Vibrational coupling effects in the resonance Raman spectra of O$_2$ adducts of heme proteins and model compounds

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Abbreviations:
CoAz_{p}\alpha
 cobalt(II) 5\alpha,15\alpha-bis[2-(2,2-dimethylpropanamido)phenyl]-10\alpha,
  20\alpha-(nonamidimido-\phi-phenylene)porphyrine
CoAz_{p}\alpha-d_8
 \beta-pyrrole oktadeuteriated CoAz_{p}\alpha
CoAz2
 cobalt(II) 5\beta,15\beta,10\beta,20\beta-bis(nonamidimido-\phi-
  phenylene)porphyrine
CoAz2-d_8
 \beta-pyrrole oktadeuteriated CoAz2
CoDe2
 cobalt(II) 5\beta,15\beta,10\beta,20\beta-bis(dodecanamidimido-\phi-
  phenylene)porphyrine
CoTPP
 cobalt(II) tetrphenylporphyrine
Co(TPP-d_8) or Co(TPP-^3H_8)
 \beta-pyrrole oktadeuteriated Co(TPP)
Co(T_{p}pp)
 cobalt(II) \sigma^4-"picket fence" porphine
DCP
 3,5-dichloropyridine
Hb
 hemoglobin
Hb_{co}
 cobalt(II)-substituted hemoglobin
IR
 infrared
Im
 imidazole
Im-d_2
 4,5-dideuterioimidazole
Im-d_4
 2,4,5-trideuterioimidazole
Mb
 myoglobin
Mb_{co}
 cobalt(II)-substituted myoglobin
py
 pyridine
py-^3H_5 or py-d_5
 perdeuteriated pyridine
RR
 resonance Raman
TNOP
 tri-\eta-octylphosphine oxide
1-Melm
 1-methylimidazole
1,2-DiMeIm
 1,2-dimethylimidazole
4-CNPy
 4-cyanopyridine
4-DMAPy
 4-(dimethylamino)pyridine
4-MI
 4-methylimidazole
4-MI-^3H_1 or 4-MI-d_1
 N-deuteriated 4-methylimidazole
4-MI-^3H_2 or 4-MI-d_2
 2,5-dideuterio-4-methylimidazole
\alpha_{co}
 cobalt(II)-substituted hemoglobin \alpha-chain
\beta_{co}
 cobalt(II)-substituted hemoglobin \beta-chain
\delta
 bending vibration
\nu
 stretching vibration

Keywords: Vibrational coupling effects; Resonance Raman spectroscopy; Dioxygen adducts; Heme proteins

1. Introduction

The mammalian oxygen transport proteins, hemoglobin (Hb) and myoglobin (Mb) are probably the most thoroughly studied of all biomolecules [1–6]. Both are members of the class of proteins, commonly called heme proteins, which contain a heme group (the iron complex of protoporphyrin IX, a tetapyrrole macrolcycle) at the active site. In the case of the oxygen transport proteins (Hb and Mb), the ferrous iron binds molecular oxygen reversibly, oxidation of the iron being inhibited by the protection afforded by the surrounding protein. While the monomeric Mb serves essentially as an oxygen storage protein within muscles, the actual oxygen
transport protein, Hb (which accounts for the bulk of the protein present in red blood cells) is a tetrameric species \((\alpha_2\beta_2)\) which exhibits cooperative ligand binding.

Despite the accumulation of a quite extensive body of knowledge, questions remain unanswered, even at an essentially fundamental level. For example, both proteins possess a (distal) histidylimidazole positioned so as to facilitate hydrogen bonding with the bound dioxygen. The importance of such H-bonding for the oxygenation process continues to attract much attention [7–13]. Perhaps even more surprising is the fact that dispute still continues concerning the number of stable structures [14–19].

The intense interest in the details of dioxygen binding to these proteins prompted an extensive effort by many research groups to develop and study model compounds in an attempt to understand the steric and electronic factors which control oxygen binding in the native systems. These elaborately designed model systems include the “picket-fence” [20–24], “capped” [25–29], “strapped” [30–35], and “jellyfish” [36–40] metalloporphyrins, among others. The essential rationale behind the use of such systems is, of course, that structural and environmental perturbations can be well-controlled and related to observed spectroscopic and functional response. Thus, in order to most effectively employ such model systems, it is important to establish reliable spectroscopic probes of the key elements (i.e., the \(\text{Fe-O}_2\) fragment) in the models and native systems.

Traditionally, vibrational spectroscopy (both infrared (IR) and Raman) has served as a powerful probe of ligand binding to metal centers in coordination and organometallic compounds [41]. Thus, it is not surprising that much effort has been expended in attempts to apply these techniques to the study of ligand binding to heme proteins and model compounds [42–45]. In principle, these methods offer great potential for detection of slight changes in bonding associated with subtle structural perturbations inasmuch as, theoretically, they may provide a direct probe of the bound \(\text{O}_2\) through detection of the fundamental vibrations of the \(\text{Fe-O}_2\) fragment (i.e., \(v(\text{O-O})\), \(v(\text{Fe-O})\) and \(\delta(\text{FeOO})\)). Unfortunately, despite this inherent potential, vibrational spectroscopic studies of these issues and these systems have been hampered by ambiguities arising from several technical and interpretational difficulties.

In the case of IR spectroscopy, the inherently weak \(v(\text{O-O})\) absorption is obscured by an envelope of features associated with vibrations of the peptide. While it is possible, in principle, to extract the weak \(v(\text{O-O})\) absorptions from the other bands in this congested spectral region by employing difference techniques, as discussed in detail by Caughey and co-workers in their report of carefully conducted IR studies [17], many factors can give rise to artifacts in these difference spectra. Thus, there is some impetus to explore the utility of Raman spectroscopy for the study of these issues.

While the normal Raman effect is notoriously weak and lacks the sensitivity to study dilute solutions, the resonance Raman (RR) effect is ideally suited to the study of heme proteins. In this technique great increases in sensitivity and selectivity are realized by using (laser) excitation wavelengths in resonance with the strong electronic absorption bands associated with the active site chromophore (i.e., the
oxygenated heme group). Since the first RR spectra of heme proteins was reported by Strekas and Spiro [46], the technique has been applied by many workers to investigate heme structure in a large number of derivatives [47–53]. Unfortunately, it has not been possible to identify conditions under which the ν(O–O) of the O₂ adducts of native Hb and Mb is effectively enhanced, thus eliminating the possibility of directly probing the Fe–O₂ fragment by observation of this mode.

A major breakthrough was achieved by Yu and co-workers [15,16,54] who demonstrated that the ν(O–O) of the corresponding cobaltous analogues is strongly enhanced by excitation near the very intense (so-called Soret) band which occurs near 400 nm, though it should be pointed out that the mechanism of enhancement is thought to be associated with an underlying charge-transfer transition ascribable to the Co–O₂ fragment. The fact that these cobalt-substituted analogues retain the gross structural and functional properties of the native system [55–59] validates the utility of RR studies of these as an effective strategy to probe the factors which influence oxygen binding.

Given the availability of an effective probe (i.e., RR spectroscopy of the cobalt analogues), systematic studies employing model compounds were undertaken by our group in order to gain insight into the steric, electronic and environmental factors which influence the vibrational spectral patterns of the model compounds so as to provide an interpretive framework for analysis of the RR spectra of the cobalt-substituted proteins.

The purpose of the present review is to summarize the results of these and related studies. As will be apparent, while the essential assumption that RR studies of model compounds are useful for the interpretation of the spectra of these proteins is demonstrated, it will also be clear that the spectra of both the proteins and model systems are severely complicated by an unusually strong (coupling) interaction between the ν(O–O) and internal modes of other fragments of the system, including the trans-axial base and intimately associated molecules of the environment.

2. Vibrationally coupled dioxygen in model compound systems

2.1. Coupling with internal modes of the trans-axial base

2.1.1. Spectral consequences of the effect

2.1.1.1. O₂ adducts of pyridine complexes: the first evidence of the effect. It is appropriate to begin with a quite simple chemical system; that of the dioxygen adduct of the pyridine complex of cobalt tetraphenylporphine, O₂CoTPP(py) [60–62]. Actually, in most of our studies we utilize the β-pyrrole deuterated analogues of the various porphyrins in order to avoid an annoying overlap of the ν(¹⁸O–¹⁸O) with a strong macrocycle mode which occurs near 1084 cm⁻¹ in the case of the natural abundance porphyrins. Thus, for studies with TPP we utilize TPP-²H₈ (abbreviated also as TPP-d₈), so the first example to be considered is O₂Co(TPP-²H₈)(pyr), whose RR spectra are shown in Fig. 1. Traces A and B give
Fig. 1. Resonance Raman spectra of Co(TPP-2H₈) in CH₂Cl₂ solution containing 3% pyridine at −90°C: (A) Co(TPP-2H₈)+no oxygen, 457.9-nm excitation; (B) Co(TPP-2H₈)+¹⁶O₂ (ca. 4 atm.), 457.9-nm excitation; (C) Co(TPP-2H₈)+no oxygen, 406.7-nm excitation; (D) Co(TPP-2H₈)+¹⁶O₂ (ca. 4 atm.), 406.7-nm excitation. "S" denotes the solvent bands (adapted from Ref. [60]).
the RR spectra of Co(TPP-^2H_8)(pyr) and the corresponding O_2 adduct [O_2Co(TPP-^2H_8)(pyr)], respectively, obtained with 457.9 nm excitation. The strong new feature at 1143 cm^{-1} appearing upon oxygenation is associated with the ν(^16O–^16O) mode (vide infra). The spectra shown in traces C and D were obtained using 406.7 nm excitation, where it is evident that the ν(O–O) band is more strongly enhanced relative to the porphyrin macrocycle and solvent bands. For this reason, the 406.7 nm line is used almost exclusively to obtain the RR spectra of the O_2 adducts discussed here.

The region wherein the ν(O–O) is expected to occur is shown in expanded scale in Fig. 2, which illustrates the spectra obtained for variously isotopically labeled derivatives. The solid line in trace A gives the spectrum for the ^16O_2 adduct obtained in CH_2Cl_2. In fact, there it is seen that the strong 1143 cm^{-1} feature possesses a high frequency shoulder which is associated with a solvent mode (the 1156 cm^{-1} feature). For this reason, the spectrum was also obtained in C_2H_2Cl_2 (abbreviated also as CD_2Cl_2) in order to eliminate this spectral overlap. As will be discussed later, this near coincidence of the ν(^16O–^16O) with the solvent mode gives rise to a quite interesting effect, but for now it is useful to avoid this complication by considering only the spectrum obtained in C_2H_2Cl_2, shown by the dashed line in trace A. Thus, in C_2H_2Cl_2, the ν(16O–16O) is observed as a strong symmetric band located at 1146 cm^{-1}.

In many, if not most, metal (M) complexes of diatomic ligands (XY) (i.e., M(XY)), the XY fragment behaves approximately as a simple harmonic oscillator. Thus, given the value of 1146 cm^{-1} for the inherent frequency of the ν(^16O–^16O), it is expected that the ν(^16O–18O) would occur at 1082 cm^{-1} (i.e., the isotopic shift, Δν(^16O/18O), is expected to be 66 cm^{-1}). However, as can be seen from inspection of trace B (solid line), two new features appear when ^16O_2 is replaced by ^18O_2. The strong band is located at 1084 cm^{-1}, while the weaker, lower frequency, feature occurs at 1067 cm^{-1}. This 1067 cm^{-1} ("satellite") feature has a frequency which is similar to that of an internal mode of pyridine [63–65] and it was suspected that it may be ascribable to the axial ligand.

