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Interactions of Cu_B with Carbon Monoxide in Cytochrome c Oxidase: Origin of the Anomalous Correlation between the Fe–CO and C–O Stretching Frequencies

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Abstract

In heme–copper oxidases, the correlation curve between the iron–CO and C–O stretching vibrational modes ($\nu_{\text{Fe–CO}}$ and $\nu_{\text{C–O}}$, respectively) is anomalous as compared to the correlation in other heme proteins. To extend the correlation curve, the resonance Raman (RR) and infrared (IR) spectra of the CO adducts of cytochrome *ba*₃ (*ba*₃) from *Thermus thermophilus* were measured. The RR spectrum has two strong $\nu_{\text{Fe–CO}}$ lines (508 and 515 cm^{−1}) and a very weak line at 526 cm^{−1}, and the IR spectrum has three $\nu_{\text{C–O}}$ lines (1966, 1973, and 1981 cm^{−1}), indicating the presence of multiple conformers. Employing photodissociation methods, the $\nu_{\text{Fe–CO}}$ RR and $\nu_{\text{C–O}}$ IR lines were assigned to each conformer, enabling the establishment of a reliable inverse correlation curve for the $\nu_{\text{Fe–CO}}$ versus the $\nu_{\text{C–O}}$ stretching frequencies. To determine the molecular basis of the correlation, a series of DFT calculations on 6-coordinate porphyrin–CO compounds and a model of the binuclear center of the heme–copper oxidases were carried out. The calculations demonstrated that the copper unit model caused significant mixing among porphyrin–CO molecular orbitals (MOs) that contribute to the Fe–C and C–O bonding interactions, and also indicated the presence of mixing between the *d*_{z² orbital of the copper and MOs that are responsible for the $\nu_{\text{Fe–CO}}$ vs $\nu_{\text{C–O}}$ inverse correlation. Together, the spectroscopic and DFT results clarify the origin of the anomaly of $\nu_{\text{Fe–CO}}$ and $\nu_{\text{C–O}}$ frequencies in the heme–copper oxidases, a long-standing issue.}

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Notes

The authors declare no competing financial interest.

Supporting Information

Supporting Figures 1–6 showing heme, CO, Cu_B geometry; band fitting analysis of IR data; analysis isotope resonance Raman data; structures and calculations of the 5-coordinate PCO complexes; and BA-1 MO pairs for each of the fluorine substituents. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcc.5b04444.

Graphical abstract

INTRODUCTION

Carbon monoxide (CO) is a very informative structural probe for the ligand-heme environment in hemeproteins.^{1–3} Most importantly, the iron–CO and C–O stretching vibrational modes ($\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$, respectively) and the Fe–C–O bending vibrational mode ($\delta_{\text{Fe-C-O}}$) of the CO adducts of heme proteins, which are detectable by resonance Raman (RR) and infrared (IR) spectroscopies, are sensitive to the electron density in the Fe–C–O moiety and the electrostatic environment on the distal side of the heme.^{1–6} When CO binds to the heme iron, it donates electron density to the iron via a σ -bond. In turn, the d_π orbital of the ferrous iron atom donates electron density back to the π^* orbital of the CO, according to the so-called “ π -back-bonding” effect.² As a result, the CO-bound heme-iron complex exists in an equilibrium between two extreme structures:

If the proximal ligand coordinated to the heme iron remains the same, the frequencies of $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ are sensitive to the electrostatic environment of the distal ligand binding pocket and are typically inversely correlated. A positive electrostatic potential in the distal pocket destabilizes form I, giving rise to a high $\nu_{\text{Fe-CO}}$ frequency and a low $\nu_{\text{C-O}}$ frequency; as such, the position of the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data point is located in the upper left corner of the correlation plot shown in Figure 1. If the electrostatic potential of the distal pocket of a protein is lowered, form I is stabilized; the data point is expected to shift along the correlation line to the lower right corner. Accordingly, the electrostatic potential of the ligand-binding site can be evaluated by the location of the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data point on the correlation line.

The electron density distribution on the Fe–C–O moiety is also modulated by the electronic properties of the proximal heme ligand. Consequently, the $\nu_{\text{C-O}}/\nu_{\text{Fe-CO}}$ correlation line of hemeproteins with histidine as the proximal ligand, such as globins (see the Mb line in Figure 1), lies on a correlation line higher than that of hemeproteins with cysteine as the proximal ligand, such as P450s (see the P450 line), as the thiolate is a stronger donor, which weakens the Fe–CO bond owing to competition for the σ -bond.^{7,8} In addition, the

correlation line defined by the mammalian nitric oxide synthases (mNOS) lies higher than the P450 line due to a slightly weaker Fe–S bond.⁹ When the fifth ligand is absent and there is no competition for the σ -bond, the $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ correlation line falls above that for Mb–CO, as shown in Figure 1, which was drawn on the basis of data from 5-coordinate CO adducts of iron–porphyrins. However, recently, whether the model complexes were in fact 5-coordinate has been called in question on the basis of new nuclear resonance vibrational spectroscopy measurements and DFT calculations, which indicate the presence of weak ligands in this class of complexes.¹⁰ Examining the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ frequency correlation data has thus been an effective way to investigate the nature of the axial ligand in heme proteins, as well as to assessing the distal polarity.^{1–6}

Although the actual bonding structures of the CO adducts of heme proteins reside somewhere between the two limits given by the canonical structures in eq 1, the σ -bond contribution to the $\nu_{\text{Fe-CO}}$ frequency (termed $\nu_{\text{Fe-CO}}^0$) can be estimated by extrapolating the $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ correlation line to the point at which the frequency of $\nu_{\text{C-O}}$ is 2145 cm^{-1} (the $\nu_{\text{C-O}}$ frequency in the gaseous CO).^{2,6} For the free gas value of 2145 cm^{-1} , the d_{π} back-donation is absent, so the only remaining interaction is the Fe–C σ -bond. This relationship may be expressed by eq 2:

$$\nu_{\text{Fe-CO}} = \nu_{\text{Fe-CO}}^0 - s[\nu_{\text{C-O}} - \nu_{\text{C-O}}^0] \quad (2)$$

For example, the Mb line shown in Figure 1 gives a $\nu_{\text{Fe-CO}}^0$ frequency of 346 cm^{-1} , which is an estimated σ -bonding contribution to the $\nu_{\text{Fe-CO}}$ frequency of each Mb–CO adduct. On the contrary, the slope, s , of the correlation line is a measure of how tightly the Fe–C π -bonding and the C–O π -antibonding orbitals are coupled.^{2,6} The parameters for the other correlation lines are listed in Table 1.

