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LETTERS

Detection of a Photostable Five-Coordinate Heme a_3-Fe−CO Species and Functional Implications of His384/α10 in CO-Bound ba_3-Cytochrome *c* Oxidase from *Thermus thermophilus*

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Resonance Raman (RR) spectra are reported for the fully reduced carbon monoxy derivative of ba_3-cytochrome *c* oxidase from *Thermus thermophilus*. The RR spectra show the formation of a photolabile six-coordinate heme−CO and a photostable five-coordinate heme Fe−CO species. The latter species is formed by the cleavage of the proximal heme Fe−His384 bond and is the first five-coordinate Fe−CO species detected in heme−copper oxidases. The frequency of the Fe−CO species observed at 526 cm^{-1} correlates with either the C−O stretching modes observed at 1967 or 1982 cm^{-1} and lie on the correlation line of ν(Fe−CO) vs ν(C−O) for all known five-coordinate heme Fe−CO complexes. The loss of intensity of the heme Fe−His384 mode observed at 193 cm^{-1} in the photostationary CO-bound spectra is attributed to the loss of the non-hydrogen bonded heme Fe−His384···Gly359 conformer. Taken together, the data indicate that the environment of the ruptured His384 that is a part of the Q-proton pathway and leads to the highly conserved among all heme−copper oxidases, H_2O pool, is disrupted upon CO binding to heme a_3.

Introduction

The chemistry of carbon monoxide (CO) and nitric oxide (NO) with the active heme centers of biological sensors that carry out important roles in biological signaling in eukaryotic and prokaryotic organisms, and with the binuclear center of heme−copper oxidases is of profound relevance.[1−5] CO has been shown to stimulate guanylate cyclase (sGC) activity and is generally believed to activate the protein in a manner similar to NO.[1−3] Activation of sGC brought about by NO is due to NO coordination to the heme followed by rupturing of the heme Fe−His bond, yielding a five-coordinate (5C) heme−NO species.[2,3] On the other hand, CO binding to sGC forms both six-coordinate (6C) and 5C heme−CO complexes.[1] Although the inhibition of heme−copper oxidases by NO plays a physiological role in controlling mitochondrial O_2 consumption, the inhibition of the respiratory enzymes by CO and the molecular mechanisms of the origin of ligand specificity are open questions.[4]

The ba_3-cytochrome *c* oxidase from the gram-negative thermophilic eubacterium *Thermus thermophilus* couples the reduction of dioxygen to proton translocation across the inner bacterial membrane and, in contrast to the eucaryotic heme−copper oxidases, catalyzes the reduction of NO to N_2O.[6,7] The enzyme contains a homodinuclear copper complex (Cu_A), one

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low-spin, 6C heme *b*, and a binuclear center that consists of Cu_B and a high-spin, 6C heme a_3 in which the Fe atom is in the plane of the heme. The distance of the heme a_3 Fe to the proximal histidine ligand (His384/α10) is 3.3 Å (Fe−N_ϵ), considerably larger than that found in *P. denitrificans* and bovine heart oxidases.[6] Furthermore, the distance from N_δ of His384 to the carbonyl of Gly359 is 3.43 Å (H-bonding distance), and the distance of N_ϵ of H384 to Asn366 (N side chain) is 3.02 Å.[6]

In this work, we have used resonance Raman (RR) spectroscopy to characterize the CO-bound complex of ba_3-oxidase. RR excitation at 413.1 nm showed the presence of both a photostable and a photolabile species. The former species is 5C heme Fe−CO in which the heme Fe−His384 bond is ruptured. The latter is a 6C His−heme Fe^{2+}−CO species.[8] The detection of the photostable 5C species is surprising and was not expected a priori since all heme−copper oxidases form a 6C heme Fe−CO species. Therefore, its characterization is important in exploring the environment of His384 that is a part of the Q-proton pathway.[6] This proton channel leads through His384, Asn366, Asp372, and the propionate of the heme a_3 pyrrole ring A to an accumulation of H_2O molecules.[6] The frequency of the 5C heme Fe^{2+}−CO species observed at 526 cm^{-1} and the C−O stretching frequencies observed at 1967 and 1982 cm^{-1} in the FTIR experiments fall on the correlation line of ν(Fe−CO) vs ν(CO) for 5C heme species.[1,5] The data reported here, in conjunction with our recent findings,[8] demonstrate that the environment of Cu_B does not control the strength of the Fe−C bond in either the 5C or 6C heme Fe−CO species. We postulate that the environment of the ruptured His 384 affects the properties of the Q-proton pathway.

