Cytochrome a_3 Structure in Carbon Monoxide–Bound Cytochrome Oxidase

Abstract. *The iron–carbon monoxide stretching mode and the iron-carbon-oxygen bending mode in carbon monoxide–bound cytochrome oxidase have been assigned at 520 and 578 cm^{-1}, respectively. The frequencies, widths, and intensities of these modes show that the Fe-C-O grouping in carbon monoxide–cytochrome a_3 is linear but tilted from the normal to the heme plane; that the iron-histidine bond in both five- and six-coordinate cytochrome a_3 is strained; and that the carbon monoxide and the proximal histidine each have characteristic, well-defined orientations in all molecules. These data can account for the binding affinities of carbon monoxide and dioxygen under physiological conditions.*

Cytochrome oxidase, the terminal enzyme in the mitochondrial respiratory chain, contains two heme a chromophores: cytochrome a and cytochrome a_3. Dioxygen binds to the cytochrome a_3 heme and is reduced to water by the transfer of four electrons (*1*). Understanding the molecular mechanism of the cytochrome oxidase function may be achieved through studies of the oxygen-bound form of the enzyme, but such studies are difficult because of the rapid reduction of O_2 and the associated oxidation of the hemes. However, carbon monoxide and the reduced enzyme form stable complexes (*2*) that contain information about ligand binding and heme pocket properties. We now present and discuss the resonance Raman spectra of the reduced enzyme with CO bound to cytochrome a_3. Isotopic substitution methods have enabled us to assign the iron-carbon stretching mode [ν(Fe-CO)] and the iron-carbon-oxygen bending mode [δ(Fe-C-O)] in the CO complex. The frequencies and intensities of these modes indicate that the CO is tilted from the normal to the heme plane and that the proximal histidine is strained in the CO complex.

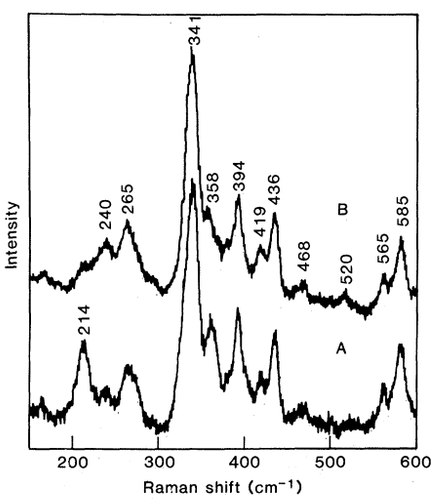


Fig. 1. Resonance Raman spectra in the low-frequency region (150 to 600 cm^{-1}) of reduced (A) and carbon monoxy (B) mammalian cytochrome oxidase. The small contribution at 214 cm^{-1} in the (CO)MOX spectrum resulted from photodissociation. Excitation wavelength, 441.6 nm; slit width, 5 cm^{-1}. The concentration of the protein was approximately 60 μM in 50 mM Hepes buffer at pH 7.4 with 1 percent dodecyl-β-D-maltoside. The spectra were averaged over ten scans.

Mammalian (beef heart) cytochrome oxidase (MOX) was separated (*3*) and frozen under liquid nitrogen until ready for use. The enzyme was buffered in 50 mM Hepes buffer at pH 7.4 with 1 percent dodecyl-β-D-maltoside for the measurements. The absorption spectra (10 μM heme) showed a Soret maximum at 444 nm for reduced MOX. When CO was bound, the Soret band shifted to 430 nm, in agreement with earlier results (*4*).

The Raman measurements were made on Raman difference instrumentation (*5*), which allows reliable detection of small differences (<0.1 cm^{-1}) in peak positions of the vibrational modes. For the isotope studies, dithionite-reduced MOX was placed in each side of a split rotating cell in a nitrogen atmosphere, and both compartments were sealed with rubber stoppers. Either $^{12}C^{16}O$ (Matheson) or $^{13}C^{16}O$ (KOR Isotopes, 90 atom-percent ^{13}C) was injected into the samples without exposure to the atmosphere. Spectra were obtained at room temperature in a few hours without detectable sample degradation. By means of a rotating cell (~30 rev/sec) and low laser power, photodissociation was minimized. None of the spectra were smoothed.