As the spectrum shown by the dashed line in trace B confirms, this satellite band disappears when pyridine-^2H_5 is used in place of pyridine, confirming that the 1067 cm^{-1} arises from the pyridine in the sample. However, it is important to note several key points. First, when the pyridine is replaced by pyr-^3H_5, not only does the 1067 cm^{-1} feature disappear, but the strong 1084 cm^{-1} mode shifts down by 2 cm^{-1} to 1082 cm^{-1}. Furthermore, it is important to note that in the case of ^16O_2 adduct (trace A), this 1067 cm^{-1} feature is not significantly enhanced. Finally, it is important to point out that the relative concentrations of pyridine in both cases (^16O_2 and ^18O_2) are approximately the same, and thus both spectra are relatively

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Fig. 2. Resonance Raman spectra (406.7-nm excitation) of O_2 adducts of Co(TPP-^2H_8) in CH_2Cl_2 solution containing 3% pyridine at -90°C under ca. 4 atm. of O_2 pressure: (A) ^16O_2, dotted line shows the position of ν(16O–16O) in C_2H_2Cl_2 at 1148 cm^{-1}; (B) ^18O_2, dotted line shows the fragment of the spectrum when pyridine-^2H_5 has been used as a base instead of neat pyridine ν(16O–16O) at 1082 cm^{-1} with pyridine-^2H_5; (C) "scrambled" O_2: ^16O_2 + 2^{18}O^{18}O + ^18O_2 (adapted from Ref. [60]).
independent of pyridine concentration (up to much higher concentrations). Essentially the same results are obtained for complexes in which molecular ratio of Co(TPP-2H₈) to pyridine is 1:1; i.e., when there is no excess pyridine in solution [61,62].

The spectrum shown in trace C corresponds to that obtained for a sample of so-called "scrambled oxygen", which contains a mixture of subO₂, ¹⁶O¹⁸O, and ¹⁸O₂. This mixture is obtained by subjecting (1:1) mixtures of ¹⁶O₂ and ¹⁸O₂ to an electrical discharge from a Tesla coil [66]. Prolonged exposure to the discharge yields the statistical distribution of ¹⁶O₂:¹⁶O¹⁸O:¹⁸O₂ (1:2:1), but abbreviated treatment can produce lower amounts of the ¹⁶O¹⁸O component. The relative concentrations can be conveniently monitored by Raman spectroscopy of the gas in the bulb and the discharge halted when the desired ratio is reached. In most cases the statistical ratio was employed, although in special situations (vidē infra) it is quite useful to employ both (1:2:1) and (1:1:1) mixtures. As can be seen in trace C, in addition to the 1143 cm⁻¹ envelope and the 1084/1067 cm⁻¹ doublet (which are associated with the ¹⁶O₂ and ¹⁸O₂ present in the sample), a strong symmetric feature is observed at 1115 cm⁻¹ which can be ascribed to ν(¹⁶O¹⁸O).

Careful consideration of the spectra shown in Fig. 2 reveals the following points. In the absence of labeling experiments (i.e., considering only the spectra shown with solid lines), it would appear that the ν(O–O) occurs at 1143 cm⁻¹ in the case of ¹⁶O₂ and shifts to 1084 cm⁻¹ upon ¹⁸O₂ substitution and that a mysterious "satellite" band occurs at 1067 cm⁻¹ for the ¹⁸O₂ adduct. Using these frequencies, non-ideal isotopic shifts are derived. Thus, the observed Δν(¹⁶O₂/¹⁸O₂) = 1143–1084 = 59 cm⁻¹ and Δν(¹⁶O₂/¹⁸O¹⁸O) = 1143–1115 = 28 cm⁻¹, while the observed Δν(¹⁶O¹⁸O/¹⁸O₂) = 1115–1084 = 31 cm⁻¹. However, upon elimination of the "interfering" modes associated with other fragments of the system (i.e., the spectra shown by dashed lines), the observed isotopic shifts are in perfect agreement with expectation. Thus, Δν(¹⁶O₂/¹⁸O¹⁸O) = 1148–1115 = 33 cm⁻¹, Δν(¹⁶O¹⁸O/¹⁸O₂) = 1115–1082 = 33 cm⁻¹ and, of course, Δν(¹⁶O₂/¹⁸O₂) = 1148–1082 = 66 cm⁻¹.

2.1.1.2. Implications for structural interpretations of spectra. The fact that such effects assume more importance than as interesting spectroscopic phenomena is perhaps best illustrated by consideration of the spectra obtained for the ¹⁶O₂ adducts of the complexes of cobalt porphyrins with 1,2-dimethylimidazole, 1,2-DiMeIm, whose spectra are shown in Figs. 3(A) and 4(A). In both cases, two relatively strong bands appear in the region near 1140–1160 cm⁻¹. In fact, in the first known report of the RR spectra of an O₂ adduct of a cobalt substituted model compound [16], the appearance of two bands for the ¹⁶O₂ was unfortunately taken as evidence for the presence of two distinct conformers. However, as evident upon inspection of the spectra of the ¹⁸O₂ analogues (Figs. 3(B) and 4(B)), only a single strong band is observed for ν(¹⁸O–¹⁸O). It should be pointed out that, in the case of ¹⁸O₂Co(T₃₄PP)(1,2-DiMeIm) (Fig. 4(B)), the weak low frequency shoulder on the ν(¹⁸O–¹⁸O) band is due to overlap with an internal mode of the porphyrin at 1078 cm⁻¹; i.e., in this case the deuteriated porphyrin was not employed. Clearly,
Fig. 3. Resonance Raman spectra (406.7-nm excitation) of dioxygen adducts of Co(THP-2H₈) in CH₂Cl₂ containing 3% of 1,2-dimethylimidazole at −90°C under ca. 4 atm. of O₂ pressure: (A) ^{16}O₂; (B) ^{18}O₂ (adapted from Ref. [60]).
Fig. 4. Resonance Raman spectra (406.7-nm excitation) of dioxygen adducts of Co(T$_{\text{piv}}$PP) in CH$_2$Cl$_2$ containing 3% of 1,2-dimethylimidazole at $-90^\circ$C under ca. 4 atm. of O$_2$ pressure: (A) $^{16}$O$_2$; (B) $^{18}$O$_2$; (C) $^{16}$O$_2$/$^{16}$O$^{18}$O/$^{18}$O$_2$=1/2/1 (adapted from Ref. [60]).
the doublet structure observed in the case of $^{16}$O$_2$ cannot be attributed to two different chemical forms.

Considering the case of Co(TPP-$^2$H$_8$) (Fig. 3), given the value of 1079 cm$^{-1}$ for the inherent frequency of $\nu$($^{16}$O-$^{16}$O), it is expected that the inherent value of $\nu$($^{16}$O-$^{16}$O) is 1145 cm$^{-1}$ (i.e., 1079 + 66 cm$^{-1}$). The base 1,2-DiMeIm is known to have an internal mode located near 1150 cm$^{-1}$ [60]. Thus, interaction of the $\nu$($^{16}$O-$^{16}$O) with this base mode induces a low frequency shift of the “$\nu$(O–O)” mode (by 5–6 cm$^{-1}$) to 1139 cm$^{-1}$ and a corresponding shift to high frequency for the “base” mode by 5–6 cm$^{-1}$ (i.e., 1150–1155 cm$^{-1}$). The point to be emphasized is that, if care is not taken to perform required isotopic labelling studies, erroneous structural interpretations can arise. Therefore, it became important to perform extensive and systematic studies on many complexes in order to assess the magnitude and clarify the details of this type of coupling interaction involving coordinated O$_2$.

2.1.1.3. Systematic model compound studies

2.1.1.3.1. 4-Methylimidazole. An example where each of the O$_2$ isotopomers can couple with a single internal mode of the trans-axial ligand (and one that is of interest from the standpoint of having direct relevance to the heme protein spectra) is the case of O$_2$ adducts of cobalt porphyrin complexes with 4-methylimidazole (4-MI), a close structural analogue of the proximal histidylimidazole of the proteins [67]. The normal Raman spectra of 4-MI, its N-deuteriated (4-MI-2H$_1$) and ring deuteriated (4-MI-3H$_2$) analogues are given in Fig. 5. There it is seen that the natural abundance 4MI possesses a band at 1107 cm$^{-1}$, whereas 4-MI-2H$_3$ (abbreviated also as 4-MI-d$_2$) exhibits no bands in the region between 1100 and 1160 cm$^{-1}$.

The spectra of the O$_2$ adducts of Co(4-MI) and Co(4-MI-d$_2$) are given in Fig. 6. In the case of the $^{16}$O$_2$ adduct in the presence of 4-MI (Fig. 6(A), solid line), the $\nu$(O–O) mode is observed at 1146 cm$^{-1}$ along with a weak feature at 1105 cm$^{-1}$. When 4-MI-d$_2$ replaces 4-MI (trace A, dotted line), the 1105 cm$^{-1}$ band disappears. Simultaneously, the $\nu$(O-$^{16}$O) downshifts by 3 cm$^{-1}$. The $\nu$($^{16}$O-$^{16}$O) mode of $^{18}$O$_2$Co(4-MI) is observed at 1077 cm$^{-1}$ as a strong feature accompanied by the weak bands at 1111 cm$^{-1}$ (trace B, solid line). Again, as in the case of $^{16}$O$_2$ dioxygen adduct, this weak band disappears (and $\nu$($^{16}$O-$^{16}$O) upshifts by 3 cm$^{-1}$) when 4-MI-d$_2$ is used. This spectral behavior is consistent with that expected for vibrationally coupled dioxygen. The $\nu$(O-$^{16}$O), having an inherent frequency of 1143 cm$^{-1}$, interacts with the 1107 cm$^{-1}$ 4-MI mode (Fig. 6(A)) giving rise to two bands at 1146 and 1105 cm$^{-1}$, respectively; i.e., the higher frequency band upshifts, while the lower frequency band downshifts by the same amount (2–3 cm$^{-1}$). Upon substituting 4-MI-d$_2$, this coupling is eliminated. The 1105 cm$^{-1}$ band disappears revealing the inherent frequency of $\nu$(O-$^{16}$O) as 1143 cm$^{-1}$. The same explanation holds for the $^{18}$O$_2$ adduct.

