The cbb_3-Type Cytochrome *c* Oxidase from *Rhodobacter capsulatus* Contains a Unique Active Site

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The novel cbb_3-type cytochrome *c* oxidase isolated from the purple photosynthetic bacteria *Rhodobacter capsulatus*[1] and *Rhodobacter sphaeroides*[2] has a low-spin heme *b* and a high-spin heme b_3-Cu_B binuclear center[3] where the reduction of dioxygen to water takes place, and thus is a member of the terminal oxidase superfamily. Similar oxidases have also been postulated to be present from deduced amino acid sequence data in the nitrogen-fixing bacteria *Bradyrhizobium japonicum*[4] and *Rhizobium meliloti*.[5] The cbb_3-type oxidase, found in organisms under conditions of low oxygen concentration and thus presumably having a high O_2 affinity, is unique in several respects. Unlike other terminal oxidases, its catalytic site contains a heme B rather than a heme A or heme O, and it is thus the first oxidase known whose heme-Cu_B binuclear center consists of a heme without a (hydroxyethyl)farnesyl side chain, demonstrating that this side chain is not essential for functional competency.[1,2] In addition, similar to the quinol oxidases (cytochrome bo_3 from *Escherichia coli*[6] and the aa_3-type oxidase from *Bacillus subtilis*[7]), this three-subunit protein does not possess a Cu_A, which is present in all other terminal oxidases having cytochrome *c* as a substrate.[8] Instead, at least two electrochemically distinct cytochromes *c* are covalently bound to subunits II and III, giving the enzyme a turnover number[1,2] of 600−900 s^{-1}. Herein we report the resonance Raman spectra of the CO-bound adduct of the cbb_3-type cytochrome *c* oxidase isolated from *R. capsulatus*. The spectra reveal that in this cbb_3-type oxidase the conformation of the Fe−CO Cu_B moiety is distinct from that in other terminal oxidases.

With vibrational spectroscopies (infrared absorption and resonance Raman scattering), the Fe−CO stretching mode (ν_{Fe-CO}), the Fe−C−O bending mode (δ_{Fe-C-O}), and the C−O stretching mode (ν_{C-O}) have been identified in a large number of porphyrins and heme proteins.[9] All terminal oxidases thus far reported exhibit relatively high frequencies for ν_{Fe-CO} (~520 cm^{-1}) and ν_{C-O} (~1963 cm^{-1}) causing the oxidase family to fall far off the well-known ν_{Fe-CO} versus ν_{C-O} frequency correlation curve characteristic of hemeproteins with a proximal histidine[9,10] as may be seen in Figure 1 (solid line), despite the fact that a fully conserved histidine has been identified as the proximal ligand in all o_3- or a_3-type oxidases.[11,12] The origin for the displacement of the oxidase family from the ν_{Fe-CO} versus ν_{C-O} correlation curve has been discussed extensively.[13] In recent investigations,[14,15] the displacement from the correlation curve was postulated to result from either a compression of the Fe−CO bond or a distortion of the Fe−C−O moiety due to a strong polar or steric effect on the heme-bound CO exerted by Cu_B.