The family of heme–copper oxidases (oxidases), which have a binuclear catalytic site consisting of a high spin heme and a copper atom (Cu_B), approximately 5 Å away from the heme iron atom, is one of a few examples among the known heme proteins that exhibit an anomalous $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ correlation plot.^{8,11,18} Despite the fact that the fifth ligand to the heme of the catalytic site (heme a_3 or heme o_3) is a histidyl imidazole, the CO adducts of the oxidases have $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data points that are significantly above the Mb line (Figure 1). In addition, some oxidases have two sets of $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data points, one of which falls near the other oxidase data points and the second of which falls on or near the correlation line with a histidine axial ligand, termed the α and β forms, respectively.^{19–22} In some cases the ratio between the two forms were shown to be pH dependent,¹² and in others the ratio could be modulated by amino acid point mutations.^{13,23} The molecular origin of the deviation of the cytochrome c oxidase points away from the correlation line with histidine as the axial ligand has been a dilemma. Several explanations have been proposed to account for this anomalous behavior: (i) The iron–histidine bond is weak, causing a stronger Fe–CO bond than in other histidine coordinated heme proteins.²⁴ (ii) The Fe–C–O moiety is bent due to the interactions with the Cu_B unit.^{12,21,24} (iii) The Cu_B and its ligands cause a compression of the Fe–C–O bonds.^{2,12,21}

Each of the postulated explanations for the anomalous inverse correlation line in oxidases has limitations. It has been noted that for the ferrous heme a_3/o_3 of oxidases the stretching frequency of the proximal histidine-iron mode ($\nu_{\text{Fe-His}}$) falls at 208–214 cm^{-1} , which is slightly lower than that in deoxy Mb ($\sim 220 \text{ cm}^{-1}$), indicating a weaker bonding interaction.^{24,25} It has been postulated that the weak Fe–His bond may cause a strengthening of the Fe–CO bond in a manner similar to that in the 5-coordinate porphyrins and hence causes the upward shifts of the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ inverse correlation line.^{11,24} However, consideration of the effect of the $\nu_{\text{Fe-His}}$ frequency in relation to variation in the α/β intensity ratio in oxidases reveals no correlation between them. Specifically, there is a large change in the α/β intensity ratio in a series of mutants of the aa_3 oxidase from *Paracoccus denitrificans*, ranging from each being dominant with the other making only a minimal contribution and *vice versa*.²³ However, despite these remarkable differences, the Fe–His vibrational modes all fall in a 4 cm^{-1} range (~ 210 – 214 cm^{-1} (H. Ji et al., unpublished results)). Similarly, the Cu_B ligand mutants, such as the H333A of bo_3 of *Escherichia coli*²⁵ or H333N of the aa_3 CcO of *Rhodobacter sphaeroides*,²⁶ have $\nu_{\text{Fe-His}}$ frequencies essentially the same as those in the corresponding wild type proteins, despite the fact that the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data points of these mutants fall on or close to the Mb line^{16,17} (Figure 1). Mutations of other amino acids, located in the heme periphery, such as the R481L mutant of bo_3 oxidase from *E. coli*, also have data points that do not fall on the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ anomalous inverse correlation curve for oxidases but the $\nu_{\text{Fe-His}}$ frequency is the same as that of the wild-type (wt) bo_3 (Egawa et al., unpublished data). Together, these findings indicate that the weakening of the histidine–iron bond in the heme–copper oxidases is not the dominant factor to cause the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ anomaly.

The distal steric hindrance induced by the proximity of Cu_B was also proposed to be the origin of the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ anomaly in the oxidases by causing either a bent Fe–C–O moiety or a compressed structure.^{2,11} The distal compression effect hypothesis was based primarily on the investigations on a heme model compound having a benzene ring strapped laterally over a porphyrin ring via short covalent tethers.²⁷ When CO was bound to such a compound with an imidazole ligand to form a 6-coordinate CO adduct, it exhibited a significant upshift in the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data point, with respect to the Mb line. Such a shift was attributed to a Fe–C–O unit compression due to the interaction with the constrained benzene ring.²⁷ Although it is an attractive possibility, the distal compression scheme does not appear to be consistent with the X-ray crystallographic studies of the CO complexes of oxidases. The Cu_B does not lie directly over the iron atom of the heme. As such, the CO is slightly bent away from the copper atom as may be seen in Figure S1 in the Supporting Information and importantly there is no evidence that the bonds are compressed. In addition, although the Fe–C–O bond is bent, it is well within the range of CO adducts in many other heme proteins.²⁸

In part, the difficulty in determining the molecular basis for the abnormal behavior of the CO-complexes of the oxidase family is the result of a limited data set for the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ modes. Most of the oxidase $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ frequencies lie in a very narrow range, making it difficult to establish an accurate correlation line. This limitation results from the availability of only one of the modes in some oxidases (either $\nu_{\text{Fe-CO}}$ or $\nu_{\text{C-O}}$) and in others the presence of multiple lines in the region of each mode indicating the existence of several

conformers, so it is not possible to define the correlated points. One approach is to correlate data points from each region based on relative intensities, but this method of assignment is not generally reliable as the intensities depend on a variety of factors that differ for regions of the spectrum and for the type of spectroscopic measurement (RR vs IR). Only when the intensities are monitored as a function of an independent variable, for example pH or mutation, can the points from the $\nu_{\text{Fe-CO}}$ region be correlated with those from the $\nu_{\text{C-O}}$ region.

To address these issues, we used CO photodissociation methods to assign the correlated modes from the ba_3 oxidase of *Thermus thermophilus* in which several modes were reported in the past.^{29,30} On the basis of this data we have been able to obtain a reliable $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ correlation line. In addition, we have carried out DFT calculations on model complexes for oxidases from which we were able to formulate a new molecular basis for the anomalous $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ correlation curve in the oxidase family of proteins, based on a direct electronic interaction between the Cu_B moiety and the bound CO.

MATERIALS AND METHODS

Oxidase and Other Chemicals

The ba_3 sample was prepared as described previously,³¹ and dissolved in phosphate buffer (100 mM sodium phosphate with 1 mM dodecyl maltoside, pH 7.5). The fully reduced ligand-free form was prepared by reducing the samples with sodium dithionite under a nitrogen atmosphere, and the CO adduct was obtained by introducing CO gas into the fully reduced samples. The isotope labeled CO gas ($^{13}\text{C}^{18}\text{O}$ with >98% ^{13}C and >98% ^{18}O) was purchased from ICON (Summit, NJ) and used without further purification.