These somewhat subtle effects of vibrationally coupled dioxygen are a consequence of rather weak coupling between modes which are relatively far separated (ca. 30–35 cm$^{-1}$). Fig. 6(C) provides a dramatic illustration of the remarkable spectro-
Fig. 5. Raman spectra of 4-methylimidazole and deuterated analogues (see text) in C\textsubscript{6}H\textsubscript{6}Cl\textsubscript{2} measured at room temperature with excitation at 406.7 nm (10 mW of laser power at the sample). Asterisks mark solvent bands (from Ref. [67]).
Fig. 6. Resonance Raman spectra of dioxygen adducts of Co(TPP-\textsuperscript{2}H\textsubscript{4}) in the presence of 4-MI (solid line) or 4-MI-\textsuperscript{2}H\textsubscript{4} (dashed line) at \(-90^\circ\text{C}\) in C\textsubscript{2}H\textsubscript{4}Cl\textsubscript{2} with excitation at 406.7 nm (10 mW of laser power at the sample): (A) \(^{16}\text{O}_2\); (B) \(^{18}\text{O}_2\); (C) \(^{16}\text{O}_2^{16}\text{O}^{18}\text{O}\) (1/2/1). Asterisks mark solvent bands (from Ref. [67]).
scopic manifestation of strong vibrational coupling; i.e., as the interacting modes become frequency matched. Given inherent \( \nu(^{16}\text{O}^{16}\text{O}) \) and \( \nu(^{18}\text{O}^{18}\text{O}) \) frequencies of 1143 and 1080 cm\(^{-1}\), \( \nu(^{16}\text{O}^{18}\text{O}) \) is predicted to occur at \( \sim 1111 \) cm\(^{-1}\), i.e., midway between 1143 and 1080 cm\(^{-1}\). Accordingly, in the case of 4-MI-d\(_3\) (no base mode is present in this region), the \( \nu(^{16}\text{O}^{18}\text{O}) \) is observed at 1111 cm\(^{-1}\) along with \( \nu(^{16}\text{O}^{12}\text{O}) \) and \( \nu(^{18}\text{O}^{18}\text{O}) \) at 1143 and 1080 cm\(^{-1}\), respectively. When 4-MI is used (trace C, solid line), the RR spectrum changes dramatically. The close matching of \( \nu(^{16}\text{O}^{18}\text{O}) \), having an inherent frequency of 1111 cm\(^{-1}\), with the interacting 4-MI mode at \( \sim 1107 \) cm\(^{-1}\) results in very efficient coupling which gives rise to two fairly strong bands, of roughly equal intensity, located at \( \pm 8 \)–10 cm\(^{-1}\) from their inherent frequencies of \( \sim 1107 \) and 1111 cm\(^{-1}\) (i.e., at 1099 and 1121 cm\(^{-1}\)).

2.1.1.3.2. 3,5-Dichloropyridine. Having documented the existence of this coupling effect in several different model systems, a system was sought which would prove useful for a thorough and systematic study. In the case of \( \text{O}_2 \) adducts of cobalt porphyrin complexes with 3,5-dichloropyridine (DCP), the vibrational interaction between \( \nu(\text{O}–\text{O}) \) and an internal mode of DCP is of adequate strength to permit an investigation of the energy-matching dependence of the interaction modes over a controlled range of frequency separations [68–71].

The spectra of the \( \text{O}_2 \) adducts of \( \text{Co}(\text{TPP-d}_8)(\text{DCP}) \) in \( \text{C}_2\text{H}_2\text{Cl}_2 \) are shown in Fig. 7. As is seen in trace A, in the spectrum of the \( ^{16}\text{O}_2 \) adduct, a strong band is observed at 1160 cm\(^{-1}\) along with a weak feature at 1112 cm\(^{-1}\). In the case of the \( ^{18}\text{O}_2 \) adduct (trace B) both the 1160 and 1112 cm\(^{-1}\) bands disappear and are replaced by bands occurring at 1121 cm\(^{-1}\) (weaker) and 1086 cm\(^{-1}\) (stronger). One possible interpretation of these observations is that the dioxygen adduct exists in two chemical (or structural) forms which exhibit \( \nu(^{16}\text{O}^{16}\text{O}) \) frequencies at 1160 and 1112 cm\(^{-1}\). The corresponding \( ^{18}\text{O}_2 \) adducts would also be expected to exhibit two \( \nu(^{18}\text{O}^{18}\text{O}) \) bands, and two bands are indeed observed at 1121 and 1086 cm\(^{-1}\). According to this interpretation, the form exhibiting the highest frequency \( \nu(\text{O}–\text{O}) \) would yield a \( \Delta \nu(^{16}\text{O}_2^{18}\text{O}_2) \) of 39 cm\(^{-1}\) (1160–1121 cm\(^{-1}\)) and the second form (lower frequency \( \nu(\text{O}–\text{O}) \)) would yield a \( \Delta \nu(^{16}\text{O}_2^{18}\text{O}_2) \) of only 26 cm\(^{-1}\) (1112–1086 cm\(^{-1}\)). These \( \Delta \nu(^{16}\text{O}_2^{18}\text{O}_2) \) values are quite small compared to that predicted (~65 cm\(^{-1}\)) by the diatomic harmonic oscillator approximation. Furthermore, given the observed intensities of these bands, this approach would also require that either the relative population or the inherent RR scattering efficiencies of the two isotopic species are different. That is, it would be necessary to consider the higher frequency form to predominate for the \( ^{16}\text{O}_2 \) adduct and the lower frequency form to be the major form for the \( ^{18}\text{O}_2 \) adduct. Alternatively, it would require that \( \nu(^{16}\text{O}^{16}\text{O}) \) has an inherently greater scattering efficiency in the second chemical form.

Such tenuous arguments can be avoided by invoking vibrational coupling of

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Fig. 7. Resonance Raman spectra of \( \text{Co}(\text{TPP-d}_8)(\text{DCP})\text{O}_2 \) in \( \text{C}_2\text{H}_2\text{Cl}_2 \) at \(-90^\circ\text{C}\) (excitation at 406.7 nm, 10 mW laser power at the sample): (A) \( ^{16}\text{O}_2 \); (B) \( ^{18}\text{O}_2 \); (C) \( ^{16}\text{O}_2^{16}\text{O}^{18}\text{O}_2 \) (1:2:1); (D) \( ^{16}\text{O}_2^{18}\text{O}^{16}\text{O}^{18}\text{O}_2 \) (1:1:1). The 1050 cm\(^{-1}\) is a solvent band (from Ref. [68]).
$\text{CoTPP-d}_8(\text{DCP})\text{O}_2$

$\text{C}_2\text{H}_2\text{Cl}_2$

A

B

C

D

$\text{INTENSITY}$

$950 \quad 1100 \quad 1250$ cm$^{-1}$

$\text{O}_2$: $^1$ $^1$ $^1$

$\text{O}_2$: $^1$ $^1$ $^1$

$\text{O}_2$: $^1$ $^1$ $^1$

$\text{O}_2$: $^1$ $^1$ $^1$
v(O–O) with an internal mode of the (trans) coordinated DCP. The Raman spectrum of free DCP in CH₂Cl₂ exhibits, among others, two strong bands of comparable intensity at 1109 and 1012 cm⁻¹. While small shifts may be associated with coordination of the DCP, it is reasonable to expect internal modes of the coordinated ligand to occur near these 1110 and 1010 cm⁻¹ regions. Inasmuch as the inherent frequencies of the v(O–O) modes for the various dioxygen isotopomers apparently occur between ~1090 and ~1160 cm⁻¹, interactions with the ~1110 cm⁻¹ ligand mode may be anticipated. As will be discussed later, such interactions may be treated quantitatively to provide estimates of induced shifts and relative intensities of the coupled modes [71]. However, the interpretation of the spectral data for this system (shown in Fig. 7) can be briefly summarized here, as follows. The strong 1160 cm⁻¹ feature (trace A) is assignable to v(¹⁶O–¹⁶O) and the weaker 1112 cm⁻¹ feature to an enhanced (coupled) internal mode of the coordinated DCP. Their inherent frequencies are 1156 and 1115 cm⁻¹, respectively; i.e., the higher frequency component is upshifted, while the lower frequency component is downshifted (by the same amount, 3–4 cm⁻¹) because of the vibrational coupling interaction. Given an inherent frequency for the v(¹⁶O–¹⁸O) mode of 1156 cm⁻¹, the harmonic approximation predicts the occurrence of v(¹⁸O–¹⁸O) at 1090 cm⁻¹. This latter frequency is also close in energy to the internal ligand mode at 1115 cm⁻¹. Interaction of these two modes yields two RR bands, both of which are shifted from their inherent frequencies by 5 ± 1 cm⁻¹ (the accuracy of the frequency measurement is ± 1 cm⁻¹); i.e., bands are observed at 1086 and 1121 cm⁻¹ (trace B).

Further confirmation of this coupling, and a clear demonstration of the spectroscopic complexity that may result from such behavior, are provided by the study of adducts formed with scrambled dioxygen (i.e., ¹⁶O₂:¹⁶O¹⁸O:¹⁸O₂). The RR spectrum of the solution containing the adduct formed in the presence of 1:2:1 scrambled dioxygen is shown in Fig. 7(C). In this figure, the band observed at 1160 cm⁻¹ is readily assignable to the ¹⁶O₂ adduct, while the 1121 and 1086 cm⁻¹ features are attributable to the ¹⁸O₂ adduct. However, new features are observed at 1132 and 1108 cm⁻¹. The 1108 cm⁻¹ band overlaps and obscures the band expected at 1112 cm⁻¹ (i.e., the weak ligand mode associated with the ¹⁶O₂ adduct). This complex spectroscopic pattern is entirely consistent with the above interpretation. Thus, given a ν(¹⁶O–¹⁶O) inherent frequency of 1156 cm⁻¹, the diatomic harmonic approximation would yield a ν(¹⁶O–¹⁸O) frequency of 1123 cm⁻¹. This frequency is within 8 cm⁻¹ of the inherent frequency of the coordinated ligand mode, and these two vibrationally couple, resulting in splitting of the coupled modes. Namely, interaction of the (high frequency) ν(¹⁶O–¹⁸O) with the 1115 cm⁻¹ DCP mode results in shifts of the coupled modes by +9 cm⁻¹ (1123–1132 cm⁻¹) and −7 cm⁻¹ (1115–1108 cm⁻¹) from their inherent frequencies. Further support for this explanation is provided by the spectrum of the 1:1:1 scrambled dioxygen adducts shown in Fig. 7(D), which confirms "linkage" of the 1132 and 1108 cm⁻¹ features. Thus, in this case the intensities of these two bands, relative to one another, remain similar to those observed in the 1:2:1 scrambled case, but intensities of both of them are proportionately decreased, relative to those associated with the ¹⁶O₂ and ¹⁸O₂ adducts.
2.1.2. The utility of coupling interactions as a structural probe

The previously discussed studies document and describe the spectral consequences of coupling interactions between ν(O–O) and internal modes of the trans-axial ligand. While it is obvious that such effects can severely complicate the RR spectra of these species, it is also true that such effects provide information which is not readily accessible by other methods. Specifically, the changes in spectral patterns which result from variations in coupling parameters are apparently greater than shifts in the inherent frequencies; i.e., slight structural perturbations which are insufficient to effect a change in vibrational frequency of the bound dioxygen are, in some cases, sufficient to induce an easily discernable change in the observed spectral pattern. Two separate studies are now summarized which nicely demonstrate this utility for detection of very subtle structural effects.

2.1.2.1. The effect of hydrogen bonding of (trans-axial) imidazole ligands on the observed spectral patterns. One control mechanism for the reactivity of heme proteins which has received considerable attention [72–76] involves alteration of the properties of the coordinated histidyl imidazole as a result of hydrogen bonding of the imidazole N–H to other active site residues or the peptide framework. The possible influence of such proximal side hydrogen bonding on the stability of O₂ adducts of Hb and Mb has been extensively discussed [72,75]. As will be seen, model compound studies clearly demonstrate the potential utility of vibrational coupling effects for revealing such subtle factors.

As will be made evident in the following discussion, the O₂ adducts of the imidazole complexes of cobalt porphyrins exhibit complicated RR spectral patterns characterized by multiple oxygen-isotope sensitive bands [77,78]. In this study we have employed the cobalt complex of a highly protected porphyrin (i.e., 5α,15α-bis[2-(2, 2-dimethylpropanamido)phenyl]-10α, 20α-(nonanediamido-o-phenylene) porphyrin, abbreviated CoAz₅₋₁₀; its structure is shown later in the paper in Fig. 12) to eliminate the direct interaction between bound O₂ and other solution components. This facilitates selective modulation of the properties of the trans-axial bound imidazole by variation of solute conditions.