Materials and Methods

Cytochrome ba_3 from *T. thermophilus* was isolated by the procedure published previously.[6] Resonance Raman spectra were obtained from 40−50 μM samples, pH 7.5, in a cylindrical quartz spinning cell maintained at 5−7 °C by a stream of cold nitrogen gas. The RR data were acquired as described elsewhere.[3,8−10] FTIR spectra were recorded from 400 μM samples at 4 cm^{-1} resolution with a BRUKER Equinox 55 FTIR spectrometer equipped with a liquid-nitrogen-cooled mercury cadmium telluride detector. The fully reduced CO samples were anaerobically loaded into a cell with CaF_2 windows and a 0.025 mm spacer. An average of 2000 scans was used for each spectrum. Optical absorption spectra were recorded before and after FTIR and Raman measurements in order to assess sample stability with a Perkin-Elmer Lamda 20 UV−visible spectrophotometer.

Results and Discussion

Figure 1A shows the low-frequency RR spectra excited at 413.1 nm of the CO-bound fully reduced ba_3 from *T. thermophilus*. For comparison we have included the 428.7 nm excited RR spectra (Figure 1B). In the 428.7 nm excitation spectra shown in Figure 1B, the difference spectrum (trace *a*) between the $^{12}C^{16}O$- and $^{13}C^{18}O$-bound states clearly demonstrates the ν(Fe−CO) and δ(Fe−C−O) of the 6C heme Fe−CO species at 507 and 568 cm^{-1}, respectively. The difference spectrum (trace *b*) obtained at higher laser power (4.5 mW) shows that the intensities of both the 507 and 568 cm^{-1} modes have diminished, indicating that the Fe−CO species is photolabile. In the 413.1 nm RR excitation spectra (Figure 1A), in addition to the 6C heme Fe−CO modes, we observe a new mode at 526 cm^{-1} (trace *a*) that shifts to 514 cm^{-1} (trace *b*) when the