Spectroscopic Measurements

The RR measurements were carried out as previously described.¹ Briefly, the 413.1 nm excitation from a Kr ion laser (Spectra-Physics, Mountain View, CA) was focused to an $\sim 30\ \mu\text{m}$ spot on the spinning quartz cell rotating at $\sim 1000\ \text{rpm}$. The scattered light, collected at a right angle to the incident laser beam, was focused on the $100\ \mu\text{m}$ wide entrance slit of a 1.25 m Spex spectrometer equipped with a 1200 grooves/mm grating (Bausch & Lomb, Analytical Systems Division, Rochester, NY), where it was dispersed and then detected by a liquid nitrogen-cooled CCD detector (Princeton Instruments, Trenton, NJ). A holographic notch filter (Kaiser Optical Systems, Ann Arbor, MI) was used to remove the laser line. The Raman shifts were calibrated with indene. The laser power was adjusted by neutral density filters, and the power at the sample point was measured by a handheld laser power meter (LaserCheck, Coherent Inc., Santa Clara, CA).

The IR spectra were recorded at $4\ \text{cm}^{-1}$ resolution on an FTIR instrument (Magna-IR 560, Nicolet, Madison, MI) with a CaF_2 IR cell ($200\ \mu\text{m}$ path). For the IR dark-light experiments, the 413.1 nm laser beam was introduced into one end of an optical fiber, and the IR cell surface ($\sim 1\ \text{cm}$ diameter) was illuminated by the laser beam dispersed from the other end of the optical fiber.

Density Functional Theory (DFT) Calculations

DFT calculations were performed with Jaguar (Schrödinger, LLC, New York, NY) version 8.0 on a High-Performance-Computing cluster at the Albert Einstein College of Medicine (Bronx, NY), and at the B3LYP/6-311G*+ functional level, unless otherwise stated.

Visualizing the molecular normal modes and isovalue surfaces of molecular orbitals (MOs) was supported by Maestro (version 9.4) from Schrödinger. Further details are given in the text or in the figure legends.

RESULTS

Assignments of the $\nu_{\text{Fe-CO}}$ RR and $\nu_{\text{C-O}}$ IR Bands

To relate the $\nu_{\text{Fe-CO}}$ modes detected in the RR spectrum to the $\nu_{\text{C-O}}$ modes detected in the IR spectrum, the intensity of the lines in the spectra were monitored as a function of CO photodissociation. It was our hypothesis that the photo-dissociation would be different for each conformer allowing for the determination of which modes originated from each conformer. Figure 2A shows RR data from the CO adducts of the ba_3 oxidase from *Thermus thermophilus*, where the data are expressed as the difference between the spectra of the $^{12}\text{C}^{16}\text{O}$ and $^{13}\text{C}^{18}\text{O}$ adducts. Hence, only isotope sensitive RR lines show up as positive ($^{12}\text{C}^{16}\text{O}$) and negative ($^{13}\text{C}^{18}\text{O}$) peaks. At relatively low Raman excitation laser power (trace a, 200 μW), the $^{12}\text{C}^{16}\text{O}$ adduct has an RR peak at 515 cm^{-1} with a shoulder at 508 cm^{-1} . The 515 cm^{-1} peak weakened (second trace) and disappeared (third trace) as the laser power was increased to 1 and 5 mW, respectively, leaving a 508/491 cm^{-1} set of ($^{12}\text{C}^{16}\text{O}/^{13}\text{C}^{18}\text{O}$) peaks. The results indicate that the CO adduct of ba_3 consists of a mixture of photodissociable and relatively photostable conformers. When the 5 mW trace was subtracted from the 200 μW trace, the $^{12}\text{C}^{16}\text{O}/^{13}\text{C}^{18}\text{O}$ difference spectra of the photodissociable conformer was identified at 515/495 cm^{-1} (trace d). The 515/495 and 508/491 cm^{-1} isotope shift values are consistent with the expected $^{12}\text{C}^{16}\text{O}/^{13}\text{C}^{18}\text{O}$ isotope shift for a Fe-CO stretching mode. Hence, we assigned the 515/495 and 508/491 cm^{-1} pairs to the $\nu_{\text{Fe-CO}}$ of the photodissociable and photostable conformers, respectively.

With 428.7 nm laser excitation, Varotsis and co-workers reported RR frequencies of $\nu_{\text{Fe-CO}}$ of ba_3 at 507 and 526 cm^{-1} ,^{29,30} the former of which is the same as the $\nu_{\text{Fe-CO}}$ frequency of the photostable conformer (508 cm^{-1}) obtained in this study, although the $\nu_{\text{Fe-CO}}$ RR line corresponding to the 515 cm^{-1} band in our investigation was not seen in their studies. On the contrary, with RR excitation of 413.1 nm, the same excitation wavelength used in the present study, they have observed another $\nu_{\text{Fe-CO}}$ RR line at 526 cm^{-1} , not clearly seen in our RR experiments, which they have attributed to a five-coordinate (5C) CO complex of heme a_3 .

To determine the origin of the differences in our results and those of Varotsis and co-workers,^{29,30} we compared the IR spectrum in the $\nu_{\text{C-O}}$ frequency range to that reported by Varotsis and co-workers.²⁹ In our IR experiments, lines were detected at 1966, 1973, 1981, and 2053 cm^{-1} (Figure 2B, trace a), among which the line at 2053 cm^{-1} is assignable to the C-O stretching mode of the Cu_B -CO species ($\nu_{\text{C-O}}^{\text{Cu}_\text{B}}$), which is in a thermal equilibrium with the heme a_3 -CO species.^{30,32} On the contrary, the frequencies of the former three bands are in very good agreement with the $\nu_{\text{C-O}}$ of the heme a_3 -CO adduct reported by Varotsis

and co-workers (1967, 1973, and 1982 cm^{-1}).^{29,30} However, the relative intensity among the three $\nu_{\text{C-O}}$ bands, especially the ratio between the highest (1981–1982 cm^{-1}) and lowest (1966–1967 cm^{-1}) frequency bands was significantly different between our investigation and theirs. Varotsis and co-workers reported that either of these two lines could originate from the 5-coordinate species.²⁹ The intensity ratio of the 1981–1966 cm^{-1} lines is ~ 1.5 in our data, whereas, the corresponding ratio in the study by Varotsis and co-workers is ~ 4 (Figure S2, Supporting Information). Importantly, both the 526 and the 1981 cm^{-1} lines have a significantly lower population in our data as compared to that of Varotsis and coworkers.²⁹ Therefore, we assign the $\nu_{\text{C-O}}$ and $\nu_{\text{Fe-CO}}$ modes at 1981–1982 and 526 cm^{-1} , respectively, as originating from a 5C species of the heme a_3 -CO complex. Because of its lower population, a $\nu_{\text{Fe-CO}}$ RR line of the 5C species is not clearly seen in our investigations; however, detailed spectral analysis suggested a minor contribution of a 5C species in the observed RR difference spectra (Figure S3, Supporting Information). At present, it is unclear why our ba_3 preparation is more resistant to the 5C formation, which is caused by cleavage of the proximal histidine-iron linkage.