The RR spectrum of the ^1⁶O₂ adduct of CoAz₅₋₁₀ exhibits two bands at 1158 and 1138 cm⁻¹ (Fig. 8(A)). The spectrum of the ^1⁸O₂ adduct is shown in the same figure in trace B. In this case, the β-pyrole deuteriated porphyrin was utilized to avoid overlap of the ν(^1⁸O–^1⁸O) with the porphyrin mode at 1078 cm⁻¹. As can be seen in trace B, two new bands appear at 1068 and 1082 cm⁻¹, the higher frequency band showing the greater intensity. Although the appearance of two bands in both traces A and B in the ν(O–O) region may suggest the presence of two structural conformers, the result shown in Fig. 8(C) demonstrates that this cannot be the correct interpretation. In this case (trace C) the solution contains the ^1⁶O₂, ^1⁸O₂ and ^1⁸O¹⁸O adducts (1:2:1 “scrambled” dioxygen). The strong symmetric band at 1111 cm⁻¹ is readily assigned to ν(^1⁶O–^1⁸O); i.e., there is no evidence for the presence of a second conformer. Instead, the appearance of multiple bands discussed in traces A and B is the result of vibrational coupling of ν(^1⁶O–^1⁸O) and ν(^1⁸O–^1⁸O) with different internal modes of coordinated imidazole.
Fig. 8. Resonance Raman spectra of O₂ adducts of CoAzₚivα complex with imidazole. Excitation at 406.7 nm (~10 mW of laser power at the sample). The 1051 cm⁻¹ band is due to C²H₅Cl₂ (from Ref. [77]).
The validity of this interpretation was supported by comparison of the corresponding spectra obtained with deuterated analogues of imidazole, where different vibrational patterns are observed for the adducts bearing axial ligated 4,5-dideuterioimidazole (Im-d₂) and 2,4,5-trideuterioimidazole (Im-d₃) [77]. The point is that these deuterated imidazole analogues have identical chemical properties and therefore (in the absence of vibrational interaction) are expected to yield identical spectra. The complex behaviour observed in the RR spectra is thus the result of complicated vibrational mode coupling. Nevertheless, it is satisfying to point out that this complete set of complex data could be reasonably explained, in an internally consistent manner, by invoking vibrational coupling interactions among the specific modes of the various imidazole and O₂ isotopomer pairs.

Returning to analysis of the spectra in Fig. 8, approximation of the bound dioxygen as a harmonic oscillator leads to an expected isotopic shift of \( \Delta \nu(^{16}O₂⁻^{18}O₂) \) of \( \approx 66 \text{ cm}^{-1} \). That is, given a \( \nu(^{16}O⁻^{18}O) \) inherent frequency of 1111 cm\(^{-1}\), the \( \nu(^{16}O⁻^{16}O) \) and \( \nu(^{18}O⁻^{18}O) \) modes are expected to occur at 1144 and 1078 cm\(^{-1}\), respectively. The spectrum of free imidazole exhibits a band near 1144 cm\(^{-1}\) that is expected to shift up to \( \approx 1150 \text{ cm}^{-1} \) upon coordination [79–81]; it was actually determined to be 1152 cm\(^{-1}\) by comparison of more extensive data [77]. Interaction of the \( \nu(^{16}O⁻^{16}O) \) mode (inherent frequency of 1144 cm\(^{-1}\)) with this mode results in the appearance of two bands shifted by \( \approx 6 \text{ cm}^{-1} \) from their inherent frequencies; i.e., the 1144 and 1152 cm\(^{-1}\) modes shift to 1138 and 1158 cm\(^{-1}\). The lower frequency mode contains the major contribution of \( \nu(O⁻O) \) and thus exhibits greater intensity.

The RR spectra presented in Fig. 8 were recorded in the presence of large excess of imidazole; ligand concentration >100× cobalt porphyrin concentration. At this high concentration in nonprotic solvents imidazole self-associates [82–84]. Thus, it is likely that spectra presented in this figure correspond to O₂ adducts in which the trans-coordinated imidazole is hydrogen bonded to excess imidazole present in the solution. Obviously, such interactions should be decreased or eliminated by increasing the temperature or lowering the concentration of imidazole. The interesting spectral patterns shown in Figs. 9 and 10 support this expectation.

In Fig. 9 are shown the spectra obtained for the \((^{16}O₂)\text{CoA₃Z₃vπxx(Im)}\) complex in the presence of a slight excess of imidazole as a function of temperature. At 185 K the spectral pattern previously shown in Fig. 8(A) is observed; i.e., two RR bands are seen at 1138 and 1158 cm\(^{-1}\) (trace A). However, at room temperature (trace D) these two bands have practically disappeared, and two new bands have emerged at 1127 and 1148 cm\(^{-1}\). At the two intermediate temperatures (traces B and C), both sets of bands are observed. Essentially the same behavior is observed as the concentration of imidazole is varied while the concentration of cobalt porphyrin is kept constant (Fig. 10). At a base concentration of 1 \times 10⁻² M, the 1138 and 1158 cm\(^{-1}\) doublet is dominant (trace A) while at 6 \times 10⁻⁴ M the 1127/1148 cm\(^{-1}\) pair dominates (trace C). It is interesting to note that at very low imidazole concentrations (traces D and E) a base-free dioxygen adduct is apparently formed exhibiting the \( \nu(^{16}O⁻^{16}O) \) at 1173 cm\(^{-1}\), which shifts to 1107 cm\(^{-1}\) upon \(^{16}O₂/^{18}O₂\) isotopic substitution [78].
Fig. 9. Influence of temperature on resonance Raman spectra of the $^{16}$O$_2$ adduct of CoAz$_{piv}\alpha\alpha$ complex with imidazole. Spectral conditions as in Fig. 8 (from Ref. [77]).
Fig. 10. Influence of imidazole concentration on resonance Raman spectra of the $^{16}$O$_2$ adduct of CoAz$_{piv}^{\alpha\alpha}$ complex with imidazole. Temperature at ~185 K. Spectral conditions as in Fig. 8 (from Ref. [77]).
The results presented in Figs. 9 and 10 indicate that the O2 adduct that has a hydrogen-bonded imidazole axial ligand gives rise to the 1138/1158 cm\(^{-1}\) pair, while the non-hydrogen-bonded analogue is characterized by the 1127/1148 cm\(^{-1}\) pair of bands. To provide further support for this interpretation, we have included a study wherein a third solution component, which is known to form a strong hydrogen bond with imidazole [83], is added. The RR spectra shown in Fig. 11 demonstrate the effect of adding tri-n-octylphosphine oxide (TONP) to a solution containing the \(^{16}\)O\(_2\) adduct at low imidazole concentration. As is evident in the figure, the 1127/1148 cm\(^{-1}\) pair, characteristic of non-hydrogen-bound imidazole, is converted to the 1138/1158 cm\(^{-1}\) doublet upon addition of TONP. Obviously, this behavior is consistent with the contention that the 1138/1158 cm\(^{-1}\) pattern is characteristic for the hydrogen-bonded imidazole dioxygen adduct.

The most reasonable interpretation of these two distinctly different spectral patterns is that the inherent frequencies of the interacting with v\(^{(16}\text{O}-^{16}\text{O})\) imidazole internal modes are different for the two types of ligated imidazole. Thus, the hydrogen-bonded form possesses an internal mode at 1152 cm\(^{-1}\) that couples with v\(^{(16}\text{O}-^{16}\text{O})\) at 1144 cm\(^{-1}\) giving rise to the pair of bands at 1138 and 1158 cm\(^{-1}\) (i.e., +6 and -6 cm\(^{-1}\) shifts, respectively). The observation of a strong 1148 cm\(^{-1}\) band along with a weak feature at 1127 cm\(^{-1}\), indicates that the non-hydrogen-bonded axial imidazole possesses an internal mode having an inherent frequency of 1131 cm\(^{-1}\). Interaction of this mode with the v\(^{(16}\text{O}-^{16}\text{O})\) mode at 1144 cm\(^{-1}\) gives rise to two bands at 1127 and 1148 cm\(^{-1}\) (i.e., shifted by -4 and +4 cm\(^{-1}\) from their inherent frequencies). It is also satisfying to note that, as expected (vid\' infra), the strength of the observed coupling is greater for the former case where the inherent frequency difference is 8 cm\(^{-1}\) (1152–1144 cm\(^{-1}\)) than for the latter, where this difference is 13 cm\(^{-1}\) (1144–1131 cm\(^{-1}\)). That is, the observed frequency perturbations are 6 and 4 cm\(^{-1}\), respectively, and the 1158/1138 cm\(^{-1}\) ratio is larger than the 1127/1148 cm\(^{-1}\) ratio. Finally, to conclude this section, it is important to emphasize that hydrogen bonding of one trans-axial imidazole with other solutes does not lead to changes in the inherent frequencies of the v(O–O). The only spectral consequence of such hydrogen bonding is the change in the observed spectral pattern that results from alteration of vibrational coupling of v(O–O) with internal modes of the trans-ligated imidazole.

2.1.2.2. Axial ligand distortions revealed by coupling effects. Another heme protein control mechanism which has attracted much attention, and which is rather difficult to probe, is the influence of steric-induced distortion of proximal histidine bonding to the heme. Though it is expected that vibrational spectroscopy would be an obviously useful probe of such effects, it has not proven possible to directly monitor the internal modes of histidine by ultraviolet RR methods and prospects do not seem hopeful [79–81]. Thus, the ability to probe internal modes of the proximal histidine indirectly via these coupling effects with v(O–O), offers a unique opportunity. In order to assess this potential, a systematic study involving several different axial ligands (pyridine, imidazole and their derivatives) and several super-structured porphyrins (which are capable of inducing distortions in axial ligand disposition)
Fig. 11. Resonance Raman spectra of the $^{16}\text{O}_2$ adduct of CoAz$_{piv\alpha\alpha}$ complex with imidazole. The effect of tri-$n$-octylphosphine oxide (TNOP); imidazole concentration $\sim 1 \times 10^{-3}$ M, TNOP concentration $\sim 5 \times 10^{-2}$ M. Temperature in both experiments $\sim 185$ K. Spectral conditions as in Fig. 8 (from Ref. [77]).
was undertaken [61]; the structures of the cobalt (II) complexes of the three superstructured porphyrins [39] utilized being given in Fig. 12.

As was shown earlier, an internal mode of pyridine, having an inherent frequency near 1070 cm\(^{-1}\) efficiently couples with \(\nu^{(18}\text{O}-^{18}\text{O})\) (Section 2.1.1.1). Thus, it is most convenient to concentrate attention on the \(^{18}\text{O}_2\) adducts. The RR spectra of the \(^{18}\text{O}_2\) adducts of the pyridine complexes with CoAz2 and CoDe2 are given in Fig. 13. As can be seen by comparison of traces A and B, an internal pyridine mode is observed at 1075 cm\(^{-1}\) (trace A). When pyridine-d\(_3\) is used this band disappears and the inherent frequency of the \(\nu^{(18}\text{O}-^{18}\text{O})\) is revealed at 1086 cm\(^{-1}\) (trace B). This 2 cm\(^{-1}\) downshift of the \(\nu^{(18}\text{O}-^{18}\text{O})\) upon removal of the coupling implies that the inherent frequency of the internal coordinated pyridine mode is 1077 cm\(^{-1}\); i.e.,

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Structures of Co(Az2), Co(De2) and Co(Az\text{-pivaa}).}
\end{figure}
the inherent frequency is expected to be 2 cm\(^{-1}\) higher than the observed 1075 cm\(^{-1}\), given the 2 cm\(^{-1}\) downshift of the \(\nu(18\text{O}-18\text{O})\). Thus, by comparison of Figs. 2(B) with 13(A), it is clear that the presence of the strap in the complex with CoAz2 imposes an encumbrance to trans-axial ligand binding which, in turn, induces a +8 cm\(^{-1}\) (1077–1069 cm\(^{-1}\)) frequency perturbation of the internal mode of the bound pyridine.

