RESONANCE RAMAN SPECTRAL ISOLATION OF THE *a* AND a_3 CHROMOPHORES IN CYTOCHROME OXIDASE

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ABSTRACT Resonance Raman spectra of reduced CO-bound cytochrome oxidase obtained at two different excitation frequencies (441.6 and 413.1 nm) are compared with the spectra of the fully reduced enzyme. In the spectra of the CO-bound complex only the cytochrome *a* modes are strongly enhanced with 441.6 nm excitation and only the modes of the CO-bound cytochrome a_3 heme are strongly enhanced with 413.1-nm excitation. In the fully reduced complex with both excitation frequencies, modes of both cytochrome *a* and a_3 are enhanced. By subtraction we are able to uncover the complete spectrum of the fully reduced ligand-free cytochrome a_3 heme. Thus, we report the discrete resonance Raman spectra of cytochromes a^{2+}, a_3^{2+}, and a_3^{2+} (CO). The spectra of fully reduced cytochrome *a* and ligand-free cytochrome a_3 are very different especially in the low frequency region. Binding CO to ferrous cytochrome a_3 results in electronic structure changes in the heme analogous to those in hemoglobin and myoglobin, from which we conclude that there is nothing electronically unique in the ferrous cytochrome a_3 heme to account for its catalytic properties.

INTRODUCTION

Cytochrome oxidase is the terminal enzyme of the mitochondrial electron transport chain, which reduces dioxygen to water and in the process generates an H^+ ion gradient across the mitochondrial membrane (for a review see reference 1). The enzyme consists of two heme *a* chromophores, referred to as cytochrome *a* and cytochrome a_3, and two copper ions embedded in the membrane bound protein. The O_2-bound reduced enzyme, although of major interest, is difficult to study because the O_2 is rapidly reduced to water. However, the first step in the reduction process is thought to be the formation of a low spin $a_3 - O_2$ complex similar to that formed by hemoglobin or myoglobin (2, 3). Since the reduced enzyme (a_3 spin, $S = 2$) forms a stable low spin complex (a_3 spin, $S = 0$) with CO, it can be studied as an analog of the initially formed O_2 complex (4). Studies of the CO complex are helpful for determining the properties of the ligand binding site as well as addressing such questions as the role of heme-heme interaction which are important for understanding the functional mechanisms.

We have been using resonance Raman scattering to study the structural changes that occur in the hemes upon ligand binding. With resonance Raman scattering the vibrational modes of the heme group may be examined. To determine the structural changes that take place in the two cytochromes upon oxidation or reduction or upon ligand binding, it is first necessary to assign the modes of cytochrome *a* and cytochrome a_3. Over the past few years the resonance Raman spectra of many complexes of cytochrome oxidase have been reported (5–7), and efforts have been made to identify the Raman modes of each of the hemes in cytochrome oxidase. These prior studies have drawn from model compound data and have reported frequencies for the modes from each of the hemes in the high-frequency region (above ~1,300 cm^{-1}) (5–9). The low-frequency region is also important to study since this region contains lines that are sensitive to the heme peripheral groups and the axial ligands.

In a previous report (10) we focussed on the geometry of the CO ligand bound to cytochrome oxidase. In a later report (11) we introduced a method for spectrally isolating the resonance Raman spectra of cytochromes a^{2+} and a_3^{2+}. In this paper we focus on the porphyrin modes in the resonance Raman spectra of CO-bound reduced cytochrome oxidase and take advantage of selective excitation to obtain spectrally isolated data from each heme. The power of selective excitation has been pointed out in the past by several authors (5, 6, 8, 11). We have observed that in the CO-bound complex only the modes of cytochrome *a* are enhanced with 441.6 nm excitation and only those of CO-bound cytochrome a_3 are enhanced with 413.1-nm excitation. In addition, by subtracting the spectrum of cytochrome *a* from the spectrum of the fully reduced enzyme at 441.6 nm, we have obtained the spectrum of fully reduced ligand free cytochrome a_3. Consequently, we are able to report the Raman spectra of cytochromes a^{2+}, a_3^{2+}, and a_3^{2+} (CO). As a result of this spectral isolation we are now able to assess the electronic structure changes that occur in cytochrome a_3 upon binding CO and sort out the lines in the entire spectrum that are sensitive to the high to low spin state change and those that are sensitive to the electronic properties of the bound carbon monoxide.