To assign other $\nu_{\text{C-O}}$ IR and $\nu_{\text{Fe-CO}}$ RR bands, the IR sample was illuminated by the 413.1 nm laser line so as to achieve a photostationary state and thereby examine the photostability of the conformers detected in the IR spectrum. The results shown in Figure 2B are expressed in the form of the dark *minus* light difference spectra; therefore, the positive and negative peaks in each spectrum represent species that disappeared and formed, respectively, upon laser illumination. Under all of the laser power conditions, positive peaks were recorded in the $\nu_{\text{C-O}}$ frequency range, and a negative peak appeared at the $\nu^{\text{CuB}}_{\text{C-O}}$ position, indicating that a substantial amount of the CO ligand moved from heme a_3 to Cu_B . The relative amplitude among the $\nu_{\text{C-O}}$ peaks changed as a function of the illuminating laser power. At the highest power (trace b: 80 mW) the difference spectrum in the 1950–2000 cm^{-1} range approached that of the dark spectrum indicating extensive photolysis of all of the species. However, in the lowest power spectrum (trace e: 2 mW; expanded by $\times 5$ in trace d), the relative peak intensity at 1966 cm^{-1} to other $\nu_{\text{C-O}}$ lines is high relative to that in the dark spectrum; on the contrary, no clear line is evident at 1981 cm^{-1} . These results demonstrate that the 1966 cm^{-1} species is the most photosensitive, whereas the 1981 cm^{-1} species is the least photosensitive. The latter observation supports our assignment of the 1981 cm^{-1} IR band to the 5C species, because the 5C species was the most photostable as compared to the six-coordinate (6C) CO adducts.²⁹ Likewise, the 1966 and 1973 cm^{-1} $\nu_{\text{C-O}}$ species are correlated with the 515 and 508 cm^{-1} $\nu_{\text{Fe-CO}}$ species, respectively, owing to the similar photo-sensitivities in the IR and RR experiments.

Correlation Parameters for the $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ Line of Heme–Copper Oxidases

On the basis of the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ data sets determined in this study, together with the established $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data from the α -forms of the oxidases, a linear least-squares line for the $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ correlation of the heme–copper oxidases is drawn in Figure 3 from which the parameters s and $\nu^0_{\text{Fe-CO}}$ were determined to be -1.1 and 327 cm^{-1} (Table 1). Very different parameters are derived if the new ba_3 frequency points are not included (dashed line). The $\nu^0_{\text{Fe-CO}}$ value is close to that for the $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ correlation in Mb (346 cm^{-1}), whereas the slope of the line ($|s| \sim 1.1$) is steeper than in Mb ($|s| \sim 0.8$). For a

comparison, the $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ correlation line of five-coordinate heme-CO adducts is also shown (thin solid line, the individual data points are not shown for clarity) and it yields s and $\nu^0_{\text{Fe-CO}}$ values of -0.33 and 463 cm^{-1} , respectively.

DFT Calculations of the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ Correlation

To investigate the origin of the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ correlation anomaly, we conducted DFT calculations on model systems that mimic the binuclear center of the heme-copper oxidases. As a first step, we sought to reproduce the typical inverse correlation between the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ frequencies in the porphyrin-CO (P-CO) complex by the DFT calculations. For this, we employed a procedure similar to that developed by Spiro and co-workers, in which instead of placing polar groups near the CO ligand, substituents are introduced on the periphery of the porphyrin ring.⁶ Such substituents either increase or decrease the electron density in the Fe-C-O moiety depending on their chemical nature. Therefore, they serve to mimic the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data shifts on the correlation line caused by polar groups in the distal environment of the CO adducts of heme proteins.^{6,33}

We selected fluorine (F) as a substituent and constructed a series of F-substituted iron-porphyrins as shown in Figure 4 (top). With the C_s symmetry along the y -axis, the structure optimization was done on 6-coordinate CO adducts of the compounds with the iron in a ferrous low-spin ($S=0$) electronic configuration. The 6-coordinate porphyrin-CO complexes (Im-P-CO), have an imidazole (Im) axial ligand, to reproduce the coordination state of the oxidases. The normal-mode analysis of the optimized structures had no imaginary frequencies for all compounds, and the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ frequencies were calculated successfully. The calculated frequencies for the Im-Fe-CO complexes yielded an inverse correlation curve (Figure 4, bottom) (its linear correlation coefficient (r) is -0.997), supporting the use of this procedure to mimic the inverse correlation resulting from the charge distribution in the distal environment. To test further the validity of the method, we also conducted DFT calculations on 5-coordinate porphyrin-CO complexes with and without the F substituents (Figure S4 Supporting Information). An inverse correlation was obtained ($r = -0.990$), and the $\nu^0_{\text{Fe-CO}}$ showed a significant upshift (493 cm^{-1}) from the value in the 6-coordinate compounds (424 cm^{-1}), consistent with the observed trends of the proximal ligand on the inverse correlation curves.

To determine the effect of the presence of Cu_B , a Cu_B unit model (Figure 5A) was introduced in the distal environment of the Im-P-CO. To keep the Im-P-CO and the Cu_B unit model stabilized, certain geometry parameters (shown in Figure 5B) were fixed during the structure optimization. The fixed values of such geometry parameters were set so that they were as close as possible to corresponding geometries of the CO form of bCcO³⁴ (3AG1; the geometry parameters are shown in Figure S1, Supporting Information) within the C_s symmetry limitation in the DFT calculations. In the optimized structure (Figure 5B), the Im-Fe-CO moiety showed slight tilting and bending deformations, which were not seen in the optimized structure of the Im-P-CO alone. Such deformations are common in the CO adducts of heme proteins, and our DFT calculation reproduced well the tilting and bending directions in the CO form of bCcO (Figure S1, Supporting Information), although the

calculated tilting (τ) and bending (β) angles (their definitions are given in the inset of Figure 5C) were slightly smaller than those in bCcO (3AG1: $\tau = 4.3^\circ$; $\beta = 6.0^\circ$).³⁴

The optimized binuclear center model exhibited three normal modes with imaginary frequencies, but all of them were of relative displacements between the Im-P-CO and Cu_B unit fragments. Therefore, the individual fragments were structurally optimized at the stationary points successfully within the geometry constraints applied to stabilize the complex. In the binuclear center model, the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data point was displaced above the inverse correlation line of the Im-P-CO compounds (Figure 4, bottom, closed circle), mimicking the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ correlation line anomaly of the heme-copper oxidases.

