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# CO as a vibrational probe of heme protein active sites

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**Abstract**

Carbon monoxide is a useful vibrational probe of heme binding sites in proteins, because FeCO backbonding is modulated by polar interactions with protein residues, and by variations in the donor strength of the *trans* ligand. This modulation is sensitively monitored by the CO and FeC stretching frequencies, which are readily detectable in infrared and resonance Raman spectra. The two frequencies are anticorrelated, and the *v*FeC/*v*CO position along the correlation line reflects the type and strength of distal polar interactions. Changes in the *trans* ligand donor strength shift the correlation to higher or lower positions. Illustrative applications of the *v*FeC/*v*CO diagram are reviewed for proteins bearing histidine and thiolate axial ligands. Steric crowding has not been found to affect the *v*FeC/*v*CO correlations significantly, except in the special case of cytochrome oxidase, where the heme-bound CO may interact with the nearby $Cu_B$ center.


*Keywords:* Carbon monoxide; Heme; Protein; Vibration; Raman

## 1. Introduction

The C–O stretching frequency has long been used as a monitor of structure and bonding in transition metal carbonyl complexes, including CO adducts of the heme group. When bound to heme, the CO frequency diminishes by ~200 $cm^{-1}$ from its gas phase value, 2143 $cm^{-1}$, and falls in a region of the vibrational spectrum which is relatively free of interferences from other molecular vibrations. At the same time the *v*CO infrared intensity is greatly augmented, as is the frequency shift in an applied elelectric field [1]. These effects are due to backbonding. Fe[II] dπ electrons are donated to the empty CO π* orbitals, thereby diminishing the CO bond order; at the same time, the transition dipole moment is increased because compressing and expanding the CO bond shifts electrons back and forth between the CO and Fe[II] orbitals.

The CO frequency is sensitive to the molecular environment of the bound CO because the extent of backbonding is readily altered. In particular, the presence of polar molecules or protein residues near the CO has a marked effect [2]. Consequently, there has been considerable interest in using CO as a probe of the protein groups in the heme binding pocket; these groups can determine the reactivity of the heme toward other ligands, particularly $O_2$ and NO.

In addition to the *v*CO frequency, the IR spectrum can provide the direction of the transition moment via polarization measurements. These have been carried out for the CO adduct of myoglobin, MbCO, in oriented single crystals [3], or via photoselection of partially photolyzed molecules, either in frozen solution [4] or by using picosecond laser pulses [5,6], which are shorter than the protein tumbling time in liquid solution. It was hoped that the polarization measurements would establish the degree of FeCO bending, a historically

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contentious issue [7], but the assumption that the transition moment lies along the CO bond vector is incorrect; due to the backbonding, CO stretching induces electrons to move throughout the π system, and the transition moment lies essentially along the FeC bond vector, as shown by DFT calculations [8,9]. Thus the observation that the transition moment is within 7° of the heme normal [3,6] is not inconsistent with modest FeCO distortion as, seen in recent crystal structures [7].

The *v*CO vibration is also influenced by the nature of the *trans* axial ligand of the heme–CO adduct [10,11]. In heme proteins, the proximal ligand is usually histidine, but other ligands are possible. In addition, there can be variations in the strength of the proximal ligand bond, due to H-bonding or mechanical strain. Measurement of *v*CO alone cannot distinguish proximal from distal effects, but these effects can be unraveled if the *v*FeC vibration is also monitored [10]. *v*FeC is difficult to detect in the IR spectrum, but it is readily observed in the resonance Raman (RR) spectrum [12], which can simultaneously reveal *v*CO. Thus RR spectroscopy has emerged as a widely used technique for studying CO adducts of heme proteins.