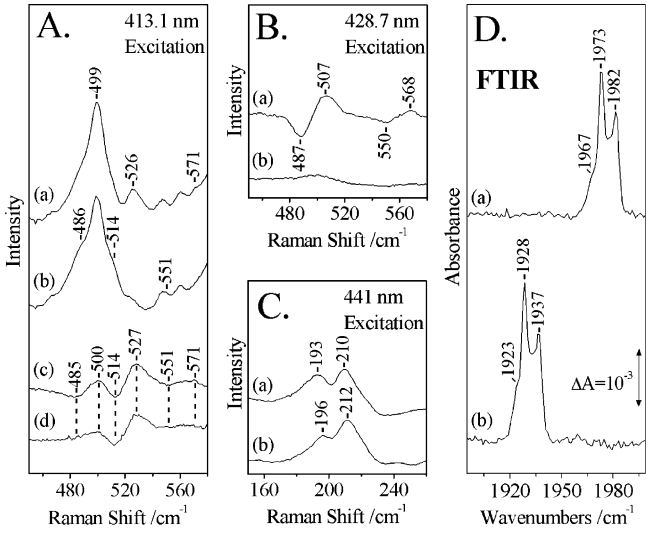

Figure 1. A. Resonance Raman spectra of CO-bound ba_3 cytochrome *c* oxidase at ambient temperature obtained with 413.1 nm excitation. The reduced samples with bound- $^{12}C^{16}O$ and $^{13}C^{18}O$ are shown in traces *a* and *b*, respectively. The difference spectrum $^{12}C^{16}O-^{13}C^{18}O$ obtained at 50 μW laser power is shown in trace *c*. Trace *d* is the difference spectrum $^{12}C^{16}O-^{13}C^{18}O$ obtained at 20 mW laser power. B. Trace *a* is the $^{12}C^{16}O-^{13}C^{18}O$ difference spectrum obtained with 428.7 nm excitation and 50 μW laser power. Trace *b* is the $^{12}C^{16}O-^{13}C^{18}O$ difference spectrum obtained at relatively high laser power (4.5 mW). C. RR spectra of fully reduced -(trace *a*) and photostationary CO-bound ba_3 (trace *b*) obtained with 441 nm excitation at 4.5 mW laser power. D. FTIR spectra of the $^{12}C^{16}O$- (trace *a*) and ^{13}CO- (trace *b*) bound fully reduced ba_3 oxidase.

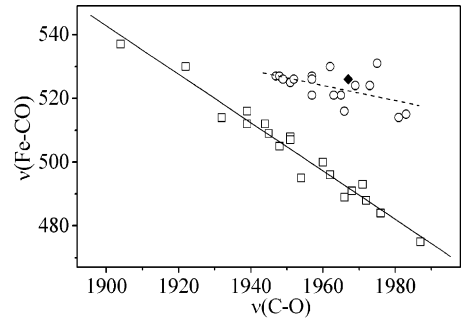

Figure 2. Correlation between frequencies of Fe−CO vs C−O stretching modes for 6C and 5C hemes and hemeproteins taken from refs 1 and 5. The open squares are data of 6C and the open circles data of 5C. ◆ represents the 5C heme Fe−CO species in ba_3-cytochrome oxidase.

experiment is repeated with $^{13}C^{18}O$. The difference spectrum (trace *c*) between the $^{12}C^{16}O$- and $^{13}C^{18}O$-bound states confirms their presence. The difference spectrum (trace *d*) obtained at high laser powers (20 mW) indicates that the 526 cm^{-1} species, in contrast to the 507 cm^{-1} species, is stable to relatively intense laser irradiation. From the resemblance of the frequency and insensitivity of the 526 cm^{-1} mode to laser power, to those of other 5C heme Fe−CO complexes,[5] we assign the 526 cm^{-1} mode to a 5C Fe−CO species in which the heme Fe−His384 bond is ruptured. Two ν(Fe−His) conformers have been detected in the RR data of ba_3 oxidase.[11] The 441 nm RR spectrum of the fully reduced enzyme (Figure 1C, trace *a*) confirms their existence at 193 and 210 cm^{-1} in our enzyme isolation. Figure 1C, trace *b*, shows the photostationary RR spectrum of the CO-bound ba_3. At first glance, it appears that the 193 cm^{-1} mode has lost intensity and upshifted 3 cm^{-1} to 196 cm^{-1} (see below). The FTIR spectra (Figure 1D) show the $^{12}C-O$ (trace *a*) and $^{13}C-O$ (trace *b*) stretching modes of heme a_3-CO.

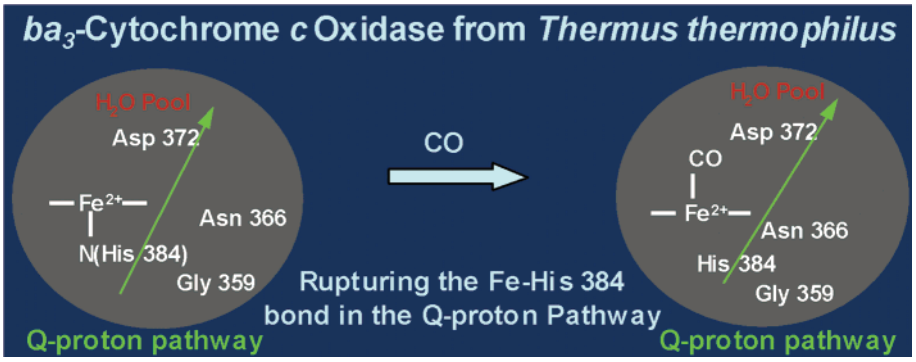

Figure 3. Schematic view of the active site in the CO complex of ba_3 oxidase. Selective residues are shown: His384 (proximal), Gly 359, Asn366, and Asp372. Part of the Q-proton pathway is shown by the arrow.

