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Two Conformations of the Catalytic Site in the aa_3-Type Cytochrome *c* Oxidase from *Rhodobacter sphaeroides*†

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ABSTRACT: Resonance Raman spectra of the carbon monoxy derivative of the aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* show two distinct Fe−CO stretching modes (519 and 493 cm^{-1}) at room temperature. The frequency of the mode at 519 cm^{-1} coincides with that of other terminal oxidases at neutral pH. Two C−O stretching modes, one at 1966 cm^{-1} and one at 1955 cm^{-1}, are also found. The splitting of the C−O stretching mode is consistent with the FTIR spectra of cytochrome *c* oxidases at cryogenic temperatures in which two different conformations (α and β) of the catalytic site of the enzyme are present. The splitting of both the Fe−CO and C−O stretching modes under our conditions indicates that these two forms of the enzyme are also present at room temperature, and with the additional information on the Fe−CO modes provided here, a structural origin for the two forms may be postulated. The α-form has the same general structure of the active site as mammalian oxidase, a structure in which the copper atom that is the part of the $Fe-Cu_B$ binuclear site interacts strongly with the bound CO. We postulate that the copper atom exerts a strong polar or steric effect on the heme-bound CO, resulting in either compression of the Fe−CO bond or distortion of the Fe−CO moiety. On the other hand, the β-form has an open structure typical of heme proteins with histidine coordination to the iron trans to a Fe−C−O moiety where there is no interaction with a copper atom in the distal pocket. The functional significance of these two forms of the enzyme remains to be determined.

Cytochrome *c* oxidase is the terminal enzyme in the electron-transfer chain. The catalytic site of the enzyme, at which oxygen is reduced to water, consists of a binuclear center formed by the iron of a heme group, cytochrome a_3, and a copper atom, Cu_B. Several different spectroscopic and structural measurements have shown that these two metals are very close to each other, being separated by 3.5−5.0 Å in the resting form of the enzyme (Powers et al., 1981; Thomson et al., 1985; Scott, 1989). It has also been demonstrated that cytochrome a_3 is coordinated to the protein by a proximal histidine residue in the ferrous ligand-free (Ogura et al., 1983; Kitagawa, 1988) and ferrous NO-bound (Stevens & Chan 1981) forms of the enzyme. Additional information concerning the properties of the active site has been provided by investigations of the stable exogenous

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ligand-bound derivatives of cytochrome *c* oxidase by vibrational spectroscopy [resonance Raman scattering and Fourier transform infrared spectroscopy (FTIR)]. The carbon monoxide-bound adduct of the protein is especially useful in this regard in that the lines involving the heme-bound CO[1] are sensitive to the properties of the endogenous proximal ligand and also serve as probes of the interactions between the exogenous CO and distal amino acid residues (Yu & Kerr, 1988).

Several important properties of mammalian cytochrome *c* oxidase have been determined from studies of its CO-bound adducts. In addition to revealing insights concerning the equilibrium structure, discussed below, photodissociation studies have shown that, by breaking the Fe−CO bond, the iron−histidine bond is strengthened transiently on a time scale of up to ~1 μs, just as in hemoglobins (Findsen et al., 1987). Furthermore, at cryogenic temperatures the CO photodissociated from the heme iron forms a stable Cu_B−CO adduct, an additional confirmation of the proximity of Cu_B to the iron of cytochrome a_3 (Alben et al., 1981; Fiamingo et al., 1982). At room temperature it is observed that CO coordinates to Cu_B in <1 ps of the photodissociation event (Dyer et al., 1991) and subsequently is released by the copper and moves out of the heme pocket in ~1.5 μs (Dyer et al., 1989a). FTIR studies of the C−O stretching mode (ν_{C-O}) at cryogenic temperatures reveal multiple conformations of the active site (Alben et al., 1981; Fiamingo et al., 1982). The major structure has been termed as the α-form, while a family of structures termed as the β-form has also been identified (Alben et al., 1981; Fiamingo et al., 1982). In the α-form of the mammalian enzyme the ν_{C-O} modes were detected at 1964 and 2065 cm^{-1} for the iron and copper carbonyls, respectively, whereas for the β-form the corresponding modes were at 1952 and 2043 cm^{-1}. The fraction of each structure depends on the preparation of the enzyme and the experimental conditions. The structural basis for the splitting of the enzyme into these forms has not been determined, and no additional information regarding the origin for the splitting of the CO adducts into two different structures has been reported.