Molecular Orbital Calculations

To obtain an understanding of the origin of the anomalous behavior of the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ correlation for CcO, the composition of the molecular orbitals was determined. The first and second HOMOs (HOMO and HOMO-1) of the porphyrin compounds without the Cu_B unit were found to be A_{1u}/A_{2u} orbitals lying in the porphyrin plane with approximate D_{4h} symmetry. In the Im-P-CO compound, two other orbitals (HOMO-2 and HOMO-3), just below the planar HOMOs in energy, were found, which were x - and y -components of a nearly x - y degenerate pair, with an isovalue surface illustrated in Figure 6, top/right. The isovalue surface image clearly shows that these near-degenerate MOs are π -bonding and π -antibonding with respect to the Fe-C and C-O linkages, respectively, and hence are termed BA-1 after their Fe-C π -Bonding and C-O π -Antibonding characteristics. Two more pairs of MOs had similar properties, BA-2 and BA-3 (Figure 6, top), although the x - y degeneracy of each pair was largely broken, because of the electron delocalization to the imidazole moiety that was different between the x - and y -components of each MO pair. These BA-type MOs appear to be the origin of the inverse correlation between $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$, because they are expected to weaken and strengthen the Fe-C and C-O bonds, respectively, when the electron density is withdrawn from the Fe-C-O moiety to the porphyrin ring.

In addition to the above MOs, additional MOs that showed significant electron density on the Fe-C-O moiety were identified. Some of them were approximately x - or y -components of a pseudo x - y degenerate pair, although the degeneracy was imperfect as in the cases of the BA-2 and BA-3 pairs. In terms of the π -bonding characteristics over the Fe-C-O moiety, we classified such MOs as AB, π -Antibonding and π -Bonding for the Fe-C and C-O bonds, respectively, and BB, Fe-C π -Bonding, and C-O π -Bonding. An example of such MOs is shown in Figure 6, top for each type.

Mulliken Bond Orders

Although the visualized images of MOs enable determination of their characteristics with respect to the Fe-C and C-O bonding, we used a parameter related to the Mulliken bond orders for a more quantitative analysis of the MO contributions, which is given by

$$P_n[A-B] = \sum_{\mu} \sum_{\nu} s_{\mu\nu}^{AB} \cdot c_{\mu n}^A \cdot c_{\nu n}^B \quad (3)$$

where $c_{\mu m}^A$ and $c_{\nu n}^B$ are MO coefficients of μ - and ν th basis functions placed at the atoms A and B, respectively, in the n th MO, and $s_{\mu\nu}^{AB}$ is the overlap integral between the corresponding basis functions. All of these values are determined by the DFT calculations. Summing the parameter P over n (that is, over all occupied MOs; with the restricted Hartree–Fock MOs, the sum is multiplied by 2) becomes the Mulliken bond order. In other words, P is a bond order contribution of an individual MO to the total bond order between the atoms A and B. In addition, by selecting basis functions in the analysis, we evaluated x - and y -polarized π - interactions independently over the Fe–C–O moiety. For example, all d_{xz} -type basis functions of Fe and all d_{xz} - and p_x -type basis functions of C within the 6-311G*+ basis set were included, when the π_x contribution over the Fe–C bond was evaluated.

The amplitudes of the parameter P for individual MOs in the Im–P–CO compound are plotted in Figure 6 (bottom). The thick pink and cyan bars show the P amplitudes for the π_x and π_y interactions, respectively, between the Fe and C atoms, and the thin red and blue lines show those between the C and O atoms. The x – y degenerate BA-1 pair has x - and y -polarized positive amplitudes of P between Fe and C, and it has negative (x - and y -polarized) values for the C–O bond. Because the positive and negative amplitudes represent, respectively, the bonding and antibonding contributions, such results are fully consistent with the Fe–C π -bonding and C–O π -antibonding characteristics that were already indicated by the MO image of BA-1. The BA-2 and BA-3 MOs also have P amplitudes that agree with their Fe–C π -bonding and C–O π -antibonding properties.

In the F-substituted Im–P–CO compounds, MOs were altered in shape and in energy from those in the Im–P–CO, but we could see MO pairs in all F-substituted Im–P–CO compounds that were compatible with the BA-1 pair of Im–P–CO (Figure S5, Supporting Information). The P values for the BA-1 MOs in the F-substituted compounds were calculated and plotted as a function of the number of F atoms (Figure 7, open circles), where $\bar{P}[\text{Fe–C}]_\pi$ and $\bar{P}[\text{C–O}]_\pi$ denote π_x and π_y sums of the P amplitudes of each x – y pseudodegenerate MO pair for the Fe–C and C–O bonds, respectively. The BA-1 shows a nearly linear decrease and increase, respectively, in $\bar{P}[\text{Fe–C}]_\pi$ and $\bar{P}[\text{C–O}]_\pi$ as the number of F atoms is increased (Figure 7). Such features are consistent with the trends in the calculated $\nu_{\text{Fe–CO}}$ and $\nu_{\text{C–O}}$ frequencies (Figure 4, bottom), which have down- and up-shifts, respectively, as the number of F atoms was increased.

Other than the BA-1 pair, no specific MO or pseudodegenerate MO pair showed a clear linear correlation of the P parameter with respect to the number of F atoms. However, the MOs altogether roughly linearly respond to the number of F atoms; i.e., just as the $\bar{P}[\text{CO}]_\pi$ increases, the C–O σ - and the other C–O π -interactions as depicted by the light gray squares and circle symbols, respectively, in Figure 7, also increase with the number of F atoms. (“Total” and “Total (– BA-1)” designate that the P values were summed over all occupied MOs of each compound and all but BA-1 pair, respectively.) Thus, such increases in P also contribute to the calculated shifts in $\nu_{\text{C–O}}$. On the contrary, the MOs other than the BA-1 pair make little contribution to the calculated $\nu_{\text{Fe–CO}}$ shifts, as demonstrated by small and scattered remaining $\bar{P}[\text{Fe–C}]_\pi$ values without BA-1 shown by the dark gray circles in Figure 7, and by nearly flat $\bar{P}[\text{Fe–C}]_\sigma$ values shown by the dark gray squares. Although the F-

substituent technique provides a series of artificial systems to mimic the actual response of Fe–C–O to the distal polarity, it is assumed that MOs corresponding to the BA-1 are also predominantly responsible for the actual distal-polarity-dependent inverse correlation in the proteins, because they are HOMOs having higher energy and are more readily perturbed as compared to other MOs.

The introduction of the Cu_B model caused changes in P as shown in Figure 7, where the data from the Im–P–CO/Cu_B complex were depicted by the filled black symbols corresponding to the data for the Im–Fe–CO in the absence of the Cu_B model. Interestingly, we also found evidence for interunit MO mixing between the d_{z^2} orbital of Cu_B and the BA-1 of Im–P–CO as illustrated by Figure 8, although the effect of the mixing on the $H[\text{Fe–C}]_{\pi}$ and $H[\text{C–O}]_{\pi}$ of BA-1 were small (Figure 7). Among the porphyrin MOs giving the bonding or antibonding characteristics for the Fe–C–O moiety, only BA-1 showed mixing with the copper d_{z^2} , possibly because of its relatively high energy level, which matches that of the copper d_{z^2} . When we tested such mixing for a complex of the 5-coordinate porphyrin–CO and the Cu_B unit, the mixing was larger (Figure S6, Supporting Information).