In addition to the 8 cm\(^{-1}\) upshift induced by the steric constrains of the strap, a detectable weakening in the strength of vibrational coupling is observed. Thus, as is shown in Fig. 13(C), when toluene-d\(_8\) is used as the solvent, no satellite feature is observed in this region. The solvent-induced shift of \(\nu(18\text{O}-18\text{O})\) from 1086 cm\(^{-1}\) (trace B) to 1096 cm\(^{-1}\) (trace C) is comparable to that observed for analogous (unprotected) CoTPP adducts [84] and implies that the strap does not impede interaction of CH\(_2\)Cl\(_2\) with the bound dioxygen. The essential point here is that, in the case of CoAz2, the bound pyridine is apparently sufficiently distorted to weaken vibrational coupling to such an extent that the two modes (1077 and 1096 cm\(^{-1}\)) are non-interacting. On the other hand, in the case of unrestrained pyridine bonding,
such as CoTPP(py)\(^{18}\text{O}_3\) complex in toluene [84], coupling is observed between the 1067 and 1094 cm\(^{-1}\) modes.

The 8 cm\(^{-1}\) upshift of the pyridine mode is apparently caused by the steric hindrance between the strap and pyridine molecule that, in turn, influences the \(\beta(\text{CCH})\) vibrations. In “unprotected” metalloporphyrins, such as CoTPP, the axial ligand is perpendicular to the mean porphyrin plane and tends to take the eclipsed orientation with respect to the N–Co–N axis [85]. In the case of the 9-carbon atom polymethylene strap (CoAz2) pyridine is too large to fit into the cavity. Surprisingly, such a distorted orientation of pyridine does not significantly influence the strength of the cobalt–oxygen linkage since the \(v(\text{Co–O})\) and \(v(\text{O–O})\) exhibit inherent frequencies similar to the dioxygen adducts of Co(\(\text{TP}^{\text{pp}}\text{PP})\) [84].

It is interesting to note that this apparent axial ligand distortion is relaxed in the case of the \(\text{O}_2\) adducts with CoDe2, which possesses a longer, more flexible strap. As shown in Fig. 13(D), the \(v(\text{O}^{18}\text{O}–\text{O}^{18}\text{O})\) is observed at 1086 cm\(^{-1}\), as a shoulder overlapped with the 1082 cm\(^{-1}\) porphyrin band and the internal pyridine mode again appears at 1067 cm\(^{-1}\) (inherent frequency of 1069 cm\(^{-1}\)).

Steric hindrance between the strap and the axial ligand also influences other physicochemical properties of metalloporphyrins. For example, Kyuno et al. [39] reported that the equilibrium constant values for pyridine and 1-methylimidazole (1-MeIm) binding to CoAz2 are about 100-fold less than those for CoDe2 or CoAz\(^{\text{pp}}\text{pax}\) complexes. They pointed out that the binding of these nitrogenuous ligands is inhibited selectively in the case of CoAz2. Debois et al. [40] showed that iron(II) complexes of CoAz\(^{\text{pp}}\text{pax}\), when reacted with 1-MeIm, formed mainly a 5-coordinate complex since the bulky pickets and a short strap restrict the axial ligand from binding inside the porphyrin “pocket”. Thus, 1-MeIm binds from the “unprotected” side of the porphyrin plane. When the 9-carbon atom polymethylene strap is replaced by a 12-carbon atom strap, a 6-coordinate complex is formed; i.e., the size of the cavity is large enough to accommodate the axial ligand. They also reported that the \(v(\text{Fe–O}_2)\) is practically insensitive to the strap length, a result which is consistent with cobalt complexes [60,61].

In conclusion, the relatively short strap of the CoAz2 complex causes distortion of the trans-axial ligand, while the longer, more flexible, strap of the CoDe2 porphyrin permits relatively unrestrained binding. The RR data not only show that the strength of the Co–O\(_2\) linkage remains relatively unaltered by this distortion (i.e., the inherent frequencies for \(v(\text{O–O})\) remain constant), but they also provide definitive spectroscopic evidence for the distortion (i.e., the ligand internal modes are strongly perturbed and thereby give rise to remarkable spectral differences).

2.1.3. Quantitative treatment of the model compound data

The previous sections summarized the results for specific examples involving several different trans-axial ligands. While the existence of the coupling effect in each of these works was clearly demonstrated by isotopic substitution, in any given case the actual number of different coupling interactions was rather small and no attempt was made to relate the observed frequency shifts and relative intensity of the coupled modes to theory, although it was noted that the behavior generally
approximated that expected for coupled oscillators [67, 68]. Having accumulated a relatively large body of data, it was then shown [71] that all of the different coupling interactions so far observed could be treated collectively and that the observed frequency shifts and relative intensities are in good agreement with the values calculated using the familiar Fermi resonance formulation [86].

2.1.3.1. Theoretical framework. As is fully described in a standard reference work on vibrational spectroscopy [87], anharmonicity may lead to interactions of normal modes giving rise to frequency perturbations of the coupled modes from their inherent frequencies and mixing of the vibrational eigenfunctions. Such interactions are adequately treated by first-order perturbation theory which leads to the following expression for the new frequencies. Here, we use a modification of Herzberg's notation.

\[ v = \nu_{ni} \pm \sqrt{1/2(4W_{ni}^2 + \delta^2)^{1/2}} \]  \hspace{1cm} (1)

In this expression

\[ \nu_{ni} = 1/2(\nu_{ni} + \nu_i) \]

i.e., the average value of the unperturbed frequencies of the coupled modes. Also,

\[ \delta = (\nu_{ni} - \nu_i) \]

i.e., the separation of the unperturbed levels. Finally, \( W_{ni} \) is the matrix element of the perturbation function \( W \) in the equation

\[ W_{ni} = \int \Psi_n^\circ \Psi_i^\circ \, d\tau \]  \hspace{1cm} (2)

where \( \Psi_n^\circ \) and \( \Psi_i^\circ \) are the nonperturbed eigenfunctions of the two vibrational levels which interact. \( W \) is determined by the anharmonic terms in the potential energy. Since \( \Psi_n^\circ \) and \( \Psi_i^\circ \) belong to the same symmetry and \( W \) is totally symmetric, these two levels will repel each other, each shifting by \( \sqrt{1/2(4W_{ni}^2 + \delta^2)^{1/2}} \) from the average value, and the shifts from the unperturbed values will increase as the separation decreases.

In Eq. (1), \( W_{ni} \) is an energy term which can be derived from known values of the perturbed and unperturbed frequencies (vide infra). Such experimentally determined values of \( W_{ni} \) include a factor which depends on the effective mass of the coupled modes. Thus, in the case of the dioxygen adducts of interest here, the derived \( W_{ni} \) will depend on the reduced mass of the \( \nu(O-O) \) and that of the internal ligand mode. Clearly, the relative \( W_{ni} \) values for \( ^{16}O_2 \) coupling to different ligand modes cannot be easily estimated since there is ambiguity in defining effective reduced masses for internal modes. On the other hand, it is useful to consider the expected relative values of \( W_{ni} \) for coupling of different dioxygen isotopomers with the same internal ligand mode. Thus, while the inherent coupling strength is independent of the oxygen isotopomer employed, the experimentally determined \( W_{ni} \) values are
expected to vary in the following manner:

\[ W_{n_1}(^{16}\text{O}_2) : W_{n_1}(^{16}\text{O}^{18}\text{O}) : W_{n_1}(^{18}\text{O}_2) \approx 1.00 : 0.985 : 0.970 \]

Utilization of these expected relative values is useful in calculating \( W_n \) and the inherent (unperturbed) frequencies of \( \nu(\text{O}-\text{O}) \) and internal ligand modes from the observed (i.e., perturbed) frequencies and intensities.

The vibrational interaction also leads to mixing of the eigenfunctions according to the following equations derived from perturbation theory [87]:

\[
\Psi_n = a\Psi_n^0 - b\Psi_i^0 \quad (3a)
\]

\[
\Psi_i = b\Psi_n^0 + a\Psi_i^0 \quad (3b)
\]

where

\[
a = \sqrt{\frac{(4W_{n_1}^2 + \delta^2)^{1/2} + \delta}{2(4W_{n_1}^2 + \delta^2)^{1/2}}}
\]

and

\[
b = \sqrt{\frac{(4W_{n_1}^2 + \delta^2)^{1/2} - \delta}{2(4W_{n_1}^2 + \delta^2)^{1/2}}}
\]

The above equations were derived for the interaction of a fundamental with an overtone or combination mode of the same symmetry to explain the appearance of two bands near 1300 cm\(^{-1}\) \([\nu(\text{CO})_{\text{sym}}]\) in the spectrum of \( \text{CO}_2 \) [86]. However, Lax [88] has shown that similar equations result for the coupling of two fundamentals in solids, and his approach has been applied to the analysis of Fermi resonance in fluid solution [89,90]. Later, Monecke [91] extended these treatments and has pointed out that, for frequencies and anharmonicities normally encountered in molecules, the new expressions yield calculated results which are insignificantly different from those calculated by using Eq. (1). Veas and McHale [69] have developed a general and rigorously correct theoretical framework for treating resonance vibrational interactions of solute modes with those of an unrestricted number \( N \) of associated solvent molecules. While this development represents an important contribution to the general problem of solute–solvent vibrational interactions (with no restrictions on \( N \)), for the special case where \( N=1 \), the equations are essentially identical with the Fermi resonance equations (i.e., Eq. (1), Eq. (3a) and Eq. (3b)). In fact, Veas and McHale [69] successfully applied their equations to the coupling of \( \nu(\text{O}-\text{O}) \) with the DCP fragment of Co(\text{TPP-d}_4)(\text{DCP})(\text{O}_2); \text{i.e.}, the experimental data presented in Section 2.1.1.3.2.

2.1.3.2. Comparison of theory and experiment. In the cases presented here, difficulty arose in applying Eqs. (1), (2), (3a) and (3b) to the \( \text{O}_2 \) adducts of interest in that the inherent frequencies of the internal ligand modes are not directly available; i.e., the mode gains intensity only as a consequence of coupling with \( \nu(\text{O}-\text{O}) \) and
only the perturbed frequencies were observed. In general, one cannot employ directly the frequency observed for the free ligand since coordination frequently shifts ligand modes by 10 cm⁻¹ or more [61,62,79–81]. Thus, successful application of the theory to interactions of this type requires a more complete data set for a given adduct. That is, either the interaction must be removed (for example by selective deuteriation of the ligand) to reveal the inherent v(O–O), or (at least two) oxygen isotopomers (each of which interacts with the same internal ligand mode) must be employed.