Information concerning the active site of the α-form of the carboxy derivative of cytochrome *c* oxidase has been deduced from studies of the proximal ligand-dependent correlation between the Fe−CO stretching mode (ν_{Fe-CO}) and ν_{C-O} frequencies in a variety of heme proteins. Despite the presence of a proximal histidine in its dithionite-reduced form, mammalian cytochrome *c* oxidase displays a unique correlation between the frequencies of ν_{Fe-CO} (520 cm^{-1}) and ν_{C-O} (1963 cm^{-1}), suggesting either an anomalous binding geometry of the histidine−Fe−CO complex (Argade et al., 1984) or the replacement of the proximal histidine by other residues in its CO adducts (Rousseau et al., 1993). The same unique correlation has also been detected in a quinol oxidase, cytochrome bo_3, another member of the terminal oxidase superfamily (Wang et al., 1993; Uno et al., 1994). On the other hand, in recent pH-dependent studies of cytochrome *c* oxidase, ν_{Fe-CO} shifts to 494 cm^{-1} when the pH is above 9.5 or below 5.5 (Rousseau et al., 1993; J. Wang, and D. L. Rousseau, unpublished results), indicating that variations in the heme environment are induced by pH-sensitive protein conformational changes. Most importantly, at these pH extremes the ν_{Fe-CO} and ν_{C-O} frequencies fall on the correlation curve for a heme coordinated to a normal histidine.

Bacterial cytochrome aa_3 from *Rhodobacter sphaeroides* belongs to the large superfamily of respiratory oxidases as it contains a high-spin heme (heme a_3) which forms a heme−copper binuclear catalytic site where oxygen is reduced to water and a low-spin heme (heme *a*) which is involved in electron transfer (Hosler et al., 1993). Just as its mammalian counterpart, the bacterial enzyme has been shown to translocate protons against a proton gradient. The three subunits of the enzyme are approximately 70% homologous to subunits I, II, and III of bovine heart cytochrome *c* oxidase (Cao et al., 1991, 1992; Shapleigh et al., 1992b). In particular, the six histidines, which are fully conserved in all aa_3 terminal oxidases and the vast majority of the members of the oxidase superfamily, have been assigned as ligands of either heme *a*, heme a_3, or Cu_B in this bacterial oxidase (Hosler et al., 1993). Thus, studies of this bacterial enzyme increase our understanding of the structural and functional properties of all terminal oxidases. It is noteworthy that FTIR data on ν_{C-O} at cryogenic temperatures demonstrate that cytochrome aa_3 from *Rb. sphaeroides* has α- and β-forms just as does the mammalian enzyme. Herein we report resonance Raman studies that reveal two distinct conformations of the CO-binding site in the aa_3-type cytochrome *c* oxidase isolated from *Rb. sphaeroides* at room temperature as well. These two conformations are detected by a splitting of both the C−O and Fe−CO stretching modes in the bacterial enzyme.

MATERIALS AND METHODS

Two independent types of preparation were used in the studies: isolation of the enzyme by ion-exchange chromatography and by histidine affinity labeling. For the ion-exchange preparation the aa_3-type cytochrome *c* oxidase from *Rb. sphaeroides* was purified from cytoplasmic membranes of strain CY91 as described in Hosler et al. (1992), except that a 15 cm × 21.5 mm DEAE-5PW (Tosoh Corp.) was used for the first ion-exchange chromatography step. In this preparation of cytochrome aa_3 from *Rb. sphaeroides*, less than 3% of heme C is present, and no detectable heme B has been found by using pyridine hemochrome analysis (Hosler et al., 1992).

For the histidine tag preparation a bacterial strain of *Rb. sphaeriodes* was used which expresses a modified subunit I containing six consecutive histidine residues genetically fused to its C-terminus as an affinity tag (D. M. Mitchell and R. B. Gennis, unpublished results). The cells were grown and harvested, and crude membranes were prepared as previously described (Hosler et al., 1992; Shapleigh et al., 1992b). The membrane pellet was rehomogenized in 10 mM Tris and 40 mM KCl, pH 8.0, and solubilized with 1% lauryl maltoside. The solubilized membranes were then centrifuged at 75000*g* for 30 min. The total amount of oxidase present in the supernatant was estimated from the dithionite-reduced minus ferricyanide-oxidized spectrum ($\Delta\epsilon_{606-630}$ = 24 mM^{-1} cm^{-1}) (Hosler et al., 1992). Imidazole was added to the solubilized membrane solution to a final concentration of 10 mM. The Ni−NTA resin was then added (0.5 mL/mg of oxidase), and the mixture was stirred at 4 °C for 1 h. The mixture was then loaded onto a gravity-flow column and washed with

[1] Abbreviations: CO, carbon monoxide; His, histidine; *Rb.*, *Rhodobacter;* FTIR, Fourier transform infrared spectroscopy.