MATERIALS AND METHODS

Mammalian cytochrome oxidase was separated by the method described by Babcock et al. (4), and was frozen under liquid N_2 until ready to use. The buffer detergent system used was 50 mM Hepes at pH 7.4 with 1% dodecyl-β-D-maltoside. For the resonance Raman measurements, 60 μM (441.6 nm excitation) or 100 μM (413.1 nm excitation) of reduced cytochrome oxidase was placed in a spinning cell and sealed under N_2. To make CO-bound reduced cytochrome oxidase, CO (Matheson) was purged on the enzyme without exposing it to the atmosphere. Use of the spinning cell along with a low laser power minimized photodissociation. The resonance Raman apparatus has been described before (12). Absorption spectra were recorded on the same samples used in Raman experiments. The spectra shown here are faithful tracings of the data, which have been background corrected but not smoothed. The peak positions are correct to ±1 cm^{-1}.

RESULTS AND DISCUSSION

The optical absorption spectra of reduced and CO-bound reduced cytochrome oxidase are shown in Fig. 1. The changes in the position and shape of the α-band in the 550–650-nm range and Soret band in the 400–450-nm range are the same as those reported previously for the reduced and CO-bound reduced enzyme (13, 14). The dashed lines in this figure show the positions of the incident laser wavelengths which we used in the present study. For the fully reduced enzyme the Soret peak at 443 nm has been shown to consist of components from cytochromes *a* and a_3 at 444 and 442.5 nm, respectively (14). Upon binding CO to the cytochrome a_3, the peak of its Soret band is shifted to 430 nm, whereas that of cytochrome *a* remains unshifted at 444 nm.

The resonance Raman spectra also confirm that the addition of CO to the fully reduced cytochrome oxidase results in a bound complex. The fully reduced spectrum

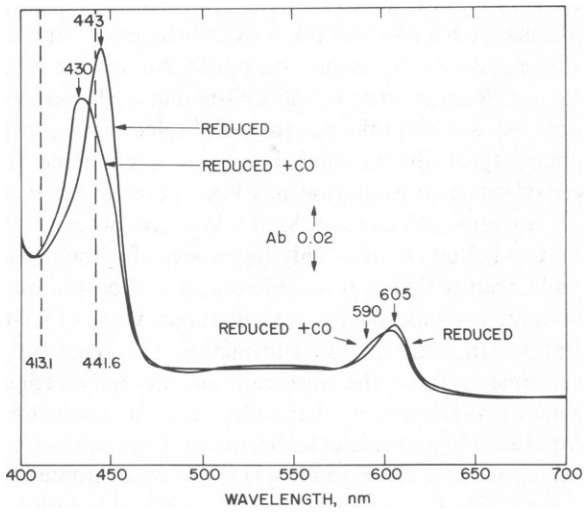

FIGURE 1 Optical absorption spectrum of (*A*) reduced mammalian cytochrome oxidase and (*B*) CO-bound reduced mammalian cytochrome oxidase. The dashed lines indicate the positions of the incident laser wavelengths used in the present study. The reduced form shows major peaks at 443 and 605 nm. The CO-bound enzyme has peaks at 430, ~443, 590, and 605 nm.

has a line at 213 cm^{-1}, which has been assigned as the iron-histidine stretching mode (7, 15). Its disappearance upon binding CO is consistent with this assignment and with the formation of the bound CO complex because in six-coordinate hemes the line in the 200–220-cm^{-1} region disappears. (See the comparison between spectra A and B in Fig. 2.)

Additional evidence that we have formed the CO complex may be seen in other lines of the Raman spectrum as well. The line in the 1,350–1,380-cm^{-1} region has been assigned as the electron density marker line (16). For both cytochrome *a* and a_3 in reduced cytochrome oxidase this line appears at 1,355 cm^{-1}. (See spectra A in Figs. 2 and 3.) Upon binding CO, a line appears at 1,368 cm^{-1} as may be seen in spectrum B of Fig. 3 obtained with 413.1-nm excitation. We assign this line as the electron density marker line for the CO bound cytochrome a_3. (See discussion below.) Its high intensity relative to a very weak shoulder at 1,356 cm^{-1} from the reduced enzyme in the spectrum obtained with 413.1-nm excitation is evidence that the laser beam is not significantly photodissociating the CO from the enzyme in this data. The degree of photodissociation with 441.6 nm was monitored by the presence of a weak iron-histidine stretching mode. Many experiments were done to assure that only minimal photodissociation occurred with both excitation frequencies.