In the low-frequency resonance Raman spectrum of reduced MOX (Fig. 1A), the strong line at 214 cm^{-1} was proposed to be the Fe-histidine stretching mode since an equivalent line between 200 and 240 cm^{-1} in hemoglobin, myoglobin, and various model compounds was assigned as this mode (*6*). This assignment was confirmed by Ogura *et al.* (*7*), who reported that in a bacterial cytochrome oxidase a line at 211 cm^{-1} exhibited an upward frequency shift by 2 cm^{-1} upon ^{54}Fe substitution. In the spectrum of (CO)MOX (Fig. 1B), the 214-cm^{-1} line disappears. This is consistent with its being the Fe-histidine stretching vibration of cytochrome a_3, since the mode is present in the spectra of five-coordinate hemes but is absent in those of six-coordinate hemes (*8*).

Comparison of these two spectra (Fig. 1, A and B) reveals evidence for a new weak line at 520 cm^{-1} with a width of about 10 cm^{-1}. Lines in other CO-bound heme proteins have been assigned in the 500- to 600-cm^{-1} region to ν(Fe-CO) and δ(Fe-C-O) by isotopic substitution studies (*9*). To determine if the line at 520 cm^{-1} in MOX involves the CO, we compared the spectrum of MOX bound to ^{12}CO to that of MOX bound to ^{13}CO (Fig. 2) and found two lines in the 550- to 590-cm^{-1} region, one of which was isotope-sensitive, and another isotope-sensitive line in the 520-cm^{-1} region. The former two lines are nearly degenerate in (^{12}CO)MOX (Fig. 2B), giving a broad line centered at 583 cm^{-1}. However, in (^{13}CO)MOX (Fig. 2C) these lines are separated into a feature at 564 cm^{-1} and one at 588 cm^{-1}. We attributed the line at 588 cm^{-1} to a porphyrin mode, and from the difference spectrum (Fig. 2D) we found that the other line shifted by 14 cm^{-1} [that is, 564 cm^{-1} in (^{13}CO)MOX and 578 cm^{-1} in (^{12}CO)MOX]. The lower frequency line shifted from 520 cm^{-1} in (^{12}CO)MOX to 516 cm^{-1} in (^{13}CO)MOX. No other lines in the 150- to 1700-cm^{-1}

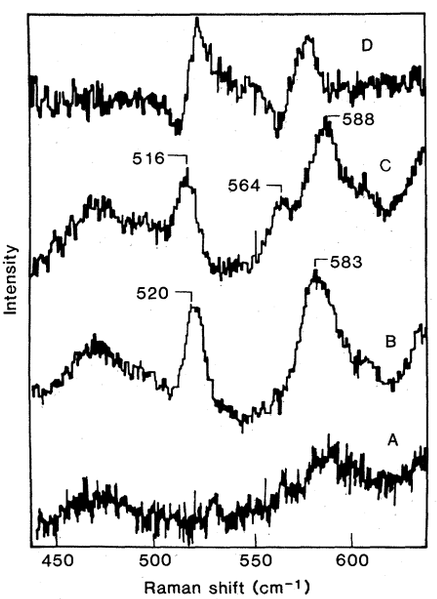
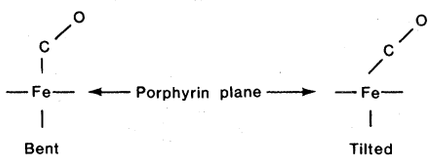


Fig. 2. Isotopic dependence of the resonance Raman spectra of carbon monoxide cytochrome oxidase. (A) Reduced MOX; (B) (^{12}CO)MOX; (C) (^{13}CO)MOX; (D) difference spectrum (B minus C) on the same scale. The solution conditions were the same as in Fig. 1 except that the MOX concentration was 160 μM. The excitation wavelength was 413.1 nm and the slit width was 6 cm^{-1}. The spectra were average over 15 scans.

range shifted on isotopic substitution. By comparing the frequency changes upon isotopic substitution in (CO)MOX to corresponding changes in a variety of proteins and model compounds (Table 1), we were able to assign the line at 520 cm^{-1} to the Fe-CO stretching mode and the line at 578 cm^{-1} to the Fe-C-O bending mode.

The frequencies and intensities of carbon monoxy modes in hemes are influenced by the characteristics of the proximal ligand, the electronic properties of the heme, and steric and polar interactions with the ligand (*10*). The steric interactions of the ligand lead to structures other than the electronically preferred linear bonding perpendicular to the heme plane and may result in either a bent or a tilted CO ligand (*11*).



The properties of the ligand in (CO)MOX may be clarified by comparison with data on other proteins and model compounds (Table 1).

The line width (<10 cm^{-1}) of the 520-cm^{-1} line is smaller than in model compounds and other proteins (*9, 12, 13*) and is consistent with the narrow width of the C-O stretching mode at 1963.5 cm^{-1} detected by infrared absorption (*14*). The narrow width of the Fe-CO stretching mode implies a homogeneous, ordered environment for the ligand and a well-defined conformation, in accord with results of infrared studies showing that the CO is in a homogeneous, hydrophobic pocket (*14*). Further, the Fe-CO and C-O vibrational frequencies are sensitive to both electronic (*15*) and steric (*12*) changes in the *trans* ligand, as inferred by the effect of changes in the proximal ligand on the properties of the Fe-C-O group in model compounds. Thus, from the narrow line widths of the Fe-CO and the C-O stretching modes, we inferred that the proximal histidine is confined to one orientation and that its configuration does not change from one molecule to another.

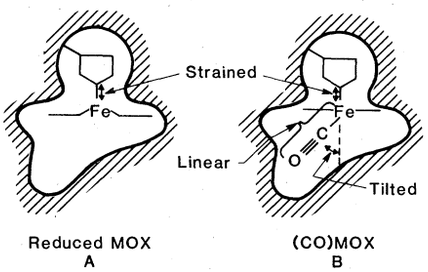


Fig. 3. Structural constraints in cytochrome a_3 heme pocket in cytochrome oxidase inferred from Raman scattering data. (A) Fully reduced cytochrome oxidase with a strained iron-histidine bond. (B) Carbon monoxide–bound cytochrome oxidase with a strained iron-histidine bond and a linear but tilted Fe-C-O unit.

From the isotopic dependence of the Fe-CO stetching mode and the C-O stretching mode, the angle of bend of the Fe-C-O bond may be determined (*13*). On the basis of our values of 520 and 516 cm^{-1} for the Fe-CO stretching mode and the frequencies of 1963.5 and 1918 cm^{-1} (*16*) for the C-O stretching mode in (^{12}CO)MOX and (^{13}CO)MOX, respectively, together with a calculation by Yu *et al.* (*13*), we determined that CO forms a linear (not bent) bond. The calculation gave an Fe-C-O angle of 175° ± 5°.

The relative intensity of the bending mode in CO hemes gives a measure of the degree of tilt (*13*). In hemes without any steric distortion the bending mode is not detected, but this mode is detected in sterically hindered porphyrins (for example, FeSP-13) (*13*). The intensity of the bending mode has been proposed to result from a charge transfer interaction between the CO and the porphyrin when the ligand is bent (*13*). This leads to enhancement of the bending mode as well as a general increase in the Fe-C-O force field. The largest intensity of the bending mode relative to the stretching mode observed heretofore is 0.25. On the basis of our observation that the intensity of the bending mode in MOX is approximately 0.5 that of the stretching mode, we propose that the Fe-C-O group is tilted with respect to the heme plane.