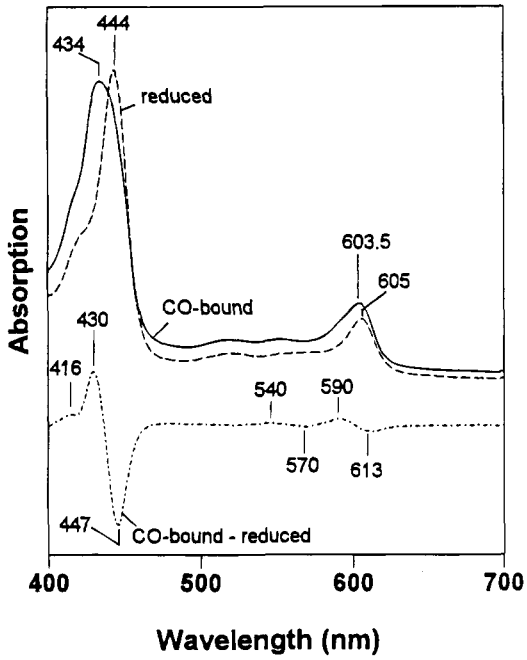

FIGURE 1: Optical absorption spectra of the dithionite-reduced (dashed line) and the CO-bound (solid line) forms of cytochrome aa_3 from *Rb. sphaeroides* in 100 mM sodium phosphate buffer with 0.1% dodecyl β-D-maltoside, pH 7.2. The difference spectrum (dot-dashed line) for the CO-bound form minus the reduced form clearly shows a conversion from a ligand-free reduced heme to the CO-bound form. The optical data were recorded before and after Raman measurements from a sealed Raman spinning cell (path length: 2 mm) which contains 100−120 μL aliquots of ~20 μM enzyme. No optical change induced by laser irradiation was observed.

10 column volumes of 10 mM Tris, 40 mM KCl, 10 mM imidazole, and 0.1% lauryl maltoside. The protein was then eluted by the buffer containing 10 mM Tris, 40 mM KCl, 100 mM imidazole, and 0.1% lauryl maltoside, at 0.1 mL/min. The purity of the preparation was confirmed spectroscopically and by SDS−PAGE analysis.

Mammalian cytochrome *c* oxidase was isolated and purified from bovine heart by previously reported procedures (Yonetani, 1960). Gases of natural and isotope-labeled CO were purchased from Matheson Gas Products (Bridgeport, NJ) and ICON Service Inc. (Summit, NJ), respectively.

The Raman data were collected as previously reported (Wang et al., 1994, 1995). Typically, CO-bound samples (130 μL, 10−50 μM) were prepared, in a sealed Raman cell, by exposing the reduced protein to the natural abundant or isotopically enriched CO gas. Optical absorption spectra were recorded both prior to and after the Raman measurements to ensure the formation and stability of the CO adducts. To minimize photodissociation of the bound CO, low laser power (0.2−3 mW) was used (excitation wavelength: 413.1 nm). The scattered light was dispersed by a 1.25-m monochromator and detected with a charge-coupled device camera. The frequencies of the Raman lines were calibrated against an indene standard or laser fluorescence lines. Spectra shown here were baseline-corrected but unsmoothed.

RESULTS

The optical absorption spectrum of dithionite-reduced bacterial aa_3 from *Rb. sphaeriodes* (Figure 1) [also see Hosler et al. (1992)] is similar to that of mammalian oxidase (Vanneste, 1966) displaying maxima at 444 and 605 nm.

