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Resonance Raman Detection of the Fe^{2+}–C–N Modes in Heme–Copper Oxidases: A Probe of the Active Site†

Eftychia Pinakoulaki, Magdalini Vamvouka, and Constantinos Varotsis*

Department of Chemistry, University of Crete, 71409 Heraklion, Crete, Greece

Received October 22, 2003

Resonance Raman spectroscopy has been employed to investigate the reduced cyano complexes of cytochrome aa_3 from bovine heart and *Rhodobacter sphaeroides* and of cytochrome bo_3 from *E. coli*. In the aa_3-type oxidases, the frequency of the Fe–CN stretching mode is located at 468 cm^{-1}, and the bending Fe–C–N vibration, at 500 cm^{-1}. The fully reduced cytochrome bo_3–CN complex gives rise to a stretching vibration at 468 cm^{-1}, a bending vibration at 491 cm^{-1}, and a stretching C–N vibration at 2037 cm^{-1}. The observed differences between aa_3 and bo_3 oxidases in the frequencies of the Fe–C–N group suggest a quantitative difference in the structure of the His–heme $a_3{}^{2+}/Cu_B{}^{1+}$ and His–heme $o_3{}^{2+}/Cu_B{}^{1+}$ binuclear pockets upon CN^- binding.

Introduction

Cytochrome aa_3 oxidase is a member of a superfamily of proton translocating proteins, terminal oxidases, that contain a heme a_3-Cu_B binuclear catalytic center in which the binding and reduction of molecular O_2 takes place.[1] On the basis of the crystal structure, the enzyme contains four redox metal centers: Cu_A, heme *a*, heme a_3, and Cu_B.[2−4] Cytochrome bo_3 is a terminal ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli*.[5−7] This membrane protein also catalyzes the four-electron reduction of molecular oxygen to water and couples the two-electron oxidation of ubiquinol-8 oxidation with dioxygen reduction to translocate protons across the energy-transducing cytoplasmic membrane, and with the bacterial and eucaryotic cytochrome *c* oxidase are grouped as the heme−copper terminal oxidases.[5−7] Unlike the aa_3-type cytochrome *c* oxidase, cytochrome bo_3 lacks the Cu_A site and contains heme *o* in the binuclear center, and it contains the three other redox-active metal centers in subunit I.[5−9]

Properties of the oxygen-binding environment in terminal oxidases may be studied by examining the carbon monoxide-,[10] nitrosyl-,[11,12] and cyanide-bound[13−18] complexes. The infrared absorption[17,18] and resonance Raman[13,15,16] (RR) spectra of the CN-bound mammalian aa_3-cytochrome *c* oxidase (CcO) have revealed previously undetermined structural characteristics of the binuclear center. These studies have suggested that CN^- is coordinated as a heme $a_3-C{\equiv}N-Cu_B$ species in the fully oxidized

* Author to whom correspondence should be addressed. E-mail: varotsis@edu.uoc.gr. Fax: +30-2810393601.

† Abbreviations: CcO = cytochrome *c* oxidase; RR = resonance Raman; FTIR = Fourier transform infrared.

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enzyme and that upon reduction of the CN-bound complex cyanide is bound to the heme a_3−iron in a bent geometry.[13,15] There are conflicting reports in the literature, however, regarding the assignment of the CN-sensitive modes in the heme a_3^{2+}−CN complex.[13,15] Kitagawa and co-workers, although their fully reduced CN samples contained the partially oxidized CN complex, reported that the RR difference spectra of reduced CcO−CN show peaks around 456−485 cm^{-1} and that a CN isotope-sensitive band was identified at 475 cm^{-1}.[13] However, Kim et al. reported the $\nu(Fe^{2+}-CN)$ at 469 cm^{-1} and $\delta(Fe^{2+}-CN)$ at 503 cm^{-1}.[15]

RR studies of the CO-bound complexes of cytochrome bo_3 and CcO revealed small frequency differences between the CO isotope-sensitive modes of heme o_3 and those of heme a_3, and accordingly, it was concluded that the binuclear center of cytochrome bo_3 is similar to that of CcO.[10] There are, however, some intriguing data regarding the properties of their active sites in the CN^--bound derivatives.[18] Recently, in a report on the FTIR spectrum of the fully reduced cyanide-bound cytochrome bo_3, the ν(C−N) was observed at 2035 cm^{-1}.[18] This mode, with a frequency 23 cm^{-1} lower than that reported for the corresponding frequency of CcO,[17] was interpreted to arise from the specific character of the cyanide binding to the binuclear center of cytochrome bo_3. The shift of the C−N (Fe) stretching mode located at 2058 cm^{-1} in heme a_3^{2+} to 2035 cm^{-1} in heme o_3^{2+} suggests a difference in the structure of the His−heme a_3^{2+}/Cu_B^{1+} and His−heme o_3^{2+}/Cu_B^{1+} binuclear pockets upon CN^- binