTAL

Cobalt(II) Porphyrin Complexes

The meso tetra ($\alpha,\alpha,\alpha,\alpha$—*o*-pivalamidophenyl) porphyrinato cobalt (II), Co(II)(TpivPP), was custom-synthesized by Midcentury, Inc. (Posen, IL) according to the method of Collman et al. (16). The sample was chromatographically pure, with very little isomerization. The cobalt tetraphenylporphyrin, Co(II)TPP, was also purchased from Midcentury. All solvents and bases were purified by distillation, including *N*-methylimidazole (Sigma Chemical Co., St. Louis, MO.) and 1,2-dimethylimidazole (Aldrich Chemical Co., Inc., Milwaukee, WI). Samples were prepared with a porphyrin concentration of $\sim 8 \times 10^{-5}$ M and ~200 times mole excess base.

To prepare the oxygenated complexes, the solution of porphyrin and base was transferred to a cylindrical Raman cell and sealed with a rubber septum. The cell was evacuated by vacuum and oxygen was introduced at ~1 atm pressure. The $^{18}O_2$ complexes were prepared in the same manner using $^{18}O_2$ (99% $^{18}O_2$; Stohler Isotope Chemicals Inc., Waltham, MA) at an initial pressure of 1 atm.

To ensure maximum oxygenation, experiments were performed at low temperatures (~7 to −80°C). When the solvent was methylene chloride, the temperature was controlled by a stream of nitrogen, which was cooled as it flowed through a cooper coil immersed in a Dewar flask of liquid nitrogen. The stream of nitrogen was directed at the rotating sample cell, which was placed inside a glass Dewar flask; the temperature was monitored with a thermocouple attached inside the flask. The benzene samples were cooled by a stream of nitrogen, which was cooled as it flowed through a cooper coil immersed in a dry ice–isopropanol bath.

Cobalt(II) Mesoporphyrin IX-Substituted Myoglobin

Mesoporphyrin IX dimethyl ester was purchased from Sigma Chemical Co., Inc., Milwaukee, WI. The porphyrin ester was hydrolyzed in 6 N HCl for 24 h with stirring at room temperature in the dark. The extent of hydrolysis was checked by thin-layer chromatography on a silica gel plate with a solvent system of 2,6—lutidine—water (20:1, v/v). The insertion of cobalt into mesoporphyrin IX free acid was carried out according to Yonetani et al. (17). The cobalt meso heme was purified on a column of silica gel 60 (70–230 mesh ASTM; Merck Chemical Div., Merck and Co., Inc., Rahway, NJ) developed with a lower layer of chloroform:pyridine:water:isooctane (10:30:10:1, by volume). After complete evaporation of the solvent, cobalt meso heme was dissolved in a small amount of glacial acetic acid, followed by filtration on a paper (No. 2; Whatman Inc., Paper Div., Clifton, NJ) The cobalt meso heme was recrystallized by adjusting the pH of the solution to 4.3 with 1 N NaOH solution on ice.

Sperm whale myoglobin was obtained from Sigma Chemical Co., Inc., and further purified in ferric form as described previously (18). The heme group was removed from metmyoglobin by the method described by Teale (19) using 2-butanone. The incorporation of cobalt meso porphyrin IX into apomyoglobin was performed by the method of Yonetani et al. (17) followed by chromatography on a CM-52 cellulose (Whatman Inc., Chemical Separation Div., Clifton, NJ) column in oxygenated form as previously reported (15). The protein concentration (heme basis) was determined spectrophotometrically in deoxygenated form. The extinction coefficient used was 15.0 mM^{-1} cm^{-1} at 542 nm (17).

INSTRUMENTATION

Experiments were performed in a sensitive multichannel, vidicon detector Raman system. It consists of a modified 1402 0.85-m Czerny-Turner double monochromator (600 grooves/mm in additive dispersion; Spex Industries, Inc., Metuchen, NJ), a PAR model 1254 cooled intensified vidicon detector (silicon intensified target), a PAR model 1216 detector controller, a Tektronix 604 monitor (Tektronix, Inc., Beaverton, OR) and a PAR model 1215 OMA 2 microprocessor-based console (Princeton Applied Research, Inc., Princeton, NJ). This system has been described in more detail elsewhere (20). Most of the experiments used a Krypton ion laser (406.7 and 413.1 nm; model 171; Spectra-Physics Inc., Mountain View, CA). Other lasers used include an Argon ion laser (457.9 and 514.5 nm; model CR-8; Coherent Radiation, Inc., Palo Alto, CA) and a CR-500K Krypton ion laser (520.8, 520.9, and 568.2 nm). Laser power at the sample was maintained between 5 and 10 mW (406.7 and 413.1 nm) or between 50 and 100 mW (457.9, 514.5, 520.8, 530.9, and 568.2 nm). The sample was kept in a spinning quartz Raman cell to prevent local heating and to minimize photodissociation. The slit to the monochromator was open 100 μm in width and 0.2 cm in height. Data integration time was 303 s (10,000 delay cycles, 100 readout scans). The spectra were wavenumber calibrated using fenchone as the standard compound (20). Reported wavenumbers are accurate within ±2 cm^{-1}.

RESULTS

The resonance Raman spectra of Co(II)(TpivPP) (*N*-MeIm)O_2 in benzene (C_6H_6) in the region of 100–700 cm^{-1} are presented in Fig. 1. The isotope sensitive line at 516 cm^{-1} is assigned as the $\nu(\text{Co—O}_2)$ stretching frequency. Fig. 2 shows oxy Co(II) mesoporphyrin IX–substituted myoglobin in the same region with the $\nu(\text{Co—O}_2)$ found at 540 cm^{-1}. The isotope shifts of 22 and 24 cm^{-1} for the picket fence complex and myoglobin, respectively, match well with the calculated shifts based on the model Im—Co—O—O with a Co—O—O angle of 130° (Yu, N.-T., and R. B. Srivastava, unpublished results). The frequencies for oxy meso CoMb agree well with those obtained from oxy Co(II) protoporphyrin IX–substituted Mb (539 cm^{-1} for $^{16}O_2$, 516 cm^{-1} for $^{18}O_2$) (15) and CoHbA (540 cm^{-1} for $^{16}O_2$ and 514 cm^{-1} for $^{18}O_2$) (15).

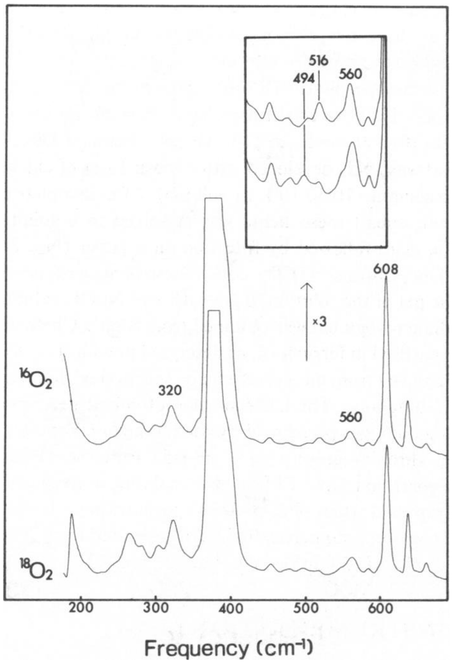

FIGURE 1 Resonance Raman spectra of Co(II)(TpivPP)(*N*-MeIm)O_2 in benzene (C_6H_6) in the 100–700 cm^{-1} region. λ_{exc} = 406.7 nm; concentration, 70 μM. *T* = room temperature.

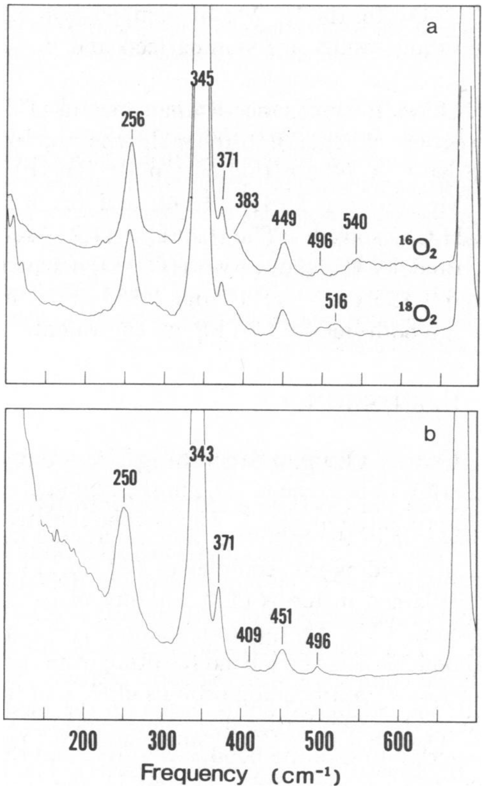

FIGURE 2 (*a*) Resonance Raman spectra of oxy meso CoMb, and (*b*) deoxy meso CoMb in the 100–700 cm^{-1} region. λ_{exc} = 406.7 nm; protein concentration 50 μM (heme basis) in 0.05 M Tris buffer pH 8.3. *T* = room temperature.