Final confirmation that the cytochrome a_3 heme has CO bound to it results from the presence of lines associated with the Fe-C-O vibrational modes. From isotopic substitution studies we recently were able to assign the lines at 520 and 578 cm^{-1} as the Fe-CO stretching and the Fe-C-O bending modes, respectively (10). The presence of these lines in the spectra confirm the presence of CO bound cytochrome oxidase. The frequencies, widths, and intensities of these modes show that the Fe-C-O grouping is linear but tilted from the normal to the heme plane and that the iron-histidine bond is strained (10).

By considering the optical absorption spectra, the relative contributions to the Raman spectra from each type of cytochrome (*a* and a_3) may be predicted to first order (8, 17, 18). The intensity of a Raman band is proportional to the square of the extinction coefficient but inversely proportional to the square of difference in energy for the electronic transition and the energy of the exciting laser photon. The 441.6-nm excitation wavelength is near the Soret maxima for both cytochromes *a* and a_3 in the unbound fully reduced state so contributions from both cytochromes would be expected in the Raman spectrum. Similarly the 413.1-nm excitation is equally far from the Soret maxima of both cytochromes in their fully reduced state so both should contribute to the spectrum. On binding CO to the cytochrome a_3 heme, the Soret peaks of *a* and a_3 become separated. The 441.6-nm excitation remains more in resonance with the cytochrome *a* than a_3 and the 413.1-nm excitation is more in resonance with the Soret transition of the CO-bound cytochrome a_3.

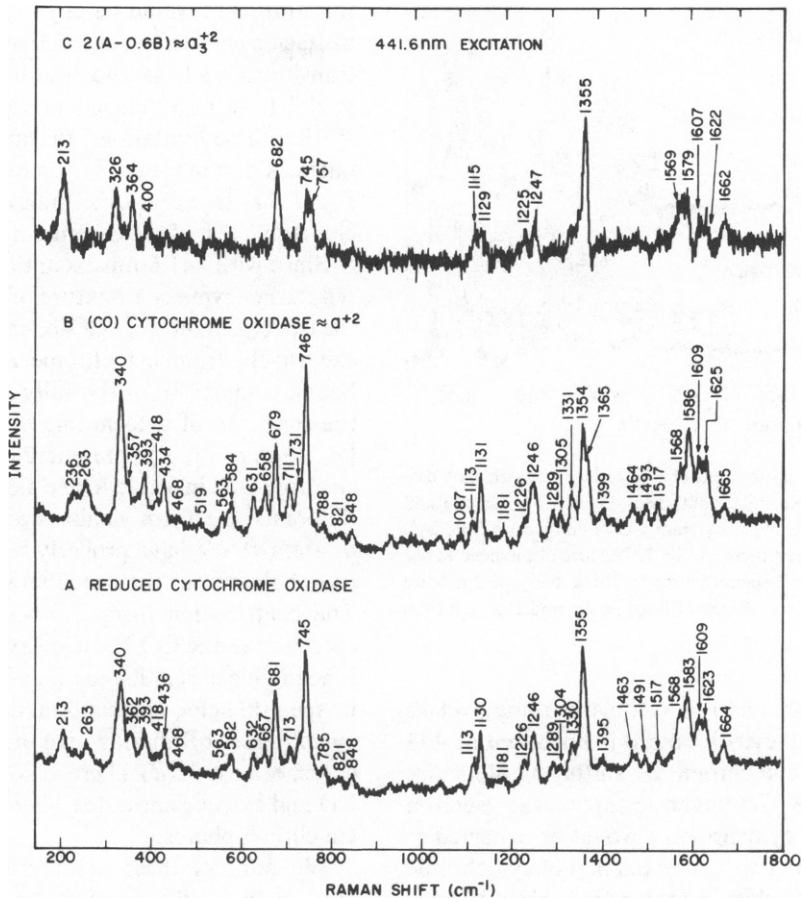

FIGURE 2 Resonance Raman spectra and difference spectrum of (*A*) reduced cytochrome oxidase and (*B*) CO-bound reduced cytochrome oxidase obtained with 441.6-nm excitation. Spectrum *B* results almost exclusively from cytochrome *a*. The difference spectrum (*C*) is displayed at twice the sensitivity as the spectra in *A* and *B*. The balance factor in the difference spectrum has been selected so as to just avoid negative peaks, thereby yielding the spectrum of cytochrome a_3^{2+}. The frequency of the formyl mode in this spectrum is 2 cm^{-1} too low due to a subtraction artifact. The spectral resolution is 5 cm^{-1}. The data were signal averaged in four scans with a total accumulation time of ~5 h.