The observed relative intensities of the perturbed v(O–O) and internal ligand modes can also be employed to help determine the coupling strength (Wni) and inherent frequencies. Thus, in the case of the O₂ adducts discussed here, a resonance enhanced v(O–O) mode interacts with a nonresonance enhanced internal axial ligand mode. The appearance of the internal ligand mode in the RR spectra is a consequence of mixing of the eigenfunctions and, accordingly, the intensity of the “mainly ligand” mode is expected to increase proportionally with b² (see Eq. (3b)). Similarly, the intensity of the “mainly v(O–O)” mode will decrease relative to its unperturbed value, the fractional intensity being given by a² (see Eq. (3a)). It is emphasized that the total scattering intensity is not affected by the interaction but is merely distributed between coupled modes. For example, in the case of exact resonance, δ = 0, a = b = (0.5)¹/², and it is seen that the perturbed levels are equal mixtures of Ψₙ⁺ and Ψₙ⁻. In this particular case, wherein the inherent intensities of the ligand mode approaches 0, the result is that two bands of equal intensity are observed and these bands are shifted by equal magnitudes (in opposite directions) from their inherent (in this case, common) frequencies.

It is worth pointing out that in special situations (such as those discussed here) where an “inactive” mode gains intensity only by virtue of coupling with an active mode, the observed relative intensities, together with the observed (perturbed) frequencies, provide sufficient information to derive inherent frequencies and Wni values. However, in practice, the small errors present in the experimental data lead to large uncertainties in the derived Wni values and calculated inherent frequencies (more than 2 cm⁻¹). Utilization of multiple dioxgen isotopomers, taken together with the knowledge of the relative Wni values for the various dioxgen isotopomers, provides additional restrictions on the combinations of inherent frequencies and Wni values which will satisfy all of the above conditions (i.e., yield correct relative intensities and Wni values of appropriate relative magnitudes).

Eqs. (1), (2), (3a) and (3b) were applied to data presented in Section 2.1.1. and 2.1.2 in the following manner. The observed and (estimated) inherent frequencies of both the v(O–O) and axial ligand modes (for two or more dioxgen isotopomers) were used to calculate Wni values and relative intensities with the use of a BASIC program based on Eq. (1) and Eqs. (3a) and (3b). The calculated intensities were then compared with those observed (integrated intensities) and the relative Wni values were also available for comparison. Slight adjustments of the input data were made until calculated intensities matched those observed and Wni values were of appropriate relative magnitude. During this fitting procedure, the perturbed (i.e., observed) frequency values which were used as input were maintained within experimental error of those actually observed. The value of the unperturbed ligand fre-
Table 1
Summary of observed and calculated RR frequencies and intensities for model compounds

<table>
<thead>
<tr>
<th>No.</th>
<th>Base</th>
<th>(v'_{O_2})</th>
<th>(v'_{L})</th>
<th>(v''_{L})</th>
<th>(v_{O_2}(I_{O_2}))</th>
<th>(v_{L}(I_{L}))</th>
<th>(v_{L}(I_{L}))</th>
<th>(W_{ni})</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>4MI</td>
<td>(^{16}O_2)</td>
<td>1143</td>
<td>1108</td>
<td>1146(0.91)</td>
<td>1105(0.09)</td>
<td>1146(0.93)</td>
<td>1105(0.07)</td>
</tr>
<tr>
<td>2</td>
<td>4MI</td>
<td>(^{16}O^{18}O)</td>
<td>1110</td>
<td>1108</td>
<td>1121(0.57)</td>
<td>1099(0.43)</td>
<td>1119.5(0.55)</td>
<td>1098.5(0.45)</td>
</tr>
<tr>
<td>3</td>
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<td>1108</td>
<td>1074(0.92)</td>
<td>1111(0.08)</td>
<td>1074(0.92)</td>
<td>1111(0.08)</td>
</tr>
<tr>
<td>4</td>
<td>DCP</td>
<td>(^{16}O_2)</td>
<td>1156</td>
<td>1115</td>
<td>1160(0.91)</td>
<td>1112(0.09)</td>
<td>1159.4(0.93)</td>
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<tr>
<td>5</td>
<td>DCP</td>
<td>(^{16}O^{18}O)</td>
<td>1123</td>
<td>1115</td>
<td>1132(0.63)</td>
<td>1108(0.37)</td>
<td>1131.9(0.65)</td>
<td>1106(0.35)</td>
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<tr>
<td>6</td>
<td>DCP</td>
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<td>1115</td>
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<td>1121(0.16)</td>
<td>1084.9(0.86)</td>
<td>1120(0.14)</td>
</tr>
<tr>
<td>7</td>
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<td>(^{16}O_2)</td>
<td>1081</td>
<td>1069</td>
<td>1084(0.88)</td>
<td>1067(0.12)</td>
<td>1084(0.83)</td>
<td>1066(0.17)</td>
</tr>
<tr>
<td>8</td>
<td>Py</td>
<td>(^{16}O^{18}O)</td>
<td>1114</td>
<td>1069</td>
<td>1115(0.93)</td>
<td>1067(0.07)</td>
<td>1115(0.98)</td>
<td>1068(0.02)</td>
</tr>
</tbody>
</table>

Co(TPP-d₈)

<table>
<thead>
<tr>
<th>No.</th>
<th>Base</th>
<th>(v'_{O_2})</th>
<th>(v'_{L})</th>
<th>(v''_{L})</th>
<th>(v_{O_2}(I_{O_2}))</th>
<th>(v_{L}(I_{L}))</th>
<th>(v_{L}(I_{L}))</th>
<th>(W_{ni})</th>
</tr>
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<tbody>
<tr>
<td>9</td>
<td>Im</td>
<td>(^{16}O_2)</td>
<td>1144</td>
<td>1153</td>
<td>1138(0.75)</td>
<td>1158(0.25)</td>
<td>1139(0.74)</td>
<td>1158(0.26)</td>
</tr>
<tr>
<td>10</td>
<td>Im*</td>
<td>(^{18}O_2)</td>
<td>1078</td>
<td>1072</td>
<td>1082(0.68)</td>
<td>1068(0.32)</td>
<td>1082(0.71)</td>
<td>1068(0.29)</td>
</tr>
<tr>
<td>11</td>
<td>Im</td>
<td>(^{16}O_2)</td>
<td>1144</td>
<td>1131</td>
<td>1148(0.84)</td>
<td>1127(0.16)</td>
<td>1148(0.81)</td>
<td>1127(0.19)</td>
</tr>
<tr>
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<td>1078</td>
<td>1067</td>
<td>1082(0.71)</td>
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<td>1082.5(0.76)</td>
<td>1063.5(0.24)</td>
</tr>
<tr>
<td>13</td>
<td>Im-d₁</td>
<td>(^{16}O_2)</td>
<td>1144</td>
<td>1150</td>
<td>1137(0.70)</td>
<td>1156(0.30)</td>
<td>1138(0.67)</td>
<td>1156(0.33)</td>
</tr>
<tr>
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<td>Im-d₁</td>
<td>(^{18}O_2)</td>
<td>1078</td>
<td>1087</td>
<td>1076(0.85)</td>
<td>1089(0.15)</td>
<td>1076(0.85)</td>
<td>1089(0.15)</td>
</tr>
<tr>
<td>15</td>
<td>Im-d₂</td>
<td>(^{16}O_2)</td>
<td>1111</td>
<td>1114</td>
<td>1106(0.62)</td>
<td>1119(0.38)</td>
<td>1106(0.62)</td>
<td>1119(0.38)</td>
</tr>
<tr>
<td>16</td>
<td>Im-d₂</td>
<td>(^{18}O_2)</td>
<td>1144</td>
<td>1117</td>
<td>1148(0.84)</td>
<td>1115(0.02)</td>
<td>1146(0.94)</td>
<td>1115(0.06)</td>
</tr>
<tr>
<td>17</td>
<td>Im-d₂</td>
<td>(^{16}O^{18}O)</td>
<td>1111</td>
<td>1117</td>
<td>1107(0.58)</td>
<td>1120(0.34)</td>
<td>1106(0.69)</td>
<td>1122(0.31)</td>
</tr>
<tr>
<td>18</td>
<td>Im-d₃</td>
<td>(^{16}O_2)</td>
<td>1114</td>
<td>1131</td>
<td>1148(0.84)</td>
<td>1127(0.14)</td>
<td>1147(0.84)</td>
<td>1128(0.16)</td>
</tr>
<tr>
<td>19</td>
<td>Im-d₃</td>
<td>(^{16}O^{18}O)</td>
<td>1111</td>
<td>1131</td>
<td>1107(0.58)</td>
<td>1132(0.08)</td>
<td>1109(0.92)</td>
<td>1133(0.08)</td>
</tr>
</tbody>
</table>

In all cases integrated intensities were used as observed intensities: asterisk indicates that Co(AZ₉₂₋₇₋₈₋₇₋₉₋₂₋₉₋₈) was used for this case.

*Imidazole free from hydrogen bonding.

quency (an unknown quantity) was also varied, keeping the same value for the different \(O_2\) isotopomers. In addition, the inherent \(v(O-O)\) values were constrained by the reduced masses relationship; i.e., \(\Delta v[^{16}O_2-^{16}O^{18}O] = \Delta v[^{16}O^{18}O-^{18}O_2] = 33 \text{ cm}^{-1}\).

The results of the application of this procedure to the \(O_2\) adducts of Co(por)(base) are summarized in Table 1. Inspection of these data clearly demonstrates that all these complicated observed RR spectral patterns are precisely those predicted by using the computer program based on Eq. (1) and Eqs. (3a) and (3b). This excellent agreement between theory and experiment is illustrated diagrammatically in Fig. 14. The greater scatter at low values of ligand intensity, \(I_L\), is merely the result of greater uncertainty in determining the experimental intensities of weak bands.

2.2. Coupling of dioxygen with an associated solvent molecule

2.2.1. Methylene chloride

As discussed in Section 2.1.1.1 (Fig. 2(A)), Bajdor et al. [60] obtained evidence for a peculiar type of vibrational coupling between the \(v(^{16}O-^{16}O)\) of the bound
Fig. 14. Plot of observed versus calculated ligand intensity $I_L$ (see Table 1 for numbering) (from Ref. [71]).