The resonance Raman spectra of oxy cobalt mesoporphyrin IX–substituted myoglobin in the region of 900–1,300 cm^{-1} are presented in Fig. 3. A number of changes are evident upon substitution with $^{18}O_2$. The most striking changes are the disappearance of the intense line at 1,137 cm^{-1} and the appearance of the two new lines at 1,068 and 1,132 cm^{-1}. In the difference spectrum, there are one strong positive peak at 1,137 cm^{-1} due to the $^{16}O_2$ complex and two strong negative peaks at 1,068 and 1,129 cm^{-1} due to the $^{18}O_2$ complex.

The resonance Raman spectra of the cobalt picket fence complexes with different proximal bases are presented in Figs. 4, 5, and 6. For the *N*-methylimidazole complex, the $\nu(^{16}O—^{16}O)$ is found at 1,153 cm^{-1}. Upon $^{18}O_2$ substitution an intense line at 1,072 cm^{-1} appears, and the region around 1,108 cm^{-1} undergoes small but real changes. The degree of oxygenation was checked by monitoring the 1,315 cm^{-1} line. In the deoxy complexes this line is at ~1,307 cm^{-1} and shifts to ~1,315 cm^{-1} upon oxygenation. For the pyridine complex, the $\nu(^{16}O—^{16}O)$ is at 1,156 cm^{-1}. Upon isotope substitution, two new lines appear at 1,067 and 1,089 cm^{-1}. The shoulder at ~1,080 cm^{-1} in the $^{18}O_2$ spectrum is probably due to some remaining deoxy complex, as evidenced by the lower frequency shoulder of the 1,318 cm^{-1} line. The 1,2-dimethylimidazole complex shows two isotope sensitive lines at 1,144 cm^{-1} and 1,158 cm^{-1} which, upon isotope substitution, disappear along

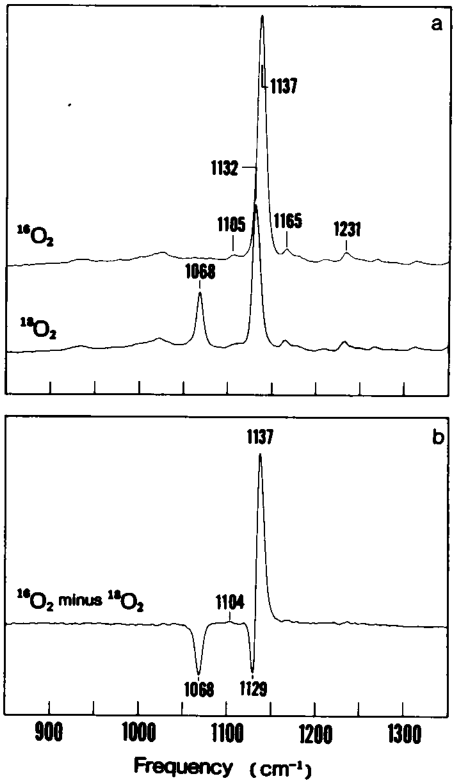

FIGURE 3 (*a*) Resonance Raman spectra of oxy meso Co Mb, and (*b*) difference spectrum ($^{16}O_2$ minus $^{18}O_2$) in the 900–1300 cm^{-1} region. Conditions same as in Fig. 2.

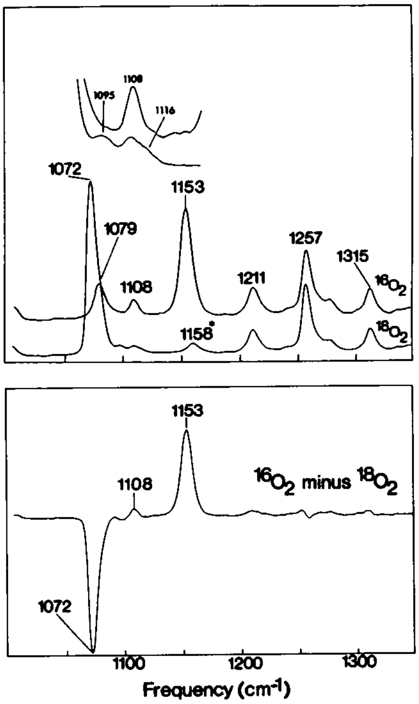

FIGURE 4 Resonance Raman spectra of Co(II)(TpivPP)(*N*-MeIm)O_2 in CH_2Cl_2 in the 1,000–1,350 cm^{-1} region. T = ~−40°C. *, Methylene chloride line.