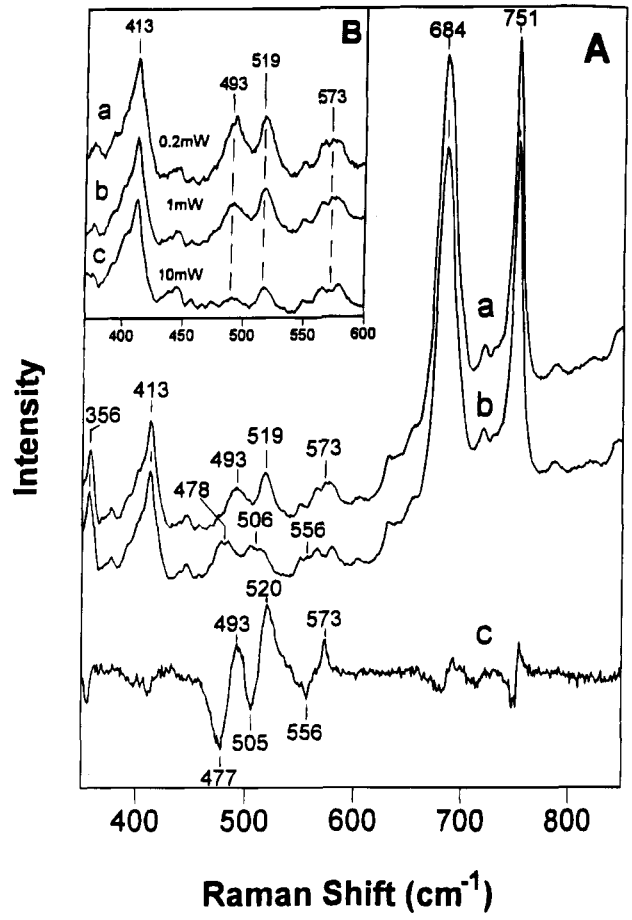

FIGURE 2: Resonance Raman spectra of the CO-bound forms of bacterial cytochrome aa_3 from *Rb. sphaeroides*, obtained at 413.1 nm excitation with defocused optics in the low-frequency region. (A) Spectra obtained at 1 mW of incident laser power. The $^{12}C^{16}O$-bound and the $^{13}C^{18}O$-bound forms at pH 7.2 are shown in traces a and b, respectively. Trace c is the difference spectrum for the $^{12}C^{16}O$-bound form (a) minus the $^{13}C^{18}O$-bound form (b). (B) Power dependence of the two Fe−CO stretching modes: (a) 0.2 mW, (b) 1 mW, and (c) 10 mW.

Flushing CO over the reduced enzyme shifts the visible band at 605 nm by 1.5 nm to 603.5 nm and the Soret maximum from 444 to 434 nm. The difference spectrum between the dithionite-reduced form and its CO adduct in the visible range presents an excellent "W" shape characteristic of binding CO to the heme. It is noteworthy that in the Soret region a trough at 447 nm appears with a positive band at 430 nm and a shoulder at 416 nm. In addition, the evidence from the pyridine hemochrome analysis, showing the absence of *b*-type heme and the presence of only 3% of *c*-type heme, rules out the possibility of any significant contribution from a cbb_3-type oxidase or denatured cytochrome *c* in our preparations (Hosler et al., 1994a).

The resonance Raman spectrum of $^{12}C^{16}O$-bound bacterial aa_3 (trace a in Figure 2A) obtained at neutral pH with 413.1 nm excitation and low laser irradiation (1 mW) exhibits peaks at 519 and 493 cm^{-1}, both of which display carbon and oxygen isotopic sensitivity by shifting to 506 and 478 cm^{-1}, respectively, in the $^{13}C^{18}O$ derivative (trace b). The shifts are more evident in the difference spectrum between $^{12}C^{16}O$-bound and $^{13}C^{18}O$-bound adducts (trace c). We assign both as Fe−CO stretching modes for the aa_3 bacterial enzyme. The ν_{Fe-CO} line at 519 cm^{-1} is very close to those of the aa_3-type cytochrome *c* oxidase from beef heart (520 cm^{-1})

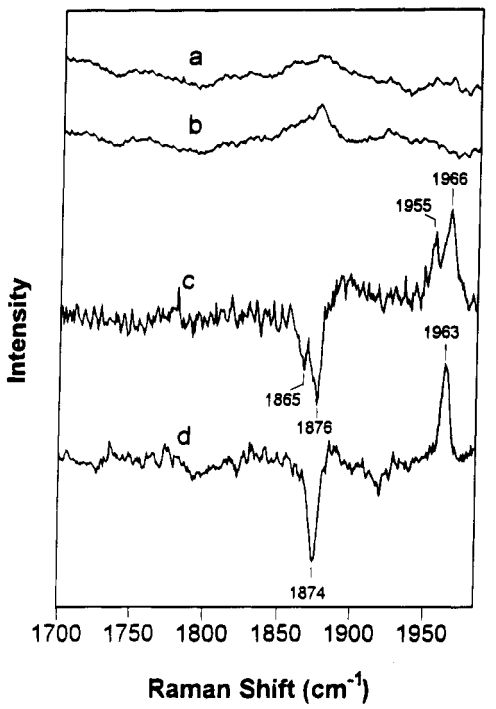

FIGURE 3: Resonance Raman spectra of *Rb. sphaeroides* cytochrome aa_3 in the C−O stretching mode region for $^{12}C^{16}O$ (a) and $^{13}C^{18}O$ (b) derivatives. The difference spectrum ($^{12}C^{16}O$ minus $^{13}C^{18}O$) is shown in trace c, and a corresponding difference spectrum for bovine cytochrome aa_3 is shown in trace d.

and cytochrome bo_3 (524 cm^{-1}), a quinol oxidase from *Escherichia coli* (Argade et al., 1984; Wang et al., 1993). In addition, the shift of a weak line at 573 cm^{-1} to 556 cm^{-1} also appears in the isotope difference spectrum. This line is attributed to a Fe−C−O bending mode (δ_{Fe-C-O}), and its frequency is about 5 and 4 cm^{-1} lower than that of mammalian cytochrome *c* oxidase (Argade et al., 1984) and cytochrome bo_3 (Wang et al., 1993), respectively. The data in Figures 1−3 were recorded on samples prepared by ion-exchange chromatography (Hosler et al., 1992), but similar results were obtained on those prepared by isolation of the histidine-derivatized enzyme on a Ni affinity column (data not shown).