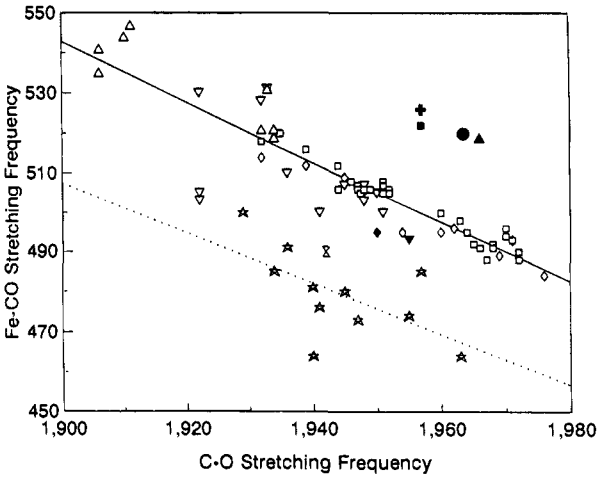

Figure 1. Correlation between frequencies of the Fe−CO versus the C−O stretching modes of hemeproteins. The dashed line is for proteins (☆) with a proximal thiolate ligand (e.g., cytochrome P-450s); the solid line is for hemeproteins (open symbols) containing a nitrogenous proximal ligand (e.g., a neutral histidine in globins and imidazolate in peroxidases). Mammalian cytochrome *c* oxidase (α-form) is designated by ●, the α-form of *R. sphaeroides* by ▲, cytochrome bo_3 by ■, and a porphyrin coordinated by a weak ligand (tetrahydrofuran) by +. Cytochrome cbb_3 (♦) from *R. capsulatus* as well as the β-form of cytochrome aa_3 (▼) from *R. sphaeroides*[14] lie on the correlation curve.

In addition to the major conformation described above (termed the α-form), another conformation of the catalytic site (β-form) with a lower C−O stretching frequency (1950 cm^{-1}) was measured in the bovine enzyme by cryogenic FTIR spectroscopy.[16] Recently, it was shown[15] that in one aa_3-type cytochrome *c* oxidase (*R. sphaeroides*) this second form (β-form) of the CO-bound heme is also present at room temperature, along with the α-form, and has ν_{Fe-CO} and ν_{C-O} frequencies of 493 and 1955 cm^{-1}, respectively. Interestingly, the frequencies

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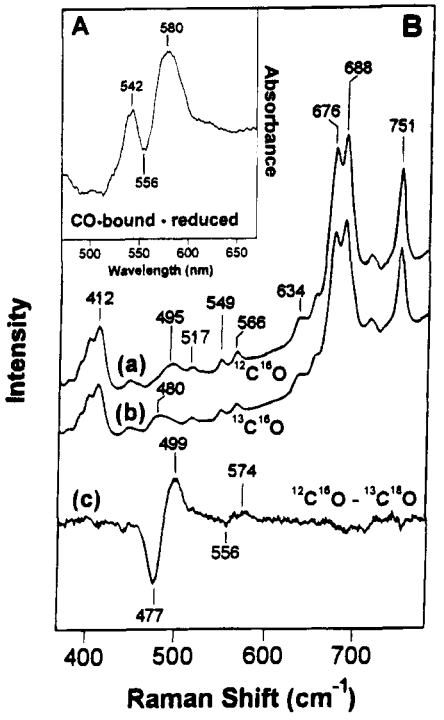

Figure 2. (A) Optical absorption difference spectrum in the visible region of CO-bound dithionite-reduced minus dithionite-reduced cbb_3-type oxidase from *R. capsulatus*. The same optical data were recorded before and after Raman measurements from a sealed Raman spinning cell containing 100−120 μL aliquots of ~50 μM enzyme, ensuring that no change was induced by laser irradiation. (B) Resonance Raman spectra[21] of the CO-bound form of cytochrome cbb_3, observed at 413.1 nm excitation with low laser power (0.15 mW) in the low-frequency region. The protein was isolated from *R. capsulatus* by a procedure described elsewhere[1] and solubilized in 100 mM sodium phosphate buffer with 0.01% dodecyl β-D-maltoside (pH 7.2). The $^{12}C^{16}O$-bound and the $^{13}C^{18}O$-bound forms are shown in a and b, respectively. Trace c is the difference spectrum of the $^{12}C^{16}O$-bound form (a) minus the $^{13}C^{18}O$-bound form (b).

for the β-form of the *R. sphaeroides* aa_3-type oxidase place it on the ν_{Fe-CO} versus ν_{C-O} correlation curve (Figure 1).