However, large effects due to the presence of Cu_B were seen in other MOs. The decrease of the total $H[\text{C–O}]_{\pi}$ without the BA-1 and the increases of the total $H[\text{Fe–C}]_{\sigma}$ and $H[\text{Fe–C}]_{\pi}$ (large circles and square) could account for the calculated downshift in $\nu_{\text{Fe–C}}$, respectively, in the presence of the Cu_B unit (Figure 4, bottom). Upon full P value analysis over all MOs (the total number of occupied MOs are 180 and 113 for the Im–Por–CO/Cu_B complex and Im–Por–CO, respectively), the above $H[\text{C–O}]_{\pi}$ decrease was found to be attributable essentially to one particular MO (MO number 116 in the Im–P–CO/Cu_B system; MO_116), which is illustrated in Figure 9A (left, side-view; right, top-view). On the basis of the electron distribution pattern over the Fe–C–O moiety, the 75th MO (MO_75) in Im–P–CO was found to correspond to the MO_116 of Im–P–CO/Cu_B (Figure 9B). Because these MOs show C–O π -bonding character (Figure 9A,B), a decrease in $H[\text{C–O}]_{\pi}$ is expected, if the electron density redistributes from the Fe–C–O moiety to the porphyrin ring in the presence of the Cu_B unit. However, comparing panels A and B of Figure 9 suggests that the alteration in the MOs in question is not simply the electron redistribution from the Fe–C–O moiety in the porphyrin plane, because the symmetry pattern of electron distribution over the porphyrin ring was changed significantly. Such a change is expected, when two (or more) MOs having different symmetry are mixed. It should be noted that there is no electron density in the Cu_B model unit in the MO_116 (Figure 9A); therefore, no MOs from the Cu_B unit participated into the possible mixing scheme. In other words, the introduction of Cu_B caused a mixing of MOs within the Im–P–CO unit. With respect to the symmetry and similar energies, MO_74 is a candidate for the MO that participates in the mixing (Figure 9C). In addition to MO_75 of Im–Por–CO, which corresponds to MO_116 of the Im–Por–CO/Cu_B complex, MO_74 in the Im–Por–CO corresponds to MO_115 in the Im–Por–CO/Cu_B complex (not shown), although its character was significantly merged into the MO_116 as compared to the MO_75 in the complex without the Cu_B unit. The MO_74 (MO_115 in the Im–Por–CO/Cu_B complex) shows only a weak bonding character for the C–O linkage. Mixing with such an MO is expected to reduce the C–O bonding character of an MO that originally has a stronger C–O bonding character.

In general, mixing two MOs yields two new MOs, which share characteristics of the original MOs, although each of the new MOs is not simply a linear combination of the originals. In the present case, we postulate that mixing MO_74 and MO_75 yields MO_115 and MO_116 in the presence of the Cu_B unit. However, the MO_115 (and all other MOs in the Por-CO/Cu_B complex) showed no significant increase in $\bar{\nu}[\text{C-O}]_{\pi}$ at the expense of the decrease in MO_116 as compared to its original (i.e., the MO_75 of Im-Por-CO), thereby the total $\bar{\nu}[\text{C-O}]_{\pi}$ decreased upon complexing with the Cu_B unit.

Similar to the $\bar{\nu}[\text{C-O}]_{\pi}$ decrease, the $\bar{\nu}[\text{Fe-C}]_{\sigma}$ increase is attributed primarily to a particular MO, in the presence (MO_150) and absence (MO_96) of the Cu_B unit, which are shown in Figure 10A,B, respectively. The MO is antibonding with respect to the Fe-C σ -interaction, and the weakening of the antibonding character in the presence of Cu_B unit resulted in the increase of total $\bar{\nu}[\text{Fe-C}]_{\sigma}$ (Figure 7, large square). However, it is unclear whether such a weakening was caused by the MO mixing, because the electron distribution pattern over the porphyrin ring was almost unchanged from the original MO (Figure 10A,B). One possibility is that the original MO was mixed with an MO that had the same or a very similar electron distribution pattern over the porphyrin ring, but it also had a Fe-C σ -bonding character instead of the antibonding character in the original MO. One example of an MO (MO_94) that fulfills such requirements is shown by Figure 10C. The MO mixing dependent changes in $\bar{\nu}[\text{C-O}]_{\pi}$ and $\bar{\nu}[\text{Fe-C}]_{\sigma}$ cause shifts of $\nu_{\text{C-O}}$ and $\nu_{\text{Fe-C}}$, respectively. It is the combination of these individual contributions that leads to the $\nu_{\text{C-O}}/\nu_{\text{Fe-C}}$ correlation anomaly (Figure 4) in the presence of Cu_B.

The MO mixing in the presence of the Cu_B unit was determined to be the origin of anomalous frequency shifts of the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$. To clarify further these effects, an attempt was made to apply the F-substituent method to the Im-P-CO/Cu_B model and determine the parameters s and $\nu^0_{\text{Fe-CO}}$. However, this approach was unsuccessful due to electrostatic interactions between the F atoms and the atoms in the Cu_B unit model, which made the complex unstable.

DISCUSSION

$\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ Inverse Correlation Line for the α -Form of Heme-Copper Oxidases

In the past the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ spectroscopic data for the α -form of CcOs have been clustered in a small frequency range limiting the ability to extract the correlation line parameters. To extend the data distribution, we have used photodissociation methods to correlate the $\nu_{\text{Fe-CO}}$ modes in the RR spectra with the $\nu_{\text{C-O}}$ modes in the FT-IR spectra in the CO adduct of the *ba*₃ oxidase from *T. thermophilus*. By adding these new $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data points to those determined previously, we were able to obtain a revised inverse correlation line for the α -form of oxidases from which reliable values of the correlation parameters, s and $\nu^0_{\text{Fe-CO}}$, were determined.