The 520-cm^{-1} frequency of the Fe-CO stretching mode is high compared to the frequency of this mode in other heme proteins and model compounds. Although such a high frequency could be a result of many factors, the data appear to be most consistent with an anomalously weak iron–proximal histidine bond in

Table 1. Frequencies (cm^{-1}) of the Fe-CO stretching mode (ν) and the Fe-C-O bending mode (δ) in CO hemes and the Fe-histidine stretching mode in reduced five-coordinate hemes compared to those in mammalian cytochrome oxidase (MOX). The $\Delta\nu$ and $\Delta\delta$ values are the isotopic frequency shifts of the Fe-CO stretching and bending modes, respectively, for ^{12}CO and ^{13}CO. The Iδ/Iν values are the intensity ratios of the bending to the stretching mode, and FWHM is the full width at half-maximum (cm^{-1}) of the Fe-CO stretching mode.

Moiety*	$\nu(\Delta\nu)$	$\delta(\Delta\delta)$	Iδ/Iν	FWHM	Fe-His	Reference
MOX	520 (4)	578 (14)	0.5	10	214	
HbA(T)	507	577	0.10	18	216(α:206; β:218)†	(*9, 24*)
HbA(R)	507 (4)	578 (15)	0.10	18	222(α:220; β:222)†	(*9, 24*)
Mb	512 (3)	577 (14)	0.25	15	221	(*9, 25*)
Lb	505	580	0.25	15	224	(*26*)
HRP					244	(*27*)
Heme-5 NMI	495 (4)	N.D.	0	17		(*13*)
FeSP-15 NMI	509 (6)	574 (11)	0.10	20‡		(*13*)
FeSP-14 NMI	512 (4)	578 (15)	0.16	22‡		(*13*)
FeSP-13 NMI	514 (4)	579 (11)	0.25	25‡		(*13*)
Tpiv PP NMI	489 (4)	N.D.	0	14	225	(*12, 28*)
Tpiv PP THF	526 (5)	N.D.	0	10		(*12*)
Tpiv PP	526	N.D.	0	11		(*12*)
Tpiv PP 1,2-DMI	496 (5)	N.D.	0	16	200	(*12, 28*)
TPP NMI	486	N.D.	0	15		(*12*)
OEP NMI	496	N.D.	0	18‡		(*12*)
PP 2MI (H_2O)					220	(*16*)
PP 2MI (det)					206	(*16*)

*Abbreviations represent the following: HbA(T) and HbA(R), T and R quaternary structure, respectively, of human adult hemoglobin; Mb, myoglobin; Lb, leghemoglobin; HRP, horseradish peroxidase; NMI, *N*-methylimidazole; 1,2-DMI, 1,2-dimethyl imidazole; THF, tetrahydrofuran; det, detergent; heme-5, dipentyl, tetramethyl, di(CH_2NHAc) porphyrin; FeSP-13, -14, -15, strapped hemes with 13-, 14-, or 15-member strap crossing CO binding site; Tpiv PP, meso-tetra (α-α-α-α-*o*-pivalamidophenyl) porphyrin; TPP, tetraphenyl porphyrin; OEP, octaethyl porphyrin; PP, protoporphyrin; and N.D., not detected. †Numbers in parentheses indicate values for distinguishable α and β chains. ‡These widths were difficult to determine because of multiple components.

cytochrome oxidase. This is supported by evidence from model compound studies (*12*), in which only those compounds with weak *trans* ligands [for example, Tpiv PP THF (CO)] or no *trans* ligand at all [for example, Tpiv PP(CO)] have frequencies as high as 520 cm^{-1}. Also, the frequency of the Fe-histidine stretching mode in reduced cytochrome oxidase is 214 cm^{-1}, a value lower than that for all other proteins except the α chains of T-state deoxyhemoglobin, which is also proposed to have a destabilized iron-histidine bond (*17*). This frequency in the reduced enzyme is therefore consistent with a strained (weak) Fe-histidine bond. Finally, in low-temperature magnetic circular dichroism (MCD) studies, the photodissociated heme in cytochrome oxidase was found to have the same MCD spectrum as the fully reduced heme (*18*). In contrast, the spectra of photodissociated hemoglobin and myoglobin differ from that of the corresponding deoxy preparations. The MCD data were interpreted (*18*) as indicating that the histidine-heme complex is confined to the same conformation when six-coordinated as it does when five-coordinated, which is the conformation with the iron atom held out of plane. This interpretation is consistent with our discovery of a weakened Fe-histidine bond in the six-coordinate heme.