Although the above analysis has been invoked to explain separation of the Raman spectra of cytochromes *a* and a_3 in the past (8, 18), the actual Raman excitation profile is a function of a variety of parameters that lead to excitation profiles that are far more complex than the absorption envelope (19, 20). In particular, high frequency modes have excitation profiles with maxima to the high energy side of the optical absorption maximum due to resonance at the 0-1 transition, i.e., one vibrational quantum above the expected energy for the absorption maximum. Furthermore, proper analysis (19, 20) of the resonance Raman intensity requires the inclusion of a distribution function of multidimensional vibrational subspace with an energy maximum above the parent state. This also contributes to an excitation profile with a maximum on the high energy side of the absorption maximum. These factors lead to a favorable situation with the CO-bound cytochrome oxidase since the 441.6-nm laser excitation wavelength is at higher energy than the cytochrome a^{2+} Soret maximum and therefore should yield maximal intensity for this species. On the other hand, 441.6 nm is to the low energy side of the cytochrome a_3^{2+} (CO) Soret peak so the Raman scattering should be far weaker. These effects are not as pronounced for the low-frequency modes but in that case the excitation profiles of the lines are narrower than the absorption profile, so again, good separation may be achieved.

The resonance Raman data in Figs. 2 and 3 obtained with 441.6- and 413.1-nm excitation, respectively, substantiate that the spectra of the different cytochromes may be enhanced by use of appropriate wavelengths. This is especially evident by following the positions of the peaks of the Soret transition and the electron density marker line in the 1,350–1,380-cm^{-1} region. In the fully reduced enzyme the Soret absorption maxima for both cytochrome *a* and a_3 are nearly the same (~444 nm) and the electron density marker line for both cytochromes appear as a single line at 1,355 cm^{-1}. In oxidized preparations the Soret transition of both cytochrome *a* and a_3 shift to shorter wavelength (~420 nm) and concomitantly, the electron density marker line shifts to 1,369 cm^{-1} (6). Thus, the electron density marker line appears to vary inversely with the Soret absorption maximum. This correlation holds for most

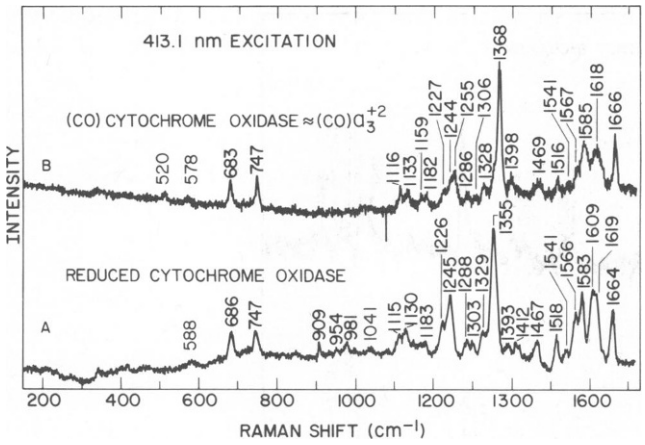

FIGURE 3 Resonance Raman spectra of (*A*) reduced cytochrome oxidase and (*B*) CO cytochrome oxidase obtained with 413.1-nm excitation. Spectrum *B* results almost exclusively from CO-bound cytochrome a_3^{2+}. The conditions are the same as those in Fig. 2. Because of the need to use low laser powers for the CO-bound enzyme these two spectra were obtained independently, *A* at ~50 mW of laser power and *B* at less than 10 mW.

heme proteins. In CO-bound reduced cytochrome oxidase the Soret transition for cytochrome *a* is unshifted at 443 nm, whereas that of cytochrome a_3 shifts to ~430 nm. Consequently, for the CO-bound complex the electron density marker line of cytochrome *a* would be expected to be unchanged at ~1,355 cm^{-1}, whereas that of cytochrome a_3 would be expected to shift to higher frequency. Indeed, with 441.6-nm excitation in the resonance Raman spectra of the CO-bound complex, in which cytochrome *a* is preferentially enhanced, a dominant line is observed at 1,354 cm^{-1} with only a weak shoulder at 1,365 cm^{-1} (Fig. 2 *B*). On the other hand, with 413.1-nm excitation in which the CO-bound cytochrome a_3 is preferentially enhanced, only a single line at 1,368 cm^{-1} is found (Fig. 3 *B*). Thus, the behavior of the electron density marker line confirms that in the CO-bound complex the CO-bound cytochrome a_3 heme is enhanced with 413.1-nm excitation and the cytochrome *a* heme is enhanced with 441.6-nm excitation. From the near-complete separation of the electron density marker lines in these spectra it is apparent that the contribution from the nonenhanced heme is very small.