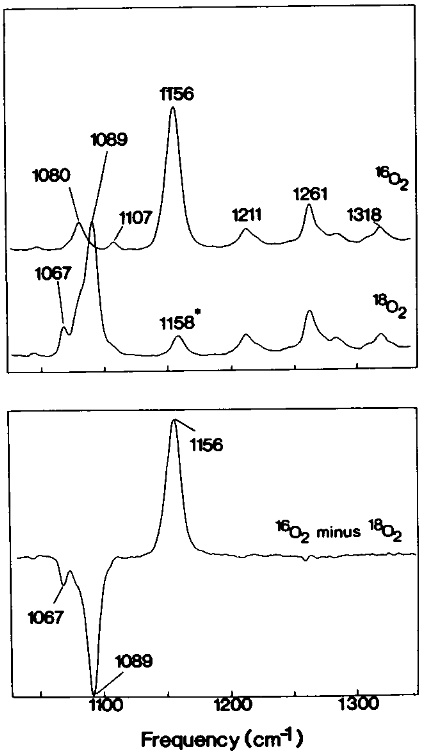

FIGURE 5 Resonance Raman spectra of Co(II)(TpivPP)(py)O_2 in CH_2Cl_2 in the 1,000–1,350 cm^{-1} region. T = −40°C ($^{16}O_2$ spectrum); −65°C ($^{18}O_2$ spectrum). *, Methylene chloride line.

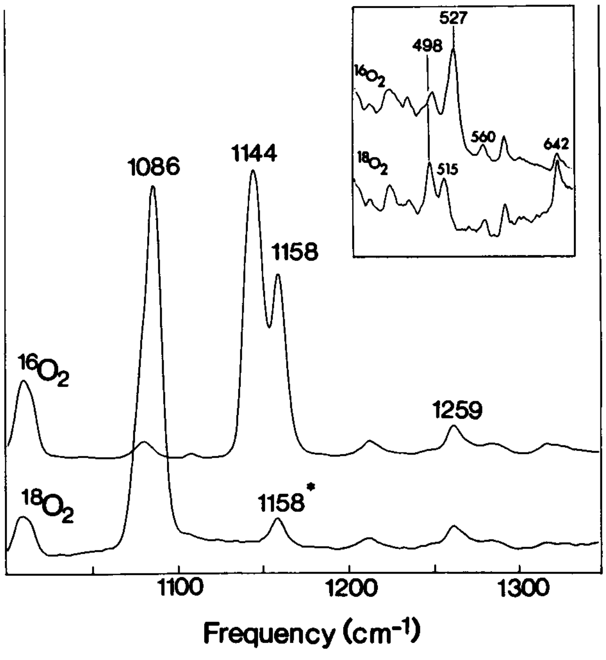

FIGURE 6 Resonance Raman spectra of Co(II)(TpivPP)(1,2-Me_2Im)O_2 in CH_2Cl_2 in the 1,000–1,350 cm^{-1} region. T = ~−80°C (except higher frequency $^{16}O_2$, T = ~−45°C). *, Methylene chloride line. Inset shows region between ~400–650 cm^{-1}.

with the appearance of an asymmetric line at 1,086 cm^{-1}. The ν(Co—O_2) for the 1,2-Me_2Im complex is at 527 cm^{-1}. The preceding results are summarized and presented in Table I.

Fig. 7 shows the resonance Raman spectra of dioxygen complexes of cobalt(II)tetraphenylporphyrin with (*a*) pyridine base, *N, N*-dimethylformamide (DMF) solvent; (*b*) 1,2 Me_2Im base, DMF solvent; and (*c*) 1,2-Me_2Im base, CH_2Cl_2 solvent. The Co(TPP)(1,2 Me_2Im)O_2/CH_2Cl_2 (and DMF) shows two ν(O—O) frequencies at 1,137 and 1,155 cm^{-1} (1,144 and 1,155 cm^{-1} in DMF) even though both sides of CoTPP are equivalent.

DISCUSSION

Cobalt-Oxygen Stretching Frequency

The ν(Co—O_2) of Co(II)(TpivPP)(*N*-MeIm)O_2 is considerably lower than the iron to oxygen stretching frequency in the corresponding iron complex (~570 cm^{-1}) (21). This can be explained in terms of the nature of the metal to oxygen bond (22). In the iron complex, the metal-to-oxygen bond consists of a σ bond resulting from the overlap of one of two π_g^* antibonding orbitals of O_2 and the metal d_{z^2} orbital. Also, the $d\pi(d_{xz}, d_{yz})$ orbital and the other π_g^* orbital overlap to give the bond π-character. In the cobalt complex, there is an extra electron in the $\pi^*(\pi_g^*/d\pi)$ antibonding molecular orbital, which is localized mainly on the dioxygen, according to electron paramagnetic resonance (EPR) studies (23). The higher nuclear charge on