The presence of peaks at 542 and 580 nm and a trough at 556 nm in the visible region difference optical absorption spectrum of CO-bound minus dithionite-reduced cytochrome cbb_3 (Figure 2A) confirms the coordination of CO to the reduced enzyme.[1,2] In the Soret region (data not shown) the difference spectrum has a maximum at 415 nm originating from the CO-bound heme. The coincidence of the laser excitation wavelength (413.1 nm) with the Soret transition from CO-bound cytochrome b_3 (415 nm) allows its resonance Raman spectrum (Figure 2B) to be significantly enhanced and, thus, the vibrational properties of the CO-bound heme to be studied. A line at 495 cm^{-1}, apparent in the resonance Raman spectrum from the $^{12}C^{16}O$-bound adduct (a), shifts to 480 cm^{-1} upon isotopic substitution by $^{13}C^{18}O$ (b) and is clearly evident in the difference spectrum (trace c in Figure 2B). In addition to the strong feature at 499/477 cm^{-1}, another isotopic-sensitive feature (at 574/556 cm^{-1}) was detected in the difference spectrum. We assign the strong line at 495 cm^{-1} (for $^{12}C^{16}O$) as ν_{Fe-CO} and the weak line at 574 cm^{-1} as δ_{Fe-C-O}. The ν_{Fe-CO} mode is very broad (FWHM = ~25 cm^{-1}) compared to that in other terminal oxidases (FWHM = ~10 cm^{-1}), and the intensity of the δ_{Fe-C-O} mode is very low. The δ_{Fe-C-O} mode frequency in heme proteins is characteristic of the type of proximal ligand:[17] thiolate, 550−560 cm^{-1}; neutral imidazole, 570−580 cm^{-1}; imidazolate, 580−590 cm^{-1}; tyrosinate, ~593 cm^{-1}. Thus, the frequency for δ_{Fe-C-O} (~574 cm^{-1}) in the cbb_3-type oxidase identifies histidine as the proximal ligand as in the other terminal oxidases, consistent with the amino acid sequence of the cbb_3-type oxidase from *B. japonicum*[4] in which the histidine assigned as the proximal ligand for the high-spin heme in aa_3-type oxidases[12] is conserved.

The Fe−CO stretching mode in the cbb_3-type oxidase from *R. capsulatus* is detected at 495 cm^{-1} with no indication of any contribution at 520 cm^{-1}. These results, along with the FTIR data[2] in which ν_{C-O} is detected at 1950 cm^{-1}, establish that a β-form rather than the α-form is the stable conformation of the cbb_3 enzyme.[18] Unlike the α-form, characteristic of all other terminal oxidases, which fails to lie on the ν_{Fe-CO} versus ν_{C-O} correlation curve, the β-form of the cbb_3 oxidase falls very close to the curve (Figure 1). The data from cytochrome cbb_3 suggest an open structure lacking the distal interactions present in the α-form thereby allowing the heme-bound CO to adopt a near-straight and perpendicular conformation with respect to the heme. The broad width of the ν_{Fe-CO} mode in cytochrome cbb_3 is an indication of the wide distribution of allowed Fe−CO conformations, the low frequency of the mode is an indication of an unstrained proximal histidine−Fe−CO complex, and the low intensity of the bending mode is an indication of a symmetric geometry and environment, all consistent with this interpretation. We conclude that the CO adduct of the cbb_3 oxidase has a unique active site with a more open pocket than that in other terminal oxidases. In certain mutant oxidases, low-frequency Fe−CO stretching modes were detected.[14] However, in those cases, the enzyme had no dioxygen reduction activity.[19] In the other case in which a β-form was detected,[15] the α-form was also present so the activity of the component with the β-form could not be assessed. Thus, the cbb_3 oxidase is the only example of a fully active oxidase with the β-form, demonstrating that the properties of the binuclear center in other oxidases resulting in the α-form are not required for enzymatic activity.

The molecular origin for the unique active site in the cbb_3 oxidase, which we ascribe as a β-form, as compared to the other members of the superfamily of oxidases is intriguing. The cbb_3 oxidase is the only documented member of the superfamily whose binuclear center does not consist of an o_3- or a_3-type heme containing a (hydroxyethyl)farnesyl side chain. The farnesyl chain could serve to anchor the heme group[20] by hydrogen bonding of its hydroxy group or by hydrophobic interactions involving its hydrocarbon chain. The crystal structure of mammalian cytochrome *c* oxidase (S. Yoshikawa, personal communication) supports this idea. There is a hydrogen bond between tyrosine-244 in helix-6, the helix in which Cu_B is coordinated, and the hydroxy group of the farnesyl chain, thereby coupling together the metals that form the binuclear center. In the absence of the farnesyl chain in the cbb_3-type oxidase, a higher degree of flexibility between the heme and the copper would be expected, leading to a less tightly structured binuclear center and consequently a more open pocket.

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