The comparison of the slope, s , and the $\nu^0_{\text{Fe-CO}}$ intercept, of -1.1 and 327 cm^{-1} , respectively, of the inverse correlation line for the α -form of oxidases with the additional data points compared to that without the new data points of -0.52 and 425 cm^{-1} , respectively, reveals a very large difference. Most significantly the $\nu^0_{\text{Fe-CO}}$ frequency of

327 cm⁻¹ is very close to that of the His-ligand (Mb) $\nu_{\text{Fe-CO}}^0$ frequency of 346 cm⁻¹. Thus, the reason the oxidase family has an anomalous inverse correlation line is not due to a change in the strength of the Fe–CO σ -bond but instead is due to slope of the curve. The larger absolute value of the slope causes the correlation line to cross the Mb line and reach a region (i.e., $\nu_{\text{Fe-CO}}$, $\nu_{\text{C-O}}$ at ~510–520, 1960–1970 cm⁻¹, respectively) near those of the 5-coordinate species. This is a consequence of a different electron density in the Fe–C–O moiety due to the electronic interaction between the CO and Cu_B. In contrast, the absolute value of the slope for the 5-coordinate CO–porphyrin complexes is much lower and the $\nu_{\text{Fe-CO}}^0$ frequency is much higher (463 cm⁻¹) due to a stronger Fe–CO σ -bond in the absence of the Fe–His bond.

DFT Calculations of the $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ Inverse Correlation Line

To determine the effect of Cu_B on the inverse correlation, we first established a correlation line by modifying the π -electron density on the iron atom of the porphyrin by introducing fluorine atoms and calculating the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ frequencies for the 6-coordinate porphyrins, and reliable inverse correlation plots were obtained. Although calculation of $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ frequency data points for the binuclear center model with the F-substituent porphyrin could not be carried out due to electrostatic interactions between the F atoms and the atoms in the Cu_B unit model, calculation of the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ frequencies for the unsubstituted porphyrin was reliable and gave a data point above the inverse correlation line, confirming that the interaction of Cu_B with the CO yields frequencies that do not lie on the histidine inverse correlation line.

Electronic Mixing Caused by Cu_B

The DFT calculations reported here give a quantitative basis for the anomalous $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ inverse correlation in the heme–copper oxidases. The calculations demonstrate the occurrence of different types of MO mixing. One is mixing among the MOs of the porphyrin–CO moiety, and the other is mixing between the d_{z^2} orbital of Cu_B and HOMO–2/HOMO–3 orbitals (BA-1) in the α -form of the oxidases. These mixing schemes work together to alter the shapes of the molecular orbitals over the Fe–C–O moiety thereby yielding the anomaly in the inverse correlation plots.

The interaction between the Fe–CO heme and Cu_B is a delicate balance so when modest structural changes occur, for example, by mutagenesis of residues, even those that are not near the binuclear center, the α/β intensity ratio changes. Similarly, the ratio is also sensitive to the pH, possibly by the protonation/deprotonation of one of the histidines that serves as a ligand to Cu_B, although changes in the protonation state of more distant residues could also alter the α/β intensity ratio.

In the case of the conventional $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ inverse correlation, electrostatic contacts from the distal environment cause electron redistribution between the Fe–C–O and porphyrin moieties within individual MOs. The MOs that play roles in this process are those of the BA type, such as the BA-1 MO pair identified in this study by the F-substitution induced frequency shifts along the linear correlation line. The BA-1 MO pair is altered in the presence of Cu_B as the Cu_B d_{z^2} orbital mixes with the BA-1 orbitals. This likely contributes

to the difference in slope of the correlation line in the oxidase family of proteins. The other effect of the Cu_B unit seen here is that it causes mixing among MOs of the Im-Por-CO. Then a question arises as to what discriminates the Cu_B from other charged groups or electric dipoles that cause the electron redistribution within an MO, rather than the MO mixing, if they are located at the distal vicinity of the CO forms of heme proteins. One possible explanation considers the charge localization of Cu_B and its juxtaposition with respect to the Fe-C-O moiety. The cuprous copper in the Cu_B unit can be regarded as a point charge, as compared to amino-acid-centered cations, such as imidazolium and guanidinium cations, in which a positive charge is delocalized over the organic groups of the residues. Furthermore, amino acid side chains are more flexible, thereby fluctuating more than the cuprous copper in the rigid Cu_B cluster. Such differences may result in the different effects on the MOs of heme-CO. Future DFT studies are needed to clarify the origin of different electrostatic effects of the amino-acid-centered cations and cuprous copper to the electronic states of heme-CO.

CONCLUSIONS

By resonance Raman (RR) and FT-IR techniques, combined with photodissociation methods, the association of the $\nu_{\text{Fe-CO}}$ RR and $\nu_{\text{C-O}}$ IR lines among the multiple lines arising from the CO adduct of cytochrome *b₅* were identified. Using the associated $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data points, reliable values of the correlation parameters, *s* and $\nu^0_{\text{Fe-CO}}$, for the $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ inverse correlation curve of the α -form of heme-copper oxidases were obtained. Furthermore, to gain a molecular understanding of the basis for the anomalous inverse correlation of the α -form of oxidases, DFT calculations on a model of the binuclear center of heme-copper oxidases were carried out.

The calculations demonstrated that the copper unit model caused significant rearrangements among certain porphyrin-CO molecular orbitals (MOs) that contribute to either the Fe-C or C-O bonding interactions, and also indicated the presence of mixing between the d_{z^2} orbital of the copper and MOs that are responsible for the $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ inverse correlation. Together, the spectroscopic and DFT results clarify the origin of the anomaly of $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ frequencies in the heme-copper oxidases, resolving a long-standing issue.

On the basis of the spectroscopic and DFT results together, we proposed an MO mixing-dependent mechanism to explain the origin of the anomalous $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ correlation curve of the heme-copper oxidases. Whereas the results obtained here account for the $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ inverse correlation line anomaly, they also suggest that electronic interactions, not just steric factors, must be considered when assessing the role played by Cu_B during the oxygen reduction chemistry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

RR

resonance Raman

IR

infrared

FTIR

Fourier transfer infrared

$\nu_{\text{Fe-CO}}$, $\nu_{\text{C-O}}$, and $\delta_{\text{Fe-C-O}}$

the iron–CO stretching vibrational mode, the C–O stretching vibrational, and the Fe– C–O bending vibrational mode

oxidases

family of heme–copper oxidases

BA

Fe–C π -bonding and C–O π -antibonding

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Figure 1.

The $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ data from the CO adducts of heme proteins and model compounds. The abbreviations and sources of the data are Mb, myoglobin (+) (ref 2 and references cited therein); 5c- Por-CO, five-coordinate porphyrin-CO compounds (x) (ref 2 and references cited therein); P450, cytochrome P450 (O) (ref 2 and references cited therein); NOS, nitric oxide synthase (●) (ref 2 and references cited therein); bCcO, bovine cytochrome *c* oxidase;¹¹ rsCcO(α) and rsCcO(β); the α and β forms, respectively, of the cytochrome *c* oxidase from *Rhodobacter sphaeroides*;¹² bo₃(α) and bo₃(β), the α and β forms, respectively, of cytochrome bo₃ from *Escherichia coli*;¹³ pdCcO, the cytochrome *c* oxidase from *Paracoccus denitrificans*;¹⁴ aa₃-600, the aa₃-type quinone oxidase from *Bacillus subtilis*;¹⁵ bo₃ H333A, an H333A mutant of the bo₃ oxidase;¹⁶ and rsCcO H333N, an H333N mutant of rsCcO.¹⁷

Figure 2.