In addition to the *v*FeC and *v*CO vibrations, the RR spectrum sometimes reveals a band near 570 $cm^{-1}$, assignable to Fe–C–O bending, δFeCO [12]. Actually this mode is an out-of-phase combination of Fe–C–O bending and Fe–C tilting coordinates, as described by Ghosh and Bocian [13], whose DFT calculation revealed a large bend–tilt interaction constant. This interaction accounts for the elevated δFeCO frequency (which had been a source of controversy over the assignment [14]). The in-phase combination is predicted [13] at a correspondingly low frequency (84 $cm^{-1}$), and has not been observed. Both modes break the 4-fold approximate symmetry of the heme group, and are expected to be RR-active only to the extent that this symmetry is lowered by the heme environment [10]. Indeed the band is not detectable in protein-free heme adducts (unless they have covalent superstructures that can interact with the bound CO [15]), or in many heme proteins. RR intensity is an indicator of off-axis interactions with nearby groups, which could be steric in character, but are more likely to be electrostatic [15]. The δFeCO values have been found to correlate with *v*FeC [16], but the variation in δFeCO is small.

In principal, the vibrational frequencies of NO and $O_2$ adducts can provide additional information about the heme environment [17]. However, these frequencies are harder to measure. The NO and OO frequencies fall in crowded regions of the protein vibrational spectrum. Moreover, the RR enhancement is lower than for CO; indeed the OO vibration is not readily assignable for heme protein $O_2$ adducts, except in special cases ([14,18] see also the paper by Egawa and Yeh in this issue). The experimental difficulties are compounded by uncertainties in interpretation [17], since the bonding is more complex for NO and $O_2$ than for CO adducts. It is also possible to detect the ligand vibrational frequencies for Fe[III]($CN^-$) adducts of heme proteins (see [19] and references therein). Although isoelectronic with Fe[II]CO adducts, Fe[III]($CN^-$) adducts experience much less backbonding, and the spread of frequencies is small [20]. However, the Fe[III]($CN^-$) is easier to bend, and bent forms can be detected, giving indications of steric and/or electrostatic distal interactions [19].

This article focuses on the uses of *v*FeC and *v*CO data to elucidate the binding site interactions in heme proteins, with special attention to the separation of distal and proximal effects. Reports of such data are already voluminous and we have not attempted a comprehensive compilation. Rather, prototypical examples have been chosen to illustrate the reasoning behind structural interpretation of the spectroscopic data.

## 2. The *v*FeC vs *v*CO backbonding correlations

A negative correlation between *v*FeC and *v*CO was noted early by several workers [16,21], and has been revisited many times. To a good approximation, the frequencies correlate linearly (as do their squares [16], which more properly represent the force constants), provided that the proximal ligand is unchanged [10]. Most of the available data is for heme proteins with proximal histidine and for protein-free hemes with imidazole or pyridine *trans* to the CO. All of these data are more or less on the same line, which covers a range of ~100 $cm^{-1}$ in *v*CO and ~70 $cm^{-1}$ in *v*FeC [10,15]; even hemes with thioether ligands fall approximately on this line [22]. Essentially all neutral *trans* ligands seem to behave similarly.

An extensive series of myoglobin variants with distal pocket mutations [2,23–38], form the most comprehensive available set ot *v*FeC/*v*CO data (Table 1) at constant *trans* ligation; these data are plotted in Fig. 1 and form the canonical Mb line, against which other adducts can be compared. The line can be expressed by the following equation [11],

$$vFeC = v^0FeC - s[vCO - v^0CO], \quad (1)$$

where $v^0$CO is the standard triple-bonded value in the gas phase, 2145 $cm^{-1}$, and $v^0$FeC is the corresponding single bonded value for Fe–C. A least squares fit gives $v^0$FeC = 361 $cm^{-1}$ for Mb, and $s = -0.73$ (Table 2). The slope represents the backbonding sensitivity of *v*FeC. For Mb, *v*FeC changes by 3/4 of the *v*CO change for a given increment of backbonding.

A large variation in backbonding is evident for the Mb variants and can be understood in terms of altered polar interactions with distal residues [2,15]. In the middle of the line [*v*CO ~ 1945 $cm^{-1}$] are the wild-type pro-