Similar CO Binding Sites in Bacterial Cytochrome *bo* and Mammalian Cytochrome *c* Oxidase

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Properties of the active site of terminal oxidases may be studied by examining the stable carbon monoxide-bound adducts. The resonance Raman[1] and the infrared absorption[2] spectra of CO-bound mammalian cytochrome *c* oxidase have revealed several structural characteristics of the heme pocket, including an indication, from the frequencies of the Fe–CO stretching mode at 520 cm^{-1} and the C–O stretching mode at 1963 cm^{-1}, that the proximal histidine, trans to the bound CO, has either atypical bonding or is not bound in the CO adduct.[1,3] In a report[4] on the Raman spectrum of a bacterial terminal oxidase (cytochrome *bo*), two Fe–CO stretching modes were identified, one at 489 and another at 523 cm^{-1}. The latter line, with a frequency very near to that reported for CO-bound cytochrome a_3 in mammalian oxidases, was interpreted as a minor component originating from an anomalous CO binding site in the bacterial enzyme.[4] The shift of the major line from 520 cm^{-1} in the mammalian enzyme to 489 cm^{-1} in the bacterial enzyme suggests a qualitative difference in the structure of the oxygen binding site. In an effort to clarify this difference, we have reexamined the Raman spectra of the CO-bound adduct of the cytochrome *bo* complex.

The cytochrome *bo* complex is a terminal ubiquinol oxidase from aerobically grown *Escherichia coli*.[5-7] The complex catalyzes the reduction of molecular oxygen by the oxidation of ubiquinol-8 and contributes to the proton gradient across the cytoplasmic membrane.[5,8] The complex contains three known redox centers: two heme groups and one copper atom.[6-9] One of the hemes, termed cytochrome $b_{563.5}$, is low spin and is analogous to cytochrome *a* in mammalian oxidases. The other heme (cytochrome *o*) and a nearby copper atom form a binuclear binding site for O_2 or CO that is analogous to the cytochrome a_3–Cu_B binuclear binding site in mammalian oxidases.[9,10]

We detect three lines in the resonance Raman spectrum (413.1-nm excitation, 15-mW power) of the CO-bound cytochrome *bo* complex which have carbon and oxygen isotopic sensitivity (Figure 1). We assign the line at 524 cm^{-1} to the Fe–CO stretching mode, the line at 577 cm^{-1} to the Fe–C–O bending mode, and the line at 1959 cm^{-1} (data not shown) to the C–O stretching mode in the $^{12}C^{16}O$-bound form of the fully reduced enzyme. In the $^{13}C^{18}O$ derivative these lines shift to 510, 559, and 1868 cm^{-1}, respectively. The frequencies of these Fe–C–O modes are all very close to those that have been reported for the CO adducts of mammalian cytochrome a_3. There are some clear differences, however. The Fe–CO stretching mode is 4 cm^{-1} higher in cytochrome *bo* than it is in cytochrome a_3, and the C–O stretching mode is 4 cm^{-1} lower. In addition, the ratio of the intensity of the Fe–C–O bending mode (I_δ) to that of the Fe–CO stretching mode (I_ν) is substantially smaller in the bacterial enzyme (I_δ/I_ν = 0.1) than that in the mammalian enzyme (I_δ/I_ν = 0.3–0.5)[1].