Resonance Raman spectra (A) and infrared absorption spectra (B) of the CO adducts of cytochrome *ba*₃ from *T. thermophilus*. The RR spectra shown are ¹²C¹⁶O–¹³C¹⁸O difference spectra, whereas the IR data are expressed in terms of the dark–light difference except for the top trace, which is an IR absorption spectrum measured in the dark. The RR spectra were excited at 413.1 nm with the indicated laser powers. The IR absorption in the light was recorded while illuminating the IR cell surface (~1 cm diameter) with the 413.1 nm laser line at the indicated powers.

Figure 3.

Updated $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ inverse correlation line of the heme-copper oxidases. The data determined in this study for ba_3 (open circles) are plotted together with the existing data from other heme-copper oxidases: bo_3 , square; aa_3 -600, inverted triangle, $bCcO$, triangle, $rsCcO(\alpha)$, diamond; $pdCcO$, closed circle. Linear least-squares fits of the data points with and without the ba_3 data are shown by solid (labeled as “CcO Extended”) and dotted (labeled as “CcO Limited”) lines, respectively. The inverse correlation lines of Mb and 5c-Por-CO, from Figure 1, are displayed for comparison.

Figure 4.

Orientation of the imidazole ligand in the iron-porphyrin compounds used for the DFT calculations (top) and the calculated $\nu_{\text{Fe-CO}}/\nu_{\text{C-O}}$ data of the CO adducts of the compounds (bottom, open circles). The $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{C-O}}$ correlation parameters, s and $\nu_{\text{Fe-CO}}^0$, were determined by a linear least-squares fit of the open circle data. The closed circle indicates the data point in the Im-P-CO and Cu_B model complexes.

Figure 5.

Cu_B unit model (A) and the optimized structures of its complex with the Im-P-CO used in the DFT calculations (B). In the geometry optimization of the complex, four geometry parameters (one distance and three angle parameters indicated in B) were fixed at constant values. In C, the tilting (τ) and bending (β) angles along the Fe-C-O moiety are indicated with their definitions in the inset.

Figure 6.

Isovalue surfaces of MOs showing either π -bonding or π -antibonding characteristics for the Fe–C–O moiety (top) and results of the Mulliken bond analysis for individual MOs (bottom) of the Im–P–CO compound. The MO images in the top are illustrated at different isovalue: 0.02, 0.03, 0.01, 0.03, and 0.04 (from left to right). Abbreviations used for the MO types are BA, Fe–C π -bonding and C–O π -antibonding; AB, Fe–C π -antibonding and C–O π -bonding; BB, Fe–C π -bonding and C–O π -bonding. In the bottom, the thick pink and cyan bars show the Mulliken bond order element (P parameter) for the π_x and π_y interactions, respectively, between the Fe and C atoms, and the thin red and blue lines show those between the C and O atoms. Basis functions in the 6-311G*+ basis set used to obtain the P values were the $x(y)$ component of $P[\text{Fe–C}]_\pi$, all $d_{xz(yz)}$ type basis functions of Fe and all $d_{xz(yz)}$ and $p_{x(y)}$ type basis functions of C; the $x(y)$ component of $P[\text{C–O}]_\pi$, all $d_{xz(yz)}$ and $p_{x(y)}$ type basis functions of C and O.

Figure 7.

Plots of P parameters as functions of the number of the fluorine atom substituents in the Im-P-CO compounds, and in response to the presence of the Cu_B model unit. Each P_{π} data is a sum of the P parameter values for the π_x and π_y interactions. Terms “Total” and “Total (–BA-1)” mean that the P values were summed over all occupied MOs of each compound and all but the BA-1 pair, respectively. The closed symbols show data in the Im-P-CO/ Cu_B complex. For $P_{\text{BA-1}}[\text{Fe-C}]_{\pi}$ and $P_{\text{BA-1}}[\text{C-O}]_{\pi}$ in the Im-P-CO/ Cu_B complex, the data are the sum of P values from the two split y-components and the x-component (Figure 8).

Figure 8. Mixing of the BA-1 MO of Im-P-CO and the d_{z^2} atomic orbital of Cu in the binuclear center model. The y -polarized component of the x - y degenerated BA-1 MO, which is shown in Figure 6, top/right, mixed with the d_{z^2} atomic orbital of copper, and split into two (A and B).

Figure 9. Effect on $\bar{A}[\text{C}-\text{O}]_{\pi}$ of the Cu_B unit induced mixing of MOs of the Im-P-CO compounds. Top and bottom show MOs in the presence and in the absence of the Cu_B model unit, respectively, of Im-P-CO compound. The left and right in each panel shows side and top views, respectively. (A) An MO that showed largest decrease (>0.02) in $\bar{A}[\text{C}-\text{O}]_{\pi}$ upon formation of the complex with the Cu_B unit compared to the value in the original MO (B) in the absence of the Cu_B unit. (C) A candidate MO that mixed with (B) to produce (A). The MO numbers for the drawings shown here are A, #116 in Im-P-CO/ Cu_B ; B, #75 in Im-P-CO; C, #74 in Im-P-CO.

Figure 10.

Effect on $P[\text{Fe-C}]_{\sigma}$ of the Cu_B unit induced mixing of MOs of the Im-P-CO compounds. Top and bottom show MOs in the presence and in the absence of the Cu_B model unit, respectively, of the Im-P-CO compound. The left and right in each panel shows side and top views, respectively. (A) MO showing the largest increase (~ 0.01) in $P[\text{Fe-C}]_{\sigma}$ upon formation of the complex with the Cu_B unit compared to the value in the original MO (B) in the absence of the Cu_B unit. (C) Candidate of MO that mixed with (B) to produce (A). The MO numbers for the drawings shown here are (A) #150 in Im-P-CO/Cu_B, (B) #96 in Im-P-CO, and (C) #94 in Im-P-CO.

Table 1Inverse Correlation Parameters^a

heme/porphyrin	$\nu_{\text{Fe-CO}}^0$ (cm⁻¹)	<i>s</i>	<i>r</i>
CcO (extended)	327	-1.1	-0.940
CcO (limited)	425	-0.52	-0.851
5C-Por-CO	463	-0.33	-0.761
Myoglobin	346	-0.81	-0.990
NO Synthase	325	-0.81	-0.949
P450	349	-0.64	-0.917

^aThe CcO (extended) and CcO (limited) are those determined in the present study with the additional data points and those reported previously, respectively. $\nu_{\text{Fe-CO}}^0$ is the limiting Fe-CO frequency; *s* is the slope; *r* is the correlation coefficient.