Both the 493 and the 519 cm^{-1} CO-bound forms of the enzyme are photolabile, but the species with the lower frequency Fe−CO stretching mode (493 cm^{-1}) is more photolabile than that with the higher frequency mode (519 cm^{-1}). This effect may be seen most clearly in Figure 2B where the spectra obtained at three different laser powers are displayed. At the lowest power (0.2 mW in trace a) the two lines have approximately equal intensity and are nearly 50% of the peak intensity of the porphyrin mode at 413 cm^{-1}. As the power is increased to 1 mW (trace b) and then to 10 mW (trace c), the intensities of both modes decrease with respect to the mode at 413 cm^{-1}, but the low-frequency mode decreases more, so it is nearly absent in the spectrum obtained at 10 mW.

In Figure 3 we present the high-frequency region of the resonance Raman spectrum, the region in which the C−O stretching mode is found. Just as for its Fe−CO stretching mode, the C−O stretching mode from the *Rb.* aa_3 enzyme is also split into two components (trace c). For $^{12}C^{16}O$ one component is centered at 1966 cm^{-1} and the other at 1955 cm^{-1}. For $^{13}C^{18}O$ these modes shift to 1876 and 1865 cm^{-1}, respectively. In addition, the line widths of both components of the ν_{C-O} lines are similar to that of ν_{C-O} in mammalian cytochrome *c* oxidase (trace d).

DISCUSSION

The data reported here show the presence of two separate frequencies for the Fe−CO and C−O stretching modes in the aa_3 oxidase from *Rb. sphaeroides* at room temperature. Two sets of C−O stretching modes were also found in FTIR measurements of the enzyme at cryogenic temperatures: 1950 and 1964 cm^{-1} when the CO is bound to the Fe of the a_3 heme (similar to the frequencies we detect at room temperature) and 2039 and 2064 cm^{-1} when the CO is bound to the Cu_B center. The splitting of these C−O modes was taken as evidence for two different conformations of the enzyme, termed the α- and β-forms (Shapleigh et al., 1992a). Our new data demonstrate that the difference detected in the C−O stretching modes is present at room temperature as well and is also reflected in the Fe−CO stretching modes. In CO-bound mammalian oxidase, ν_{Fe-CO} is located at 520 cm^{-1} and ν_{C-O} at 1964 and 2065 cm^{-1} for CO bound to the Fe and Cu, respectively. The Soret transition is found at 430 nm, although a second form of the enzyme was detected in the cryogenic FTIR measurements. We assign the CO-bound form of the bacterial enzyme with lines at 519 (ν_{Fe-CO}), 573 (δ_{Fe-C-O}), and 1966 cm^{-1} (ν_{C-O}) as the α-form and posit that it has the same structure as that of the major form of the mammalian enzyme, and we assign the CO-bound form with frequencies at 493 (ν_{Fe-CO}) and 1955 cm^{-1} (ν_{C-O}) as originating from the β-form of the enzyme. These assignments are listed in Table 1 along with the frequencies of cytochrome bo_3 and the two forms of mammalian cytochrome *c* oxidase. In a prior report of the Raman spectrum of the CO adduct of the cytochrome aa_3 oxidase from *Rb. sphaeroides*, the low-frequency component of the

Table 1: Spectral Features of the Two Forms of CO-Bound aa_3-Type Cytochrome *c* Oxidase from *Rb. sphaeroides* Compared with the Cytochrome aa_3 from Beef Heart, Cytochrome bo_3 from *E. coli*, and Cytochrome cbb_3 from *Rb. capsulatus*