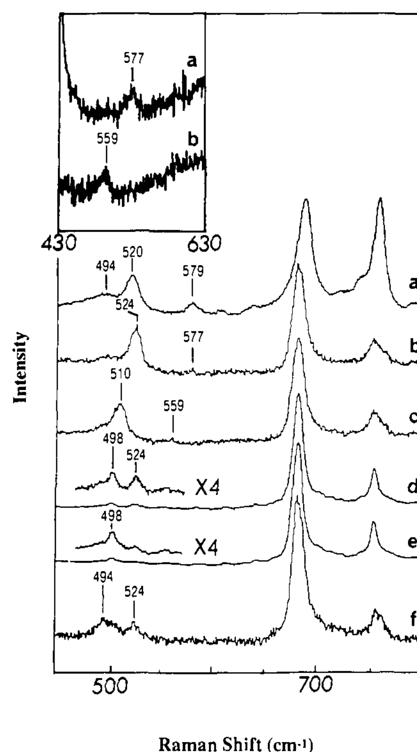

Figure 1. Resonance Raman spectra of bacterial cytochrome *bo* in the Fe–CO stretching mode and Fe–C–O bending mode region. For comparison, the spectrum of $^{12}C^{16}O$-bound mammalian cytochrome *c* oxidase (a) is included. The spectra of $^{12}C^{16}O$-bound and $^{13}C^{18}O$-bound reduced cytochrome *bo* are shown at low laser power density (15–20 mW of power with a defocused spot on the sample in the spinning cell) in b and c, respectively, and at high laser power density (200 mW of power with a focused spot) in d and e, respectively. The sample used for spectrum f was the same as that used for spectrum b, but it was partially oxidized by exposure to oxygen. (inset) Resonance Raman spectra of $^{12}C^{16}O$-bound (a) and $^{13}C^{18}O$-bound (b) fully reduced cytochrome *bo* in the Fe–C–O bending region. The cytochrome *bo* complex was isolated as described previously[7] and solubilized at a concentration of 60 μM in 100 mM Tris-HCl, 0.05% sarkosyl, pH 7.5. The samples were prepared by anaerobically, reducing the enzyme with dithionite followed by exposing the solution to either a $^{12}C^{16}O$ or a $^{13}C^{18}O$ atmosphere. The data were recorded on Raman instrumentation described in detail elsewhere[15] with krypton ion laser excitation at 413.1 nm.

The species with the Fe–CO stretching mode at 524 cm^{-1} was found to be photolabile as illustrated in Figure 1 (trace d), where the power of the excitation laser was 200 mW. The line at 524 cm^{-1} virtually disappeared, with a line at 498 cm^{-1} remaining. No isotopic shifts were detected in the mode at 498 cm^{-1}, indicating that it is a porphyrin macrocycle mode rather than a mode involving the CO (Figure 1, trace e). The photolability of the species with the Fe–CO stretching mode at 524 cm^{-1} is fully reversible. By reducing the laser power to 15 mW, the low-power Raman spectrum was completely reestablished.

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Several measurements were made to determine if the preparation with the Fe–CO stretching mode at 524 cm^{-1} originates from a major or a minor CO-bound species. First, the Raman spectrum of the C–O stretching mode (1959 cm^{-1}) has the same frequency as the dominant C–O mode found in infrared absorption measurements on the CO-bound enzyme and assigned as originating from the fully intact cytochrome *bo* complex.[10,11] Second, there is full reversibility of the photodissociation, demonstrating that the laser has not damaged the samples. Third, the absence of lines from the ferric form of the enzyme in our spectra confirms complete reduction of our samples. We conclude that the spectra reported here result from the major, and in our hands the only, CO-bound adduct of cytochrome *bo*.

Uno et al.[4] reported the major Fe–CO stretching mode at 489 cm^{-1}. If correct, this would indicate that the active site of the bacterial terminal oxidase is qualitatively different from that of mammalian oxidases. In our reduced preparations, no line at 489 cm^{-1} is detected. However, when the fully reduced CO-bound enzyme is partially oxidized by exposure to oxygen, the spectrum changes, yielding a broad line at 494 cm^{-1} in the Fe–CO stretching region (Figure 1, trace f). The spectrum of the fully oxidized preparation in the absence of CO, has a line at this same frequency. Thus, we assign the line at 489 cm^{-1} reported by Uno et al. to a porphyrin mode of the oxidized enzyme.

The frequencies of the CO-sensitive modes which we detect in cytochrome *bo* are qualitatively similar to the corresponding frequencies found in CO-bound cytochrome a_3.[1-3] Thus, these data confirm the similarity in the properties of the active sites in these two terminal oxidases. The data substantiate the validity of utilizing site-directed mutagenesis experiments in cytochrome *bo* to draw inferences concerning the properties of cytochrome aa_3.[12] Furthermore, the frequencies of the modes involving the bound CO underscore the conclusion that if the proximal ligand in the CO adduct is histidine, features of its bonding to the iron atom of the heme are unique among the histidine-coordinated heme proteins that have been studied to date.[1,3,13] The relatively high frequencies for both the Fe–CO and the C–O stretching modes indicate an anomalously weak proximal ligand–iron bond.

The small frequency differences between the CO-isotope-sensitive modes in cytochrome *bo* and cytochrome aa_3 do indicate that the binuclear site of the bacterial enzyme, while similar, is not identical to its mammalian counterpart. In addition, the intensity of the Fe–C–O bending mode is much weaker than that of the stretching mode in cytochrome *bo*, in contrast to CO-bound cytochrome a_3, in which the bending mode is very strong.[1] Model compound studies have demonstrated that the intensity of the bending mode is proportional to the sterically induced tilting of the CO group away from the heme axis.[14] Therefore, the Fe–C–O moiety in this bacterial enzyme is closer to being perpendicular to the heme plane than it is in the mammalian enzyme, where it is tilted significantly.[1]

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