O$_2$ and an internal mode of solvent molecules (CH$_2$Cl$_2$) at $\sim$1156 cm$^{-1}$. Briefly, it was observed that the RR spectrum of Co(TPP-d$_{6}$) in a CH$_2$Cl$_2$ solution with added pyridine and $^{16}$O$_2$ exhibited two unresolved bands at $\sim$1155 and 1143 cm$^{-1}$. Upon solvent replacement with CD$_2$Cl$_2$, the 1155 cm$^{-1}$ shoulder (which was assignable to an internal mode of the solvent) disappeared and, most significantly, the 1143 cm$^{-1}$ band assigned to the $\nu(16\text{O}^{-16}\text{O})$ mode was shifted by 5 cm$^{-1}$ to higher frequency. The displacement of the $\nu(\text{O}^{-\text{O}})$ from its inherent frequency of 1148 cm$^{-1}$ is due to vibrational coupling, rather than to any possible chemical effects of the solvent change, since no similar shift was observed in the case of the $\nu(18\text{O}^{-18}\text{O})$ frequency. These experiments provided evidence of selective enhancement of the 1155 cm$^{-1}$ band as a result of this coupling since this band was only very weakly observed in the experiment with $^{18}$O$_2$, though all components were present in comparable concentrations [60] for both the $^{18}$O$_2$ and $^{18}$O$_2$ cases. Comparison of RR spectra of the complexes in different solvents and the use of various porphyrins indicated that such coupling is facilitated by intimate association of a CH$_2$Cl$_2$ molecule (presumably via hydrogen bonding) with the bound O$_2$ [60].
2.2.2. Toluene

A more interesting case of selective enhancement of internal modes of associated solvent molecules is observed in the case of six-coordinate dioxygen adducts of cobalt porphyrins measured in toluene [60,84,92]. As can be seen in trace A of Fig. 15, two unresolved bands at 1151 and 1160 cm\(^{-1}\) are observed in the region where \(\nu(16O-16O)\) is expected to occur. The appearance of two bands in this region is not due to the presence of two types of oxygen adducts since only one intense band is observed (at 1094 cm\(^{-1}\)) when \(^{18}\text{O}_2\) is used (trace B). The weak band at 1067 cm\(^{-1}\) in this spectrum is due to enhancement of an internal mode of pyridine [60–62] as was shown earlier. The observation of a strong band at 1151 cm\(^{-1}\) in trace A is seen to be the result of a selective enhancement of an internal mode of toluene, because when toluene-\(^2\text{H}_8\) is used instead of natural abundance toluene, only one strong band, assignable to \(\nu(16O-16O)\), is observed at 1159 cm\(^{-1}\) (trace C). The small shoulder at 1174 cm\(^{-1}\) is due to an internal mode of the bulk solvent, toluene-\(^2\text{H}_8\); i.e., its intensity is not enhanced relative to other solvent modes.

Natural abundance toluene exhibits three bands in this region at 1155, 1178 and 1210 cm\(^{-1}\) [63–65]. The relative intensities of these three bands in neat toluene are similar to those observed in Fig. 15(B); i.e., approximately 1:1:9. Thus, it is apparent that the occurrence of \(\nu(16O-16O)\) in the vicinity of the internal modes of toluene gives rise to an enhancement of the 1178 and 1155 cm\(^{-1}\) toluene bands relative to the band observed at 1210 cm\(^{-1}\). It is equally apparent that if the \(\nu(\text{O-O})\) is displaced to a much lower frequency by substitution of \(^{18}\text{O}_2\), the bands corresponding to these internal modes are not enhanced significantly. Therefore, the mechanism by which the internal modes of the solvent molecules are selectively enhanced by interaction with \(\nu(\text{O-O})\) is critically dependent upon energy matching of the two sets of modes as explained in the previous section. The sensitivity of this energy matching was documented [84] by carrying out a set of experiments designed to “fine tune” the \(\nu(16O-16O)\) so as to vary its frequency systematically between the two toluene modes at 1155 and 1178 cm\(^{-1}\). Such variation was accomplished by employing a number of different nitrogeneous bases as the axial ligand. Since the bonding in these \(\text{O}_2\) adducts can generally be formulated as \(\text{Co}^{5+}-\text{O}_2\), it is expected that the stronger is the axial ligand, the stronger the \(\text{Co-O}_2\) bond and the weaker the \(\text{O-O}\) bond. Although other factors may be important, if these are held constant, the \(\nu(\text{O-O})\) should be sensitive to the nature (basicity) of the axial ligand. Indeed, the \(\nu(\text{O-O})\) was varied between 1169 and 1147 cm\(^{-1}\) as the \(pK_a\) of the particular pyridine derivative as the axial ligand varied between 0.67 and 9.70. As is clearly demonstrated in Fig. 16, the 1155 cm\(^{-1}\) toluene mode gains intensity, while the 1178 cm\(^{-1}\) mode experienced smaller and smaller enhancement relative to the 1210 cm\(^{-1}\), as the \(\nu(\text{O-O})\) shifts from 1169 to 1147 cm\(^{-1}\).

It is important to emphasize the fact that this type of vibrational coupling with “solvent” is actually the result of intimate association of an individual solvent molecule with the resonantly enhanced \(\text{Co-O}_2\) fragment. This point is clearly demonstrated by the spectra given in Fig. 17. Trace A shows significant enhancement of the toluene modes (at \(\sim 1180\) and 1153 cm\(^{-1}\)). As is shown in trace B, the spectrum of the dioxygen adduct of \(\text{Co}(\text{TPy}_{4})(4\text{-cyanopyridine})\), obtained under identical
Fig. 15. Resonance Raman spectra of Co(TPP-2H₈) in toluene containing 3% pyridine at -85°C under ~4 atm of O₂ pressure: (A) ¹⁶O₂; (B) ¹⁸O₂; (C) ¹⁶O₂, toluene-²H₈ (from Ref. [84]).
Fig. 16. Resonance Raman spectra of Co(THP-2H₆) in toluene containing 3% 4-cyanopyridine (A), pyridine (B), and 4-dimethylaminopyridine (C) at −85°C under ~4 atm of ¹⁸O₂ pressure (from Ref. [84]).
conditions, exhibits only one strong band at 1159 cm$^{-1}$. This latter porphyrin is a (so-called) super-structured porphyrin which possesses bulky pivaloyl groups on one side only of the macrocycle. It is generally accepted that these groups restrict close association of all but very small molecules with the bound molecular oxygen. Clearly, restricted access of the toluene molecule to the bound O$_2$ prevents the vibrational coupling interaction and eliminates selective enhancement of the solvent modes. In a later study by Nakamoto and co-workers [92], other atropisomers of such super-structured porphyrins were employed to show that, as this restriction is incrementally lifted (by using macrocycles with 4, 3 or 2 bulky groups on one side), the coupling interaction is restored.
3. Applications to heme proteins

3.1. Dioxoxygen adducts of cobalt-substituted hemoglobin and myoglobin

The concepts and quantitative treatment (Section 2.1.3.1) outlined above for analysis of these complex vibrational interactions can, in principle, be applied to the RR spectra of the $O_2$ adducts of heme protein systems. However, it must be pointed out that such attempts are naturally somewhat hindered by the inability to eliminate or control the vibrational coupling as was accomplished in the studies of the model compounds.

Thus in the model systems, fully deuteriated ligands can be used to reveal the inherent $\nu$(O–O) and deuteriated porphyrins (e.g., Co(TPP-d₈)) can be employed to remove overlapping macrocycle modes. Such manipulations are not easily accomplished in the case of protein systems. In addition, the inherent frequencies of the internal modes of the coordinated histidylimidazole fragment are not known since conditions for direct resonance enhancement of these have not been identified. While systematic application of the procedure to the proteins is thus hampered by the considerations outlined above, it is satisfying to point out that the observed frequencies and intensities can be closely approximated with Eqs. (1), (2), (3a) and (3b) by making several assumptions which are well-supported by existing data.

The RR spectra of six different cobalt-substituted oxymeme proteins (Hb$_{Co}$, Mb$_{Co}$, $\alpha_{Co}$, $\beta_{Co}$, $\alpha_{Fe}$,$\beta_{Co}$, and $\alpha_{Co}$,$\beta_{Fe}$) were first presented and discussed qualitatively in an early work [67]. The spectral data for Hb$_{Co}$ was later analyzed quantitatively [71] by the method outlined in Section 2.1.3.1 and this analysis is briefly summarized below.

The RR spectra of the $^{16}$O$_2$, $^{16}$O$^{18}$O, and $^{18}$O$_2$ adducts of Hb$_{Co}$ measured in aqueous and deuteriated (D$_2$O) buffers are presented in Fig. 18. In order to clarify the complicated multiple interactions, we have diagrammatically represented the observed spectra in Fig. 18, including separate sets of vertical lines for each component of the spectrum (i.e., sets for the histidine and $\nu$(O–O) modes). In the figure, the inherent (i.e., unperturbed) frequencies of coordinated histidine and dioxygen are depicted with dotted and broken lines, respectively, while the solid lines represent the calculated perturbed frequencies. It should be noted that this diagram is not intended to provide an accurate representation of the calculated relative intensities. Those are listed in Table 2.

As was shown in our earlier work [71], these complicated spectral patterns, including the observed RR frequencies and their approximate relative intensities are, in fact, precisely those predicted by Eq. (1) and Eqs. (3a) and (3b), given the following few, well supported, assumptions. There is only one O$_2$ conformer.

---

Fig. 18. Resonance Raman spectra of cobalt-substituted hemoglobin A, Hb$_{Co}$, in 50 mM Tris–HCl buffer (H$_2$O or $^2$H$_2$O) pH (pD) 8.2, excitation at 406.7 nm. Vertical lines represent the following: dotted (· · ·), inherent frequencies of coordinated histidine; broken (· · ·), inherent frequencies of coordinated O$_2$; and solid lines (———), calculated perturbed frequencies of coordinated O$_2$ and histidine (from Ref. [71]).
Table 2
Observed and calculated RR frequencies and intensities for cobalt-substituted oxyhemoglobin in H\textsubscript{2}O and \textsuperscript{2}H\textsubscript{2}O

<table>
<thead>
<tr>
<th>O\textsubscript{2} isotopomer</th>
<th>(\nu_{e})</th>
<th>(\nu_{L})</th>
<th>Observed</th>
<th>Calculated</th>
<th>(W_{ni})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\nu_{e}(I_{e}))</td>
<td>(\nu_{L}(I_{L}))</td>
<td>(\nu_{e}(I_{e}))</td>
<td>(\nu_{L}(I_{L}))</td>
<td>(\nu_{e}(I_{e}))</td>
</tr>
<tr>
<td>(1) \textsuperscript{16}O\textsubscript{2}</td>
<td>1139</td>
<td>1148</td>
<td>1136(0.86)\textsuperscript{a}</td>
<td>1150(0.14)\textsuperscript{a}</td>
<td>1136(0.80)</td>
</tr>
<tr>
<td>(2) \textsuperscript{16}O\textsubscript{2}</td>
<td>1139</td>
<td>1109</td>
<td>(\ldots)\textsuperscript{b}</td>
<td>1107(weak)</td>
<td>1141(0.94)</td>
</tr>
<tr>
<td>(3) \textsuperscript{16}O\textsubscript{18}O</td>
<td>1106</td>
<td>1109</td>
<td>1098(?)</td>
<td>(\ldots)\textsuperscript{c}</td>
<td>1098(0.58)</td>
</tr>
<tr>
<td>(4) \textsuperscript{18}O\textsubscript{18}O</td>
<td>1106</td>
<td>1085</td>
<td>(\ldots)\textsuperscript{b}</td>
<td>1080(weak)</td>
<td>1113(0.80)</td>
</tr>
<tr>
<td>(5) \textsuperscript{18}O\textsubscript{2}</td>
<td>1073</td>
<td>1085</td>
<td>1063(0.78)</td>
<td>1093(0.22)</td>
<td>1064(0.70)</td>
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<td>(6) \textsuperscript{16}O\textsubscript{2}</td>
<td>1139</td>
<td>1109</td>
<td>1140(?)</td>
<td>1108(weak)</td>
<td>1141(0.94)</td>
</tr>
<tr>
<td>(7) \textsuperscript{16}O\textsubscript{18}O</td>
<td>1106</td>
<td>1109</td>
<td>1098(?)</td>
<td>(\ldots)\textsuperscript{c}</td>
<td>1098(0.58)</td>
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<tr>
<td>(8) \textsuperscript{18}O\textsubscript{18}O</td>
<td>1106</td>
<td>1089</td>
<td>(\ldots)\textsuperscript{b}</td>
<td>1089(weak)</td>
<td>1109(0.87)</td>
</tr>
<tr>
<td>(9) \textsuperscript{18}O\textsubscript{2}</td>
<td>1073</td>
<td>1089</td>
<td>1070(0.79)</td>
<td>1093(0.21)</td>
<td>1070(0.86)</td>
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</tbody>
</table>

In all cases integrated intensities were used as observed intensities. Entries 1–5 obtained from H\textsubscript{2}O solution, entries 6–9 obtained from \textsuperscript{2}H\textsubscript{2}O solution.