	Rb. sph. (aa_3)		bovine (aa_3)		*E. coli* (bo_3)	*Rb. cap.* (cbb_3)
transition[n]	α	β	α	β	α	β
Soret (nm)	430 (300 K)	?	430 (300 K)	?	416 (300 K)	415[j,k] (300 K)
ν_{Fe-CO} (cm^{-1})	519[a] (300 K)	493[a] (300 K)	520[b] (300 K)	?	524[f] (300 K)	495[i] (300 K)
δ_{Fe-C-O} (cm^{-1})	573[a] (300 K)	?	578[b] (300 K)	?	577[f] (300 K)	574[i] (300 K)
$\nu_{C-O(Fe)}$ (cm^{-1})	1966[a] (300 K)	1955[a] (300 K)	1963.6[a,c] (300 K)	1952[e] (80 K)	1960[h] (300 K)	1950[k] (15 K)
$\nu_{C-O(Cu)}$ (cm^{-1})	2064[m] (10 K)	2039[m] (10 K)	2065[d] (300 K)	2043[g] (10 K)	2065[i] (15 K)	2065[k] (15 K)

[a] This work. [b] Argade et al., 1984. [c] Caughey et al., 1993. [d] Dyer et al., 1989a. [e] Alben et al., 1981. [f] Wang et al., 1993. [g] Fiamingo et al., 1982. [h] Uno et al., 1994. [i] Hill et al., 1992. [j] Gray et al., 1994. [k] Garcia-Horsman et al., 1994. [l] J. Wang, D. L. Rousseau, K. A. Gray, and F. Daldal, unpublished results. [m] Shapleigh et al., 1992a. [n] ν_{Fe-CO} and δ_{Fe-C-O} correspond to the Fe−CO stretching and Fe−C−O bending modes, respectively. $\nu_{C-O(Fe)}$ and $\nu_{C-O(Cu)}$ are the C−O stretching vibrations for the iron-bound and the copper-bound species of the CO.

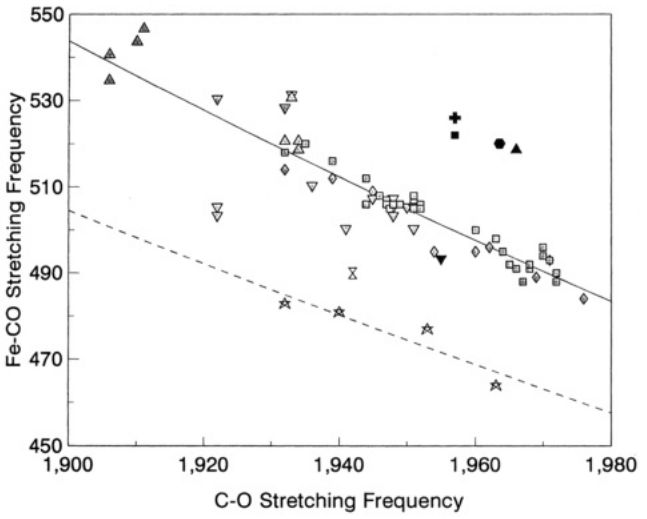

FIGURE 4: Correlation of ν_{Fe-CO} versus ν_{C-O} frequencies. The α-forms of oxidases are all clustered together off the correlation for the case when a neutral imidazole is the proximal ligand. Mammalian cytochrome *c* oxidase is designated by a darkened hexagon (⬢), the α-form of *Rb. sphaeroides* by a darkened triangle (▲), cytochrome bo_3 by a darkened square (■), and a porphyrin coordinated by a weak ligand (tetrahydrofuran) by a darkened cross (+). The points on the correlation line for proximal histidine coordination (solid) are from hemoglobins, myoglobins, peroxidases, and porphyrins with neutral imidazoles. The β-form of cytochrome aa_3 from *Rb. sphaeroides* designated by the darkened inverted triangle (▼) lies on the correlation line. The points (stars) on the correlation for a proximal thiolate ligand (dashed) are from cytochrome P-450s.

ν_{Fe-CO} mode was not detected (Hosler et al., 1994b). We attribute its absence in that case to either the use of a different laser excitation frequency or power level since we have found that the species with the 493 cm^{-1} frequency is very photolabile. An additional entry in Table 1 lists the frequencies for CO-bound cytochrome cbb_3, a novel oxidase from *Rhodobacter capsulatus* (Garcia-Horsman et al., 1994; Gray et al., 1994) which was proposed recently to have only a β-conformation (J. Wang, D. L. Rousseau, K. A. Gray, and F. Daldal, unpublished results).