The behavior of the carbonyl stretching mode (1,666 cm^{-1}) of the formyl group (21, 22) of the cytochrome a_3 (see discussion below) is also consistent with complete spectral separation. It is very weak for the CO-bound reduced enzyme with 441.6-nm excitation in Fig. 2 *B*. In contrast with 413.1-nm excitation it is the second strongest line in the spectrum. Thus, under the assumption that the other lines in the spectrum scale with these two cytochrome a_3 marker lines, the spectra of the CO-bound complex has contributions from only cytochrome a^{2+} with 441.6-nm excitation and contributions from only cytochrome (CO) a_3^{2+} with 413.1-nm excitation. Possible exceptions to this argument would be any modes that may be enhanced by electronic states other than the porphyrin π to π^* transitions such as the iron-ligand stretching modes or modes from other chromophores such as the Cu centers. Within these limitations, the spectrum in Fig. 2 *B* may be taken as that of reduced cytochrome *a* and the spectrum in Fig. 3 *B* as that of CO-bound cytochrome a_3. The frequencies from each of these hemes are listed in Table I.

Since with 441.6-nm excitation the spectrum of the fully reduced enzyme is a mixture of cytochromes *a* and a_3 and the spectrum of the CO-bound complex results nearly exclusively from cytochrome *a*, subtraction of the CO-bound complex from the fully reduced preparation yields the spectrum of cytochrome a_3. This difference spectrum [$A - nB$, $n = 0.6$] is presented in Fig. 2 *C* and the frequencies are listed in Table I. The assumptions under which this difference spectrum yields a spectrum of pure cytochrome a_3 are: (*a*) We have properly selected the coefficient, *n*, so as to balance out the contribution from cytochrome *a*. (*b*) The contribution from CO-bound cytochrome a_3 in the spectrum of the CO-bound enzyme at 441.6-nm excitation is negligible in relation to that of cytochrome *a*. (*c*) There is no spectral heme-heme interaction [i.e., perturbation of the cytochrome *a* Raman active modes due to binding CO to cytochrome a_3]. (*d*) There is no direct interaction between CO and cytochrome *a*. (*e*) There are no contributions from Cu chromophores.

We address these assumptions below. (*a*) We have obtained a family of subtractions in which the coefficient, *n*, was varied. The spectrum shown has a value of 0.6 in Fig. 2 *C*. At lower values of *n*, the same lines appear in the spectrum as in the spectrum of the fully reduced enzyme. At high values of *n* negative deflections appear. In addition, changes in *n* near the 0.6 value (±0.1) affected some relative intensities but had very little effect on the peak frequencies in Fig. 2 *C*. We recognize that different regions of the spectrum may be best balanced with different values of *n* since the relative enhancements may be different. However, this effect appears to be small so we have used only one balancing factor. (*b*) The contribution of the CO-bound cytochrome a_3 heme in the spectrum of the CO-bound complex at 441.6-nm excitation may be estimated by the intensity of the formyl mode (1,666 cm^{-1}) and the electron density marker line (1,368 cm^{-1}). We estimate that the contribution from CO-bound cytochrome a_3 to this spectrum is roughly 10–20%. Thus, it should not significantly alter our difference spectrum. Moreover, as pointed out by Ondrias and Babcock (18), the contribution of each cytochrome to the spectrum may be calculated to first order. From this calculation we find that the a_3 (CO) should contribute <30% of the cytochrome a^{2+} intensity. Our observation that it is even less is consistent with our previous discussion of the more complex nature of the excitation profile. (*c*) The effect on the optical spectrum of cytochrome *a* upon changing the redox state of cytochrome a_3 or the redox states of the copper atoms have been studied