\textsuperscript{a}Intensity calculated from data in Ref. [15].

\textsuperscript{b}These features derived from a second interaction. Observed features are determined by the primary interaction.

\textsuperscript{c}Band obscured by overlap with porphyrin macrocycle mode.

\textsuperscript{d}Calculated frequencies and intensities were obtained using \(W_{ni}\) values derived from the \(W_{ni}\) value of the corresponding dioxygen isotopomer (e.g., 9.52 obtained as 9.38 \times 1.0/0.985).

The frequencies and intensities are calculated using the same \(W_{ni}\) as was used for H\textsubscript{2}O (i.e., 9.52).

present whose inherent \(\nu(O-O)\) frequencies are: \(\nu(16O-16O)=1139\text{ cm}^{-1}\), \(\nu(16O-18O)=1106\text{ cm}^{-1}\), and \(\nu(18O-18O)=1073\text{ cm}^{-1}\) (note the enforced agreement of the dioxygen isotopic shift of 33 cm\(^{-1}\)). The histidylimidazole fragment possesses internal modes at 1148, 1109, and 1085 cm\(^{-1}\) in H\textsubscript{2}O, but upon exchange (NH/ND) in D\textsubscript{2}O the first two modes are downshifted to 1109 and 1089 cm\(^{-1}\), respectively, while the 1085 cm\(^{-1}\) mode moves down below 1045 cm\(^{-1}\). These assumed histidylimidazole modes are entirely consistent with previously reported data [93,94]. Thus, free histidine in H\textsubscript{2}O (pH 8.2) exhibits four bands in this region: 1158 (broad), 1108, 1093, and 1070 cm\(^{-1}\). In D\textsubscript{2}O, only two bands, at 1097 cm\(^{-1}\) (strong) and 1107 cm\(^{-1}\) (weak), are observed in this same range. All of these modes are apparently of A’ symmetry, thus they can couple with the \(\nu(O-O)\) mode [93,94]. As has been previously demonstrated [79–81], these histidylimidazole modes may shift slightly upon coordination.

Hydrogen bond formation between bound dioxygen and the distal histidyl NH is now generally accepted as a potentially important factor in the control of O\textsubscript{2} binding [6–13]. As was made clear in our work published earlier [67,71], H\textsubscript{2}O/D\textsubscript{2}O exchange (which leads to NH/ND exchange of the proximal, as well as the distal, histidylimidazole fragments) may differentially alter the vibrational interaction between the various \(\nu(O-O)\) isotopomers and the internal modes of the proximal histidylimidazoles, which results in a wide variation in observed RR spectral patterns and apparent "shifts". To the extent that such "shifts" cannot then be unambiguously ascribed to
changes in distal side hydrogen bond strength, they cannot be taken as evidence for distal side hydrogen bonding, as had been proposed in an early study of the RR spectra of oxygenated adducts of cobalt-substituted heme proteins [95]. However, we wish to emphasize that the interpretation which invokes vibrationally coupled dioxygen does not imply that distal side hydrogen bonding is nonexistent, but simply that the observed $H_2O/D_2O$ shifts are the result of altered vibrational coupling patterns rather than altered hydrogen bond strengths [60,67,71,77,78].

3.2. Dioxygen adducts of cytochrome P450: coupling of $v(O\u2013O)$ with the internal mode of the substrate

As is discussed in Section 2.2, internal modes of solvent or other solute molecules can be resonance enhanced by specific association and interaction with the bound dioxygen. This enhancement requires close association of the molecule in question with the bound dioxygen, and is critically dependent upon energy matching of the mode with the $v(O\u2013O)$. The RR spectra of the dioxygen adduct of adamantanone-bound cytochrome P450 from Pseudomonas putida [96] provide an interesting example of selective enhancement of the internal modes of an enzyme-bound substrate molecule.

Cytochromes P450 is a family of heme monooxygenase enzymes involved in a variety of oxidative reactions [97]. Among various cytochromes P450, the one isolated from the bacterium Pseudomonas putida is induced by camphor and is known as cytochrome P450$_{cam}$. It has been the most extensively studied member of this class of enzymes owing to its relative ease of handling and its ready availability [97]. This cytochrome is the terminal oxidase in the $d$-camphor hydroxylase system and catalyzes hydroxylation of this substrate with exquisite regio- and stereospecificity; i.e., the substrate is held in a fixed position near the (hydroxylating) heme active site. The molecular structure of P450$_{cam}$ has been well established [97–101]. Unlike the majority of other heme proteins, which utilize histidylimidazole as an axial ligand, cytochrome P450$_{cam}$ possesses thiolate (Cys357) as the proximal axial ligand that binds to the heme iron. In the substrate-free form, a cluster of water molecules occupies the substrate pocket, one of which serves as the distal-side axial ligand. Upon the binding of camphor, the water cluster is expelled from the substrate binding site and the heme assumes a pentacoordinated, high-spin configuration. The substrate is in close proximity to the heme iron on the distal side and is held in a relatively fixed position by a hydrogen bonding interaction between its carbonyl oxygen and the hydroxyl group of the side chain Tyr96. In addition, there is a “lock-and key” hydrophobic interaction between the camphor gem-dimethyl groups and Leu244 and Val295. Thus, the substrate is positioned in the enzyme active site in a way that it can interact with a molecular oxygen molecule that binds to the heme iron at the distal side [97–101].

Figs. 19 and 20 show the RR spectra of dioxygen adducts of camphor- and adamantanone-bound cytochrome P450$_{cam}$. Comparison of these figures illustrates the effect of substrate replacement on the $v(O\u2013O)$ mode. It is emphasized that the electronic absorption spectrum and heme RR core vibrations of the dioxygen adducts
Fig. 19. Resonance Raman spectra of dioxygen adduct of camphor-bound cytochrome P450_{cam} in the ν(O–O) region: trace A, $^{16}$O$_2$; trace B, $^{18}$O$_2$. Excitation at 413.1 nm (from Ref. [96]).
Fig. 20. Resonance Raman spectra of dioxygen adduct of adamantaneone-bound cytochrome P450cam at −80°C in the ν(O=O) region: trace A, 16O2; trace B, 18O2. Excitation at 413.1 nm (from Ref. [96]).

are identical for both substrates [96]. However, the spectral pattern in the ν(O=O) region exhibits a dramatic change. While a single strong band is observed at 1140 cm⁻¹ in Fig. 19(A), in Fig. 20(A), two bands of nearly equal intensity are
observed at 1139 and 1147 cm\(^{-1}\). The assignment of these bands to the \(\nu(16\text{O}--16\text{O})\) mode is confirmed by the shifts observed upon \(16\text{O}_2/18\text{O}_2\) substitution (traces B of Figs. 19 and 20). The magnitudes of the observed isotopic shifts (67 and 65 cm\(^{-1}\), respectively) are in good agreement with those expected for the diatomic harmonic oscillator (i.e., 65 cm\(^{-1}\)).

Fig. 21 shows the difference spectra of the \(16\text{O}_2\) and \(18\text{O}_2\) adducts of cytochrome P450\(_{cam}\) in the presence of camphor (trace A) and adamantaneone (trace B) [96]. These spectra give a clear-cut comparison of the band intensities and widths of the \(\nu(\text{O}--\text{O})\) modes for both substrate-bound adducts, because the interfering heme modes are cancelled out. The \(\nu(16\text{O}--16\text{O})\) and \(\nu(18\text{O}--18\text{O})\) of P450\(_{cam}\) are observed, at 1140 cm\(^{-1}\) (positive peak) and 1074 cm\(^{-1}\) (negative peak), as symmetric RR bands; an observation that is in agreement with data presented in early work on these systems [102–104]. In trace B of Fig. 21 it is noted that both the \(\nu(16\text{O}--16\text{O})\) and \(\nu(18\text{O}--18\text{O})\) are broader (each is a doublet at 1139/1147 and 1074/1080 cm\(^{-1}\), respectively) for the adamantaneone-bound adduct, relative to camphor. These multiple lines can be attributed to the known conformational disorder [99] of the Fe--O linkage caused by different orientations of adamantaneone within the heme pocket [96]. However, it is especially important to note that, in addition to the broadening caused by the multiple conformers, the bandwidth of the unresolved features in the \(\nu(\text{O}--\text{O})\) region of the \(16\text{O}_2\) adduct of the adamantaneone-bound enzyme is significantly broader than that of the \(18\text{O}_2\) adduct, though the integrated areas of the positive and negative peaks are identical within experimental error. The observation of selective band broadening of only one isotopeomer \(16\text{O}_2\) is most reasonably ascribed to a vibrational coupling interaction and enhancement of an internal mode of the bound adamantaneone. As is seen in trace C, several Raman bands occur in this region; in particular, the mode at 1140 cm\(^{-1}\) is closely energy-matched with the \(\nu(16\text{O}--16\text{O})\) frequency. The natural substrate, camphor, does not exhibit any band in this region and no evidence of a perturbed spectral pattern is indicated in trace A.

As we discussed in a previous section (Section 2.2), RR enhancement of the solvent or solute molecule internal mode via such vibrational coupling interactions requires close association of the molecule in question with the bound dioxygen and is critically dependent upon energy matching of the mode with \(\nu(\text{O}--\text{O})\). Clearly, these two requirements are satisfied for the dioxygen adduct of adamantaneone-bound cytochrome P450, and may be responsible for the observed band broadening in the case of \(16\text{O}_2\) adduct, although further studies (employing deuteriated substrate) would be needed to unambiguously confirm such coupling.

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Fig. 21. Difference RR spectra of the \(16\text{O}_2\) and \(18\text{O}_2\) adducts of cytochrome P450 from Pseudomonas putida in the presence of camphor (trace A) and adamantaneone (trace B) obtained by normalizing the respective RR spectra to the 1374 cm\(^{-1}\) band. Trace C: Raman spectrum (413.1 nm excitation) of adamantaneone dissolved in CH\(_2\)Cl\(_2\). The solvent band at 1157 cm\(^{-1}\) is subtracted (from Ref. [96]).
4. Summary and conclusions

The complex spectral patterns observed in the RR spectra of dioxygen adducts of cobalt-substituted heme proteins and model compounds are shown to result from vibrational coupling of $\nu$(O–O) with internal modes of the trans-axial ligand or intimately associated solvent or solute molecules. The conventional Fermi resonance equations, applied to data obtained for multiple isotopomers, can be employed to extract the actual inherent frequencies of the $\nu$(O–O) and the coupled internal modes of the ligand, solute or substrate. Recent theoretical treatments validate the use of these equations for the interaction of two fundamental modes within different fragments of intermolecular complexes [88–91,105]. The excellent agreement between theory and experiment is illustrated in Fig. 14, as presented earlier in the paper.

While the existence of such complications underscore the need for high-quality spectra and cautious interpretation, the studies summarized here clearly demonstrate the potential utility of such vibrational coupling interactions. Thus, even quite subtle structural perturbations, which may not be sufficiently strong to produce shifts in the inherent frequency of the $\nu$(O–O) mode, can be documented by the (sometimes quite remarkable) effects such structural changes produce in the observed resonance Raman spectral patterns.

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