The frequencies and intensities of the Fe−CO and C−O stretching modes, as well as the correlation between them, are of great help in the determination of the local heme environment of the CO adducts and thereby allow inferences concerning possible structures of the catalytic site of the terminal oxidases to be made. In particular, when histidine is the proximal ligand in heme proteins (or imidazole in porphyrins), there is an excellent correlation between the frequencies of ν_{Fe-CO} and ν_{C-O} (Figure 4). A different correlation is obtained when thiolate is the proximal ligand to the iron (Li & Spiro, 1988; Rousseau et al., 1993). The frequencies of mammalian cytochrome *c* oxidase and cytochrome bo_3 both lie off the correlation curve for proteins with a trans histidine even though the Fe−His stretching mode (ν_{Fe-His}), an indication of the existence of a proximal histidine in the ligand-free ferrous form of the enzymes, has been identified in both proteins (Salmeen et al., 1978; Ogura et al., 1983; Tsubaki et al., 1994). The α-form of *Rb. sphaeroides* cytochrome aa_3 also lies off the correlation curve (Hosler et al., 1994b, and Figure 4) and is therefore part of the family of oxidases with this anomalous feature in its CO derivative. However, the frequencies of the β-form of the enzyme place it on the correlation curve (Figure 4) just as in the high pH form of mammalian oxidase (J. Wang, and D. L. Rousseau, unpublished data).

The apparent anomalous frequencies for the Fe−C−O modes in mammalian oxidase have been a conundrum and subject of debate for a number of years. When the frequency of the Fe−CO stretching mode was detected originally at 520 cm^{-1}, it was proposed that this high frequency was a consequence of an anomalously weak proximal histidine bond (Argade et al., 1984). Later the possibility that the histidine was replaced by a tyrosine in the CO-bound form of the enzyme was raised (Rousseau et al., 1993). More recently, arguments were made that the high frequency of the Fe−CO stretching mode and the position of oxidases off the ν_{Fe-CO} frequency versus the ν_{C-O} frequency correlation curve can be attributed to distal effects involving the interaction of Cu_B with the bound CO based on a lowering of the ν_{Fe-CO} frequency in mutated forms of the enzyme in which a residue that coordinates to Cu_B was replaced (Hosler et al., 1994b; Uno et al., 1994). Although the present data do not distinguish between these proposals, the site-directed mutagenesis studies and the polarized spectroscopy study from which an angle of 21° for the CO (with respect to the heme normal) was demonstrated (Dyer et al., 1989b) clearly indicate that the distal environment plays a role in the structure of this coordinated ligand.

We consider first the role the distal environment may play in the α-form of the CO-bound enzyme. In the resting enzyme the Fe−Cu separation at the binuclear site has been shown to be in the 3.5−5.0 Å range (Powers et al., 1981; Thomson et al., 1985; Scott, 1989). The presence of such a constricted pocket will not allow CO to coordinate to the iron without strong steric interactions, which could alter the Fe−C−O conformation by compressing the Fe−C and C−O bonds or by distorting the Fe−C−O group from its axial symmetry. In addition, the Cu atom would be expected to exert strong electrostatic effects on the bound CO. A compression force pushing on the bound CO was studied recently in highly constrained model complexes in which it was found that the distal force could shorten the Fe−CO bond, thereby raising its frequency (Ray et al., 1994). The distal constraint in cytochrome oxidase could bring about a similar increase in the frequency of the Fe−CO stretching mode. In addition to the bond compression, the strong steric interaction could distort the Fe−C−O moiety from its preferred axial symmetry into a bent or tilted conformation. The C−O stretching frequency would be expected to be relatively insensitive to either of these geometry changes, because of its large force constant, whereas the Fe−CO frequency could be sensitive to both. Furthermore, electrostatic forces, shown to significantly affect the CO vibrational frequencies in mutant myoglobins (Li et al., 1994), may also play a role in oxidase.

Additional information concerning the distal pocket is provided by the intensity of Fe−C−O bending mode (δ_{Fe-C-O}), which is very strong for the α-form of the terminal oxidases (Argade et al., 1984). For conventional resonance Raman scattering mechanisms, if the Fe−C−O group is perpendicular to the heme plane and has a linear geometry, δ_{Fe-C-O} should not be enhanced in the Raman spectrum due to symmetry. In the past it was argued that it became enhanced due to steric forces which caused it to be bent or tilted (Argade et al., 1984). More recently, it was pointed

out that electrostatic interactions, such as polar or H-bonding interactions, may equally well serve to lower its symmetry so that δ_{Fe-C-O} may be enhanced (Li et al., 1994; Ray et al., 1994). Unlike peroxidases in which H-bonding has been demonstrated (Satterlee & Erman, 1984; Edwards & Poulos, 1990; Smulevich et al., 1991), recent resonance Raman results (data not shown) showing no frequency shifts of ν_{Fe-CO} upon solubilization of beef heart cytochrome *c* oxidase in D_2O at neutral pH argue against the possibility of H-bonding between the bound CO and residues within the distal pocket. Thus, the high intensity of δ_{Fe-C-O} in the α-form of the terminal family is an additional indication of a strong interaction between the CO and a group in the distal pocket such as Cu_B. For δ_{Fe-C-O} to become Raman activated by Cu_B, the copper atom has to break the Fe−C−O symmetry electrostatically by residing off the Fe−C−O axis or sterically by pushing the bound CO off-axis, a structure which was suggested originally for the α-form of the CO−heme complex of mammalian oxidase obtained at neutral pH (Argade et al., 1984). The juxtaposition of the CO and Cu_B, which would result from the very short off-axis distance (Cu_B to heme normal) reported in the EXAFS experiments, would offer just the type of off-axis interaction presumably needed to enhance the bending mode.