TABLE I
RAMAN FREQUENCIES FOR HEMES IN CYTOCHROME OXIDASE

Assignment	Cytochrome a^{2+}	Cytochrome a_3^{2+}	(CO) Cytochrome a_3^{2+}
	cm^{-1}	cm^{-1}	cm^{-1}
$\nu_{Fe\text{-}His}$	—	213	—
	236	—	—
Pyr. tilt	263	—	—
$\delta_{C_bC_\alpha C_\beta(2)}$	—	326	—
ν_8	340	—	—
ν_{35}	357	364	—
ν_{C_bS}	393	—	—
$\delta_{C_bC_\alpha C_\beta(1)}$	418	400	—
Pyr. fold	434	—	—
Pyr. fold	468	—	—
$\nu_{C\text{-}O}$	—	—	520
ν_{49}	563	—	—
$\delta_{Fe\text{-}C\text{-}O}$	—	—	578
ν_{48}	584	—	—
δ_{CO} (formyl)	631	—	—
	658	—	—
ν_7	679	682	683
	711	—	—
	731	—	—
ν_{16}	746	745	747
	—	757	—
ν_{32}	788	—	—
ν_6	821	—	—
δ_{C_mH}	848	—	—
$\delta_{as\text{-}CH_2}$	1,087	—	—
ν_{22}	1,113	1,115	1,116
$\nu_6 + \nu_8$	1,131	1,129	1,133
ν_{30}?	—	—	1,159
ν_{30}?	1,181	—	1,182
ν_{13}	1,226	1,225	1,227
$\nu_5 + \nu_9$	1,246	1,247	1,244
$\nu_5 + \nu_9$?	—	—	1,255
ν_{42}	1,289	—	1,286
ν_{21} or $\delta_{CH\text{-}}$	1,305	—	1,306
$\delta_{S\text{-}CH_2}$	1,331	—	1,328
ν_4	1,354	1,355	1,368
ν_{20}, ν_{29} or $\delta_{C\text{-}H}$	1,399	—	1,398
ν_{28}	1,464	—	1,469
ν_3	1,493	—	—
ν_{11}	1,517	—	1,516
	—	—	1,541
ν_{37}?	1,568	1,569	1,567
ν_2	1,586	1,579	1,585
ν_{10}	1,609	1,607	—
$\nu_{C\text{-}C}$ (vinyl)	1,625	1,622	1,618
$\nu_{C\text{-}O}$ (formyl)	—	1,662	1,666

The normal modes are from references 9 and 33. The cytochrome *a* and cytochrome a_3 frequencies are from Figs. 2 *B* and *C*, respectively. The (CO) cytochrome a_3 frequencies are from Fig. 3 *B*. The 1,662 cm^{-1} C–O (formyl) frequency in cytochrome a_3 is 2 cm^{-1} low due to a subtraction artifact. The question marks designate uncertainty in the assignment. Pyr., Pyrrole.

in detail by Blair et al. (23) who concluded that all of their data on the optical absorption spectra could be accounted for by treating the cytochrome *a* heme as being spectrally isolated. Moreover, with mixed valence forms of CN^--bound cytochrome oxidase, using similar methods we have been able to separate the contribution from cytochrome *a* that agrees well with the spectrum in Fig. 2 *B*, indicating that the spectrum of cytochrome *a* is unaffected by the nature of the ligand and the oxidation state of cytochrome a_3 in these two cases (11). (*d*) There is no evidence for any direct interaction between CO and cytochrome *a*. (*e*) The question of contributions from Cu centers to our observed spectra, cannot be addressed from our present results.

Another difference between 413.1-nm and 441.6-nm excitation lies in the range of frequencies of the modes which are enhanced. With 441.6-nm excitation, lines throughout the 200–1,700 cm^{-1} region are enhanced. In contrast, with 413.1-nm excitation, which lies to the high-energy side of both Soret bands, the low-frequency spectrum is very weak, and strong lines are seen in the high-frequency region only. This behavior is consistent with enhancement via 0 → 0 transitions with 441.6-nm excitation and 0 → 1 transitions with 413.1 nm excitation (6, 17).

With isolated spectra available for reduced cytochromes *a* and a_3 and for CO-bound cytochrome a_3, the differences between the resonance Raman spectra of these hemes may now be identified and their implications considered. We start with the line at 1,664 cm^{-1} in the reduced enzyme, which has been assigned (21, 22) as the C = O stretching vibration of the formyl group (—CHO). The behavior of this line in different oxidation and spin states is important to determine since it has been proposed to modulate the redox potential (5, 24) and also take part in proton transport (25). This line has also been seen in model compounds and was observed to shift from 1,660 to 1,644 cm^{-1} on conversion from high-spin five-coordinate heme *a* (2 methyl-imidazole) to low-spin six-coordinate heme *a* (bis-imidazole) (7, 9). This spin sensitivity has been attributed to coupling between the porphyrin orbitals and the orbitals of the formyl group. Thus, the changes in the porphyrin electronic structure that accompany the spin-state change in cytochrome oxidase upon binding CO would be expected to result in electronic changes in the formyl group as well and cause a decrease in its frequency. We also note that this band is sensitive to the oxidation state of the iron (6). The formyl line at 1,670 cm^{-1} in oxidized cytochrome oxidase shifts to 1,664 cm^{-1} upon reduction. Thus, the formyl line is sensitive to both the electron density and the spin state of the iron.