TABLE I
COBALT-OXYGEN AND DIOXYGEN STRETCHING FREQUENCIES (cm^{-1})

Oxy-Cobalt complexes	$\nu(Co—^{16}O)$	$\nu(Co—^{18}O)$	$\nu(^{16}O—^{16}O)$	$\nu(^{18}O—^{18}O)$
meso $CoMbO_2$	540	516	~1,125 (1,104, 1,137)*	1,068
$Co^{II}(TpivPP)(N\text{-}MeIm)O_2$	516	494	1,153 ~1,185‡	1,072 ~1,108 (1,095, 1,116)*
$Co^{II}(TpivPP)(1,2\text{-}Me_2Im)O_2$	527	498	1,144 1,158	1,086
$Co^{II}(TpivPP)(py)O_2$	§	§	1,156	~1,080 (1,067, 1,089)*

*Numbers in parentheses indicate the vibrationally perturbed lines actually observed.
‡Implied from $^{18}O_2$ data.
§Not observed.

cobalt contracts and lowers the energy of the d-orbitals. This results in weaker π-interaction between $d\pi$ and π_g^*, which leads to a lower value for the stretching frequency.

There is a 24 cm^{-1} difference between the $\nu(Co—O_2)$ in $Co(II)(TpivPP)(N\text{-}MeIm)O_2$ (516 cm^{-1}) and oxy meso CoMb (540 cm^{-1}). However, the $\nu(Fe—O_2)$ frequencies are nearly identical in $Fe(II)(TpivPP)(N\text{-}MeIm)O_2$ (571 cm^{-1}) and oxy FeMb (573 cm^{-1}). This suggests that oxygen can bind to the iron in the protein in much the same manner as in the sterically unhindered picket fence complex. What effect does the protein have on the $(Co—O_2)$ moiety that would increase $\nu(Co—O_2)$? Stretching frequency is dependent upon both bond strength and geometry. If the protein distorted the Co—O—O by decreasing the angle, then the effective reduced mass of the $Co—O_2$ is reduced. This effect could increase the stretching frequency, assuming the bond force constants are relatively unchanged. This relationship between protein and model complex was also found in $Fe(II)(TpivPP)(N\text{-}MeIm)CO$ [$\nu(Fe—CO)$] = 489 cm^{-1} (24, 25), and MbCO (ν = 512 cm^{-1}) (24), where the CO is believed to be bent (26) or tilted (27), rather than linear and perpendicular to the porphyrin plane as in the model compound (28, 29). According to recent EPR studies on single crystals of oxy CoMb (30), the Co—O bond is not strictly perpendicular to the porphyrin plane, but inclines ~8° from the normal of porphyrin plane with the Co—O—O angle of 129°.

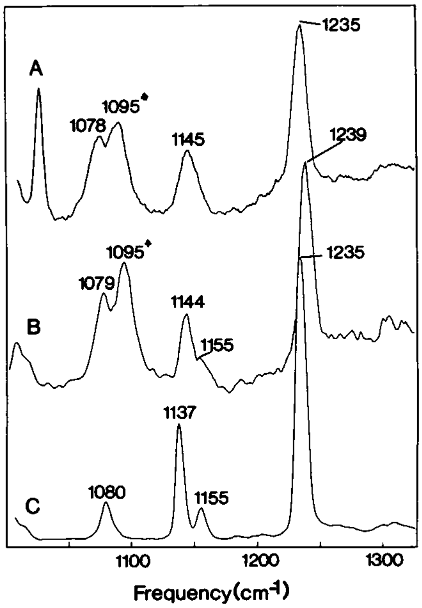

FIGURE 7 Resonance Raman spectra of (*a*) $Co(II)TPP(py)O_2/DMF$, T = ~−40°C; (*b*) $Co(II)TPP(1,2\text{-}Me_2Im)O_2/DMF$, T = ~−40°C; (*c*) $Co(II)TPP(1,2\ Me_2Im)O_2/CH_2Cl_2$, T = ~−80°C (contribution from methylene chloride subtracted out). ‡; DMF line.

The $\nu(Co—O_2)$ at 527 cm^{-1} for the low affinity $Co(II)(TpivPP)(1,2\text{-}Me_2Im)O_2$ complex is 11 cm^{-1} higher than the 516 cm^{-1} value for the high affinity $Co(II)(TpivPP)(N\text{-}MeIm)O_2$ complex. The O_2 binding affinity is lowered by a factor of 6.4 (31) on going from the unstrained (N-MeIm) complex to the (1,2—Me_2Im) complex. But, because the overall free energy change ($\Delta G°$) accompanying the O_2 binding, which determines the binding affinity, need not be localized at the Co—O bond, the Co—O bond strength alone should not be a good indicator of binding affinity.