In the β-form of cytochrome aa_3 oxidase from *Rb. sphaeriodes*, the much lower frequency for ν_{Fe-CO} allows the protein to fall on the Fe−CO *vs* the C−O stretching frequency correlation curve, indicating that the interaction present in the α-form of the enzyme is absent (Figure 4). This is consistent with a movement of the Cu atom away from the bound CO since in the report by Hosler et al. (1994b) when a ligand that coordinates to Cu_B was changed to a noncoordinating residue by site-directed mutagenesis, the Fe−CO stretching frequency was observed to decrease significantly. In the β-form of the enzyme, the frequencies of the modes involving CO are similar to those of model compounds and heme proteins with imidazole or histidine as the proximal ligand and in which CO can bind to the iron without anomalous polar and steric interactions; *i.e.*, the frequency of 493 cm^{-1} for the ν_{Fe-CO} of the β-form of aa_3, together with the ν_{C-O} frequency of 1955 cm^{-1}, places it on the ν_{Fe-CO} *vs* ν_{C-O} correlation curve (Figure 4) with a normal trans histidine.

While we are unable to assess unambiguously the intensity of the bending mode in the β-form of the bacterial enzyme, we note that the relative intensity of the line at 573 cm^{-1} to that at 519 cm^{-1} in trace c in Figure 2A is identical to that observed from mammalian oxidase at neutral pH (Argade et al., 1984), suggesting that the β-form of aa_3 (ν_{Fe-CO} at 493 cm^{-1}) makes a negligible contribution to the total intensity of δ_{Fe-C-O} at 573 cm^{-1}. Furthermore, the low intensity of δ_{Fe-C-O} in the β-form is also consistent with the absence of strong distal polar or steric interactions between the CO and the copper atom. These results resemble the low intensity of δ_{Fe-C-O} in the CO adducts of mammalian oxidase recorded at high or low pH where the stretching modes are found at 494 cm^{-1} (ν_{Fe-CO}) and 1968 cm^{-1} (ν_{C-O}) (Caughey et al., 1993; J. Wang, and D. L. Rousseau, unpublished data). Similarly, in resonance Raman measurements of *Rb. sphaeroides* cytochrome aa_3 oxidase obtained at high pH where the ν_{Fe-CO} mode of the α-form at 519 cm^{-1} completely shifted to 493 cm^{-1} similar to the β-form reported here, the δ_{Fe-C-O} at 574 cm^{-1} was extremely weak (J. Wang, and D. L. Rousseau, unpublished results). However, the line widths and frequencies of ν_{C-O} obtained from the high pH form and the β-form appear to be quite different, so the relationship between the β-form and the high pH form of the enzyme remains to be clarified.

The α- and β-forms of oxidases have only been detected in the CO-bound adduct of the enzyme. Equivalent differences have not been reported in other coordination and oxidation states of the enzyme, although the difference between the pulsed and the resting forms of mammalian oxidase has been attributed to differences in the distal environment as well. It is interesting to speculate that in these oxidized forms of the oxidase a change in the position of Cu_B is also taking place. It may very well be that only for very specific ligand binding states can the difference in the position of the copper be detected since it interacts with the exogenous ligands but not with the heme directly. Thus, the postulated difference in the coordination of pulsed and resting oxidase could be a consequence of the same structural difference that gives rise to the α- and β-forms of the CO-bound adducts. It will be interesting to determine if there are associated variations in the catalytic function between these forms since the interaction of the copper atom with heme-bound oxygen intermediates in the binuclear site could be quite different for these two forms of the enzyme. Finally, we must consider the possibility that changes in the position of Cu_B are a consequence of a change in coordination (or ligand exchange) to the copper. Ligand exchanges on Cu_B associated with the redox cycle and a possible coupling between such changes and proton translocation have been proposed (Hosler et al., 1993; Wikstrom et al., 1994). Additional studies are needed to determine if the two forms of the CO-bound enzyme are a reflection of such ligand exchanges. If so, data on the CO-bound adduct of oxidases could be important for elucidating the mechanism of proton translocation.

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