The variation in protonation state of the proximal heme Fe−His384 with Gly359 is invoked to account for the occurrence of the split Fe−His stretching mode, which has components at 193 and 210 cm^{-1}. The conformer with the weaker (or absent) H-bond is expected to have the weaker Fe−His bond and the lower frequency vibration at 193 cm^{-1}. The more strongly H-bonded conformer contributes to the 210 cm^{-1}. From the present experiments we conclude that the reduced intensity of the 193 cm^{-1} mode in the photostationary experiments is the result of the loss of the non-Hydrogen bonded conformer. However, time-resolved RR experiments, which are in progress, are required to explore the dynamics of the H- and non H-bonded conformers. The frequencies of the CO-bound form of fully reduced heme−copper oxidases lie off the correlation line for proteins with a trans His ligand even though the Fe−N(His) stretching mode has been identified in the ferrous form of the enzymes.[9,10,12] Recently,[9,10] we have demonstrated that the high frequency of the Fe−CO stretching frequency and the position of the heme−copper oxidases on the correlation line is not due, as previously thought,[12] to distal effects involving the interaction of Cu_B with the bound CO to the heme. The present data further support this conclusion since the frequencies of the Fe−CO and C−O stretching modes are similar to those observed in any 5C heme−CO protein (Figure 2). It should be noted that the Fe−CO mode observed at 526 cm^{-1} can correlate with either the C−O modes observed at 1967 or 1982 cm^{-1} but not with that observed at 1973 cm^{-1}. The latter C−O mode correlates with the 6C Fe−CO mode observed at 506 cm^{-1}, and together they constitute the *a*-form of the enzyme.[8,9,12] Finally, it seems remarkable that all 5C heme−CO species have similar ν(Fe−CO) frequencies, as the distal pockets of ba_3 and other heme proteins have different polarities.[5]

Figure 3 shows a schematic view of the rupturing of the Fe−His384 bond in the Q-proton pathway. The data presented here demonstrate that addition of CO to the fully reduced enzyme causes the rupture of the heme Fe−His bond yielding a 5C heme Fe−CO photostable species. In general, CO does not compete effectively with endogenous ligands in heme proteins. Thus, we can exclude the possibility that CO binds to the proximal site of heme a_3 by displacing His384. On the basis of the crystal structure,[6] we suggest that upon CO binding there is a communication linkage between Asn366 and His384 that causes the rupture of the Fe−His384 bond. We also exclude the possibility (see below) that the photostable 5C species is the result of the fast rebinding of CO to the heme Fe because the decay of the transient Cu_B-CO is 34.5 s^{-1} and rebinding to heme a_3 occurs with $k_2 = 28.6\ s^{-1}$ ($t_{1/2} = 24.2$ ms).[13] If the 5C species is photo dissociable then we expect the same time scales for the initial and final events to occur in the photodynamics of the 6C and 5C species.[11,13] On the basis of the absence of an activation barrier to the migration of CO from heme a_3 to Cu_B, and on the slow rebinding of CO to the heme, we conclude that the 5C heme Fe−CO species is photostable.

The proximal His384 is conserved in all structurally known heme−copper oxidases, and thus, we suggest that our data do not reflect any specific properties of ba_3 oxidase. The environment of His384, that is part of the Q-proton pathway is disrupted upon CO binding, causing the rupturing of the heme Fe−His384 bond. We suggest that the conformational change induced by such an event will affect the relationship between His384 and Asn366 both of which constitute a part of the Q-proton pathway, and/or that of His384 and Gly359. Such conformational changes can act as the molecular switch for further conformational changes in the propionate of the heme a_3 pyrrole ring A and Asp372.[14] All of the above-mentioned residues are part of the Q-channel that leads to the highly conserved among all structurally known heme−copper oxidases, the H_2O pool. Experiments to address these issues are in progress.

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