Our conclusions as to these structures (See Fig. 3) clarify some of the physiological properties of cytochrome oxidase binding to both oxygen and carbon monoxide. The affinity of MOX for CO is lower than that of myoglobin (*19*). We found that the bound CO is tilted in the MOX presumably because of an amino acid residue present in the distal pocket. This should result in a restricted and unfavored entry to the binding site for the CO and consequently in a low on-rate and low affinity. The measured on-rate for CO ($\sim 1 \times 10^5\ M^{-1}\ sec^{-1}$) (*19*) is lower than that of myoglobin ($\sim 5 \times 10^5\ M^{-1}\ sec^{-1}$)] (*20*). The off-rate, which results from a unimolecular dissociation, would be expected and is observed to be similar in MOX and myoglobin since the Fe-C bond strengths are comparable (that is, the stretching frequencies are both in the 500-cm^{-1} range). The steric interaction in the binding site would not affect the oxygen binding since it binds preferentially in a bent orientation.

The intrinsic affinity for oxygen binding to MOX is difficult to measure because the oxygen is reduced by the oxidase and the cytochrome a_3 is oxidized. Values for the association constant have been reported to be from 10^6 to 10^8 (*21*), although the results of low-temperature experiments have been interpreted (*22, 23*) as evidence for a lower value for the actual binding to the iron. The apparent high affinity under physiological conditions has been described as a consequence of secondary reactions that are subsequent to binding to the iron and culminate in the oxygen reduction (*23*). Our data on (CO)MOX indicate that the Fe-histidine bond is strained when the oxidase is six-coordinated. We interpret this strain as evidence that the iron movement into the heme plane is restricted upon oxygen binding and causes a weak iron-dioxygen bond. These data support proposals that oxygen binds only weakly to the iron in cytochrome oxidase and that the apparent high affinity for oxygen results from additional reactions.

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Mechanical Wounding Induces the Formation of Extensive Coated Membranes in Giant Algal Cells

Abstract. *Mechanically wounding giant cells of* Boergesenia forbesii *induces the formation of bristle-coated, plasma-membrane invaginations (coated pits) and coated vesicles, easily providing a plentiful source of coated membranes in a clean cellular system unencumbered by other tissues. Contractions evoked by wounding partition the cytoplasm into hundreds of spherical protoplasts with approximately 40 percent less total plasma-membrane surface area than the original cell. Ferritin labeling and the appearance of numerous large coated pits and vesicles at the peak period of contraction indicate that these organelles play a role in extensive endocytosis of the plasma membrane.*

Giant cells of plants (*1–3*), animals (*4*), and protists (*5*) heal wounds through the activity of putative contractile mechanisms (*3, 6*). In some plant cells (*1, 2*), being wounded induces cytoplasmic contractions that result in the complete segregation of cytoplasm into numerous spherical protoplasts within 90 minutes (Fig. 1, A to E). Ultrastructural studies of this phenomenon in the giant-celled green alga *Boergesenia forbesii* (*7*) reveal that approximately 45 minutes after wounding, extensive coated pits and vesicles become evident in and near the plasma membrane (Fig. 1F–H). They continue to be abundant as long as 75 minutes after wounding, whereafter they are only infrequently observed. The 30-minute interval of their observed occurrence corresponds to the peak period of cytoplasmic contraction that leads to protoplast formation (*2*). The average sizes of coated pits and coated vesicles are 250 nm (range, 80 to 625 nm) and 175 nm (100 to 350 nm, outside diameter), respectively. This variability in size is