In x-ray studies of Co(II)(TPP)(N-MeIm) (32) and $Co(II)(TPP)(1,2\text{-}Me_2Im)$ (33), the $Co—N_\epsilon$ bond lengths were found to be 2.16 and 2.20 Å, respectively. This steric interaction, caused by the 2-methyl group, results in a higher $\nu(Co—O_2)$. However, in the corresponding iron complexes, the reverse behavior is observed, i.e., the $\nu(Fe—O_2)$ decreases for the $(1,2\text{-}Me_2Im)$ complexes (25, 34). This can be explained in terms of decreased σ and π-donation from the imidazole. In the cobalt complexes, the primary effect is the increase in σ-bonding between Co and O_2, which is caused by the decrease in the $Co—N_\epsilon$ σ-bonding because the σ-donations from both sides are competing for the same d_{z^2} orbital. Because the overlap between the imidazole π-orbital and the π*-orbital of O_2 is not as extensive as in the case of iron complexes, a decrease in π-donation from $1,2\text{-}Me_2Im$ produces a relatively small decrease in π-bonding between Co and O_2. Thus, the weakening of the $Co—N_\epsilon$ bond in the $(1,2\text{-}Me_2Im)$ complex strengthens the Co—O bond. On the other hand, there may be an extensive overlap in the iron complexes between the imidazole π and $O_2\pi^*$ orbitals (6). This is indicated by the fact that O_2 does not bind well if the proximal base is pyridine (35), which is known to be a poor π-donor. However, O_2 does bind to cobalt with pyridine as proximal base. The importance of π-donation from imidazole to iron $d\pi$ and to O_2 π^* orbital in the Fe—O_2 bonding makes it likely that the much greater decrease in π-bonding between Fe and O_2 more than offsets the increase in σ bonding, resulting in the observed decrease in ν(Fe—O_2) frequency for the (1,2-Me_2Im) complex.

Resonance Interaction Between ν(O—O) and Porphyrin Ring Modes

Examination of the dioxygen stretching region of the resonance Raman spectra of oxy meso CoMb and several cobalt picket fence porphyrin complexes provides evidence for resonance intereaction between ν(O—O) and porphyrin ring modes. This type of interaction was first observed in the resonance Raman spectra of oxy CoMb and oxy CoHbA (15).

In oxy meso CoMb, the $\nu(^{16}O—^{16}O)$ stretching vibration at ~1,125 cm^{-1} is involved in resonance interaction with the porphyrin ring mode at 1,132 cm^{-1}. The result of this interaction is the complete disappearance of the 1,132 cm^{-1} line and the appearance of an intense symmetric line at 1,137 cm^{-1} and a very weak line at 1,104 cm^{-1} (see Fig. 3). The 1,104 cm^{-1} lines in the spectra of oxy CoMb/MbA (15) are somewhat stronger. Upon $^{18}O_2$ substitution, the ν(O—O) shifts to 1,068 cm^{-1}, and the porphyrin mode at 1,132 cm^{-1} clearly reappears because of the removal of resonance conditions. These results are completely analogous to those found in oxy CoMb/HbA (15) except for the absence of signals at ~1,152 cm^{-1} ($^{16}O_2$ spectrum) and ~1,095 cm^{-1} ($^{18}O_2$ spectrum) due to the ν(O—O) of a second Co—O_2 conformer. The residual intensities at ~1,125 cm^{-1} in the spectra of oxy CoMb/HbA (15) are due to the porphyrin ring mode of the second conformer (with ν(O—O) at ~1,152 cm^{-1}), which does not participate in resonance interaction. Before perturbation, the $\nu(^{16}O—^{16}O)$ at ~1,125 cm^{-1} in the spectrum of oxy meso CoMb (Fig. 3) should have an intensity comparable to that of the $\nu(^{18}O—^{18}O)$ at 1,068 cm^{-1}.