In CO cytochrome oxidase we observe the formyl stretching mode at 1,666 cm^{-1}, only 2 cm^{-1} higher than its frequency in the fully reduced enzyme (Fig. 3 *B*). Thus, the expected shift to lower frequency associated with the spin state change in the model compounds upon the high to low spin state conversion is not observed in the enzyme. We

propose that this observation may be explained as follows: binding of oxygen or carbon monoxide to heme proteins has been shown to decrease the electron density in the porphyrin to a degree similar to that upon oxidation of the heme iron (16). This removes electron density from antibonding orbitals and causes skeletal modes to increase in frequency. Just as the skeletal modes, the formyl stretching mode also appears to be sensitive to electron density and therefore, oxidizing the reduced enzyme increases the frequency of the formyl stretching mode. In contrast, in the reduced enzyme a high spin to low spin transition shifts this mode to a lower frequency. Thus, we propose that upon CO binding the change in porphyrin electronic properties due to the ligand binding (a general increase in frequency) approximately counterbalances the frequency shift in this mode due to the spin state change (a general decrease in frequency), resulting in only a 2-cm^{-1} shift to higher frequency. The behavior of the electron density marker line in cytochrome oxidase is consistent with these ideas as pointed out above. In addition, on formation of the bis-imidazole complex of heme *a*, a small change is detected in the electron density marker line indicating that the π-electron density is not significantly changed (7, 9). Similar observations have been made in the CN^- complex of cytochrome oxidase in which the formyl stretching mode behaves as expected for a spin state change alone (the line shifts from 1,664 cm^{-1} in the reduced enzyme to 1,644 cm^{-1} in the CN-bound form) and there is no change in the electron density marker line (11). From the foregoing analysis, it is clear that the formyl stretching mode by itself is not a reliable spin state marker line, but the formyl stretching mode along with the absorption spectrum and the electron density marker line can be used to infer spin state changes.

In CO cytochrome oxidase a line grows in at 1,255 cm^{-1} which is absent in the spectrum of the fully reduced enzyme (Fig. 3 *B*). It should be pointed out that in this region, there are both porphyrin modes and the porphyrin-formyl group stretching mode (9). Since this line is present in the CO-bound enzyme and the CN^--bound enzyme (11) even though the formyl C-O stretching mode behaves very differently, we conclude that the appearance of a line at 1,255 cm^{-1} results from a porphyrin mode and not the formyl group stretching mode. The presence of this mode in both CN^- and CO-bound reduced enzyme appears characteristic of the formation of a low-spin six-coordinate cytochrome a_3 irrespective of the axial ligand. Thus, it could serve as a useful spin-state marker line for the heme in the ferrous case.

A line at 1,622 cm^{-1} in hemoglobin and myoglobin that has been assigned to the vinyl stretching mode (26–28) is found to be insensitive to the oxidation state or spin state of the iron. Similarly, a line at ~1,620 cm^{-1} for cytochrome oxidase is found to be insensitive to the oxidation state, spin state of the iron, or the state of ligation. This behavior, which is distinct from the other modes, may be rationalized by considering that the other high-frequency modes have normal coordinates consisting primarily of the skeletal atoms of the porphyrin macrocycle. Since the vinyl group is not a part of that macrocycle, its unique behavior is not entirely unexpected. Thus, we assign the mode at ~1,620 cm^{-1} to the vinyl stretching mode consistent with the assignment of Choi et al. (9). The insensitivity of the vinyl mode suggests that it does not become enhanced by ground state electron delocalization but instead is enhanced by another mechanism. Choi et al. (9) have proposed that this mode is enhanced by an electronic interaction of the porphyrin and vinyl π systems in the excited state. An alternate mechanism is enhancement through a dipole-dipole interaction (29). In this mechanism the interaction between a system of two weakly coupled components is treated as a perturbation. One of the components absorbs the incident light (the porphyrin) and the other does not (the vinyl). It was shown that the interaction can induce large enhancements (29). This mechanism of enhancement also bears on the intensity of the Fe-C-O bending mode in cytochrome oxidase as well as in other heme proteins. These modes have been shown to be enhanced only when the Fe-C-O grouping is tilted with respect to the heme plane (10). Its high intensity in cytochrome oxidase indicates a significantly tilted grouping. Since an orientation dependence of the enhancement is consistent with this mechanism (29), it could account for the intensity behavior of this line in the Raman spectrum.