In the case of cobalt picket fence complexes, resonance interaction occurs between the $\nu(^{18}O—^{18}O)$ and a porphyrin mode at ~1,080 cm^{-1}. By varying the axial ligand, the ν(O—O) is changed slightly, but enough to show very different degrees of interaction. The strongest evidence for the resonance interaction involves the pyridine complex with $\nu(^{16}O—^{16}O)$ = 1,156 cm^{-1} (Fig. 5). Upon substitution with $^{18}O_2$, the ν(O—O) interacts with the 1,080 cm^{-1} porphyrin mode, resulting in two lines at 1,067 and 1,089 cm^{-1}. For the *N*-methylimidazole complex a $\nu(^{16}O—^{16}O)$ is observed at 1,153 cm^{-1}, 3 cm^{-1} lower than the pyridine complex. Upon substitution with $^{18}O_2$, this line shifts to 1,072 cm^{-1}. Most likely the $\nu(^{18}O—^{18}O)$ simply overlaps with the 1,079 cm^{-1} porphyrin mode, but a small degree of perturbation is possible, especially since the difference spectrum (Fig. 4) shows that intensity is not conserved.[1] Note also that while the $\nu(^{16}O—^{16}O)$'s exhibit a 3 cm^{-1} difference between the two complexes (*N*-MeIm and 1,2-Me_2Im), the $\nu(^{18}O—^{18}O)$'s at 1,072 and 1,089 cm^{-1} differ by 17 cm^{-1}. Also, there is some interaction occurring around the 1,108 cm^{-1} porphyrin mode in the *N*-MeIm complex, which may be due to a second conformer's $\nu(^{18}O^{18}O)$. However, due to the lack of enhancement of the corresponding $\nu(^{16}O—^{16}O)$ expected at ~1,185 cm^{-1}, definitive assignment is not warranted.

There is some evidence of two distinct dioxygen stretching frequencies in the 1,2-Me_2Im complexes. The resonance Raman spectrum of Co(II)(TpivPP)(1,2-Me_2Im)O_2 shows two isotope sensitive bands at 1,144 and 1,158 cm^{-1}. When $^{16}O_2$ is replaced with $^{18}O_2$, a very intense asymmetric line appears at 1,086 cm^{-1}. The question may arise that the two isotope sensitive lines are due to two different dioxygen complexes, i.e., oxygen is bound to the side protected by the picket fence in some of the molecules and bound to the unprotected side in others. To resolve this question, we examined the oxy cobalt tetraphenyl porphyrin (CoTPP) complexes, as shown in Fig. 7. Even though both sides of Co(TPP) are equivalent, Co(II)(TPP)(1,2-Me_2Im) $^{16}O_2$ shows two lines due to $^{16}O_2$ at 1,144 and 1,155 cm^{-1} in dimethylformamide and 1,137 and 1,155 cm^{-1} in methylene chloride. Hence, the ν(O—O)'s in the 1,2-Me_2Im complexes may arise from two conformers of the same complex although the nature of these conformers is not known.

The dioxygen stretching region in these complexes has proven to be very complex and it is difficult to explain exactly the nature of the interaction. One possibility is that a type of Fermi resonance is operative. However, Fermi resonance is usually considered to be the interaction between a fundamental and overtone or combination modes that are accidentally degenerate and of the same symmetry type (36). The perturbation function is given by the anharmonic terms in the potential energy. The interaction leads to a perturbation of the energy levels and a mixing of the eigenfunctions. The mixing usually results in the weaker overtone gaining intensity from the stronger fundamental mode. On the other hand, the interaction in the oxy cobalt hemes appears to involve two fundamentals of comparable intensities. After the perturbation occurs, the result is always one strong, higher energy line and one weak, lower energy line.

The resonance intereaction referred to here is a general resonance phenomenon and does not require the anhar-

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[1] Without vibrational perturbation, the intensity of a resonance Raman line may be changed by isotopic substitution when such a substitution alters the form of normal coordinate so that a significant change occurs in the projection of this mode on the geometry change in the excited state. This does not appear to be the case here.

monic potential. The interaction occurs when a molecule contains several groups of similar frequencies so that the splitting is produced by a repulsion (a type of vibration-mixing) (36). An alternate explanation invokes a dipole coupling mechanism. This is analogous to exciton splitting (37) in visible absorption spectroscopy, where two or more transition dipoles perturb one another depending on the distance between the dipoles and the relative orientation. In vibrational spectroscopy, this type of interaction was observed in the amide I mode of oriented polypeptides (38). The dioxygen acquires its transition dipole through its binding to the metal, although it may be small. However, the separation between the interacting dipoles in the oxy cobalt hemes can be as small as 1.8 Å. According to this mechanism, it is necessary that only accidentally degenerate IR active ring modes (Raman inactive under D_{4h}) can interact with the ν(O—O) vibration. The interacting dipoles may oscillate either in phase or out of phase, resulting in a splitting of the energy levels and intensity variations in the resonance Raman effect. But, because the interaction observed here seems to involve Raman active modes, this mechanism does not appear likely. Again, the exact nature of the interaction between the ν(O—O) of oxy cobalt hemes and nearby porphyrin modes is not fully understood at this time and further experiments should help to clarify this area of the spectrum.