The overall effect of binding CO to cytochrome a_3 may be seen by comparing the difference spectrum generated in Fig. 2 *C* (fully reduced cytochrome a_3) to that of the CO-bound complex obtained with 413.1-nm excitation, Fig. 3 *B*. From this comparison we find that upon binding CO to cytochrome a_3, a new line grows in at 1,255 cm^{-1}, the electron density marker line shifts from 1,355 to 1,368 cm^{-1}, and the line at 1,569 cm^{-1} decreases in intensity. (In part, some of the changes in the intensity could result from the different laser excitation frequency.) The behavior of these lines in cytochrome oxidase is more similar to the changes upon CO binding to hemoglobin or myoglobin (26, 30) than to the changes observed when the spin state in model compounds is changed (7, 9) (low-spin bis-imidazole vs. high-spin 2-methyl imidazole). For example, upon converting deoxyhemoglobin to CO hemoglobin, changes occur in the 1,215–1,225 cm^{-1} region, and modes at 1,357- and 1,567-cm^{-1} shift to 1,373 and 1,584 cm^{-1}, respectively (26). Similar changes occur in myoglobin (30). In the above-mentioned model compounds upon going from high spin to low spin (five-coordinate to six-coordinate) (7, 9), the line at 1,357 cm^{-1} is nearly unchanged and the line at 1,473 cm^{-1} shifts to 1,493 cm^{-1} in contrast to the behavior of cytochrome oxidase or hemoglobin and myoglobin. The vinyl mode in the 1,618–1,622 cm^{-1} region appears insensitive to spin state and

oxidation state changes for all of these proteins. In the low-frequency region we cannot make comparisons between the CO-bound and unliganded cytochrome a_3 since the low-frequency lines of the CO-bound heme are not enhanced by 413.1-nm excitation. However, the comparison between fully reduced cytochrome oxidase and deoxyhemoglobin and deoxymyoglobin shows striking similarities. In the oxidase we detect lines at 213, 326, 364, and 400 cm^{-1}. In deoxyhemoglobin (26) and deoxymyoglobin (31) the strongest lines are at 216–221 cm^{-1}, 301–306 cm^{-1}, 341–344 cm^{-1}, 366–372 cm^{-1}, and 406 cm^{-1}. The line at ~342 cm^{-1} (absent in oxidase) has been found to be very structure-sensitive in hemoglobin (32). The frequencies of the remaining lines of these proteins agree quite well although there are some intensity differences. From the above comparisons we conclude that the structure in unliganded cytochrome a_3^{2+} as well as changes in cytochrome a_3 upon binding CO are similar to those that occur in the heme-*b*–containing proteins, hemoglobin and myoglobin. Thus, the electronic structure changes which take place upon CO binding appear to result in hemes with similar properties; i.e., the population of the heme orbitals due to the low-spin iron atom and the π-electron density in the porphyrin due to the CO binding have the same influence in heme a_3 of cytochrome oxidase as in heme *b* of hemoglobin or myoglobin. This behavior contrasts to molecular structural differences between hemoglobin or myoglobin and cytochrome oxidase in that the Fe-C-O grouping in cytochrome oxidase is tilted from the normal to the heme plane and the iron-histidine bond is strained (10). Therefore, our data suggest that any unique catalytic activity in the ferrous state does not result from special electronic properties of the cytochrome a_3 heme but instead may be influenced by the stereochemical properties of the binding site.

In conclusion, we have obtained resonance Raman spectra of cytochrome oxidase under conditions in which we can spectrally isolate the contributions from cytochromes a^{2+}, a_3^{2+}, and a_3^{2+} (CO). Since the cytochrome *a* is a six-coordinate low spin heme and the cytochrome a_3 is a five-coordinate high spin heme we find that their Raman spectra are quite different, especially in the low-frequency region. The identification of all of the lines that are associated with each of the hemes now makes it possible to follow the properties of each heme independently as the various oxidation states and states of ligand binding of this enzyme are examined. It opens the door to being able to determine the mechanism of heme-heme interaction as well as the molecular structure of the heme groups in the various functional intermediates. Application to the CO binding allows identification of spin state marker lines as well as demonstrating that the electronic changes that occur in the cytochrome a_3 are analogous to those that occur in the heme-*b*–containing proteins, hemoglobin and myoglobin. Since the changes in cytochrome a_3 on binding ligand are the same as those in heme-*b*–containing oxygen carrier and storage proteins, there appears to be nothing electronically unique in ferrous cytochrome a_3 to account for the catalytic properties of cytochrome oxidase.

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