Comparison of ν(O—O)'s Between Hemoproteins and Model Compounds

There may be two conformers in both oxy Co- and oxy Fe-Mb/HbA. Tsubaki and Yu (15) reported two ν(O—O) frequencies at ~1,125 and 1,152 cm^{-1} in the resonance Raman spectra of oxy CoMb/HbA. However, in the present study, we detect only one ν(O—O) frequency at ~1,125 cm^{-1} in oxy meso CoMb. The evidence for the presence of two conformers in oxy FeMb/HbA comes from a combination of resonance Raman and infrared data. The intensity increase at 1,125 cm^{-1} in the resonance Raman spectrum of oxy FeMb upon $^{16}O_2 \rightarrow {}^{18}O_2$ substitution (15) indicates the presence of a ν(O—O) frequency at ~1,125 cm^{-1}, which is involved in resonance interaction with the 1,125 cm^{-1} porphyrin ring mode. On the other hand, infrared data indicate the presence of a second ν(O—O) at 1,148 cm^{-1} (oxy FeMb) (39) and 1,156 cm^{-1} (oxy HbA) (13). One of the conformers in oxy Co- and oxy FeMb/HbA has its ν(O—O) at ~1,150 cm^{-1}, similar to the values found in Co(TpivPP)(*N*-MeIm)O_2 at 1,153 cm^{-1} (see Fig. 4) and Fe(TpivPP)(*N*-MeIm)O_2 at 1,160 cm^{-1} (1). However, the other conformer (possibly the major conformer in hemoproteins) gives rise to its ν(O—O) ~25 cm^{-1} lower, which suggests an additional interaction between bound molecular oxygen and distal residues. The exact nature of the interaction is not known although the sp^2 donation from distal histidine (E7) to the π^* orbital of O_2 was suggested (15). Recent neutron diffraction studies of oxy FeMb (40) reveal an oxygen-histidine hydrogen bond, which could lower the ν(O—O) frequency. EPR measurements (41) have also shown that a hydrogen bond is formed between bound oxygen and the distal histidyl group in oxy CoMb.

Evidence for a Charge-Transfer Band Underlying the Soret

The fact that the ν(O—O) vibration in oxy Co-hemes, but not in oxy Fe-hemes, can be resonance enhanced with excitation in the Soret region suggests the existence of a charge-transfer (CT) transition underlying the intense Soret. Tsubaki and Yu (15) have suggested that this CT transition may be of the type $\pi^*(\pi_g^*O_2/d_{xz}) \rightarrow \sigma^*(d_{z^2}Co/\pi_g^*)$. The electron localized on the dioxygen (23) can be promoted to the antibonding σ^*molecular orbital involving $d_{z^2}Co$ and $\pi_g^*O_2$. This electronic displacement is expected to cause elongation of the Co—O bond and contraction of the O—O bond in the excited state, which could be effective in shifting the origin of the potential energy curve along these coordinates. It is believed that the Franck-Condon scattering mechanism (42) has contributed primarily to the observed ν(Co—O_2) and ν(O—O) intensities. The ν(O—O) at 1,154 cm^{-1} in Co(II)(TpivPP)(*N*-MeIm)O_2/C_6H_6 is most intense with excitation at 413.1 nm, close to the Soret maximum at 412 nm. With excitation in the Q-band region (500–570 nm), neither ν(Co—O_2) nor ν(O—O) vibrations have been detected in oxy CoMb or Co(II)(TpivPP) (*N*-MeIm)O_2.

In contrast, the ν(Fe—O_2) vibration in oxy Fe-heme can be observed with excitation in the Soret and Q-band region. The Raman excitation profile appears to follow the absorption spectrum of oxy Mb/HbA (24, 43). Other Fe-ligand vibrations with similar excitation profiles include ν(Fe—CO) and ν(Fe—NO) (24). It has been suggested (24) that these vibrations are enhanced by the $\pi \rightarrow \pi^*$ transition. Since the (O_2, CO, or NO) π^* orbital is competing with the π^* porphyrin orbital for the iron $d\pi$ electrons, laser excitation into the porphyrin $\pi \rightarrow \pi^*$ transition may cause the elongation of the Fe-ligand bond in the excited state. It is interesting to note that such a mechanism is not effective in bringing out the ν(O—O) vibration in oxy Fe-hemes.

The enhancement of ν(Co—O_2) in oxy Co-hemes with Soret excitation may have some contribution from the porphyrin $\pi \rightarrow \pi^*$ transition mechanism. However, the absence of the ν(Co—O_2) enhancement in the Q-band region indicates the insignificance of such a contribution. Thus, we believe that both ν(Co—O_2) and ν(O—O) modes are enhanced primarily via a charge transfer transition, which is likely of the $\pi^*(\pi_g^*O_2/d_{xz}) \rightarrow \sigma^*(d_{z^2}Co/\pi_g^*O_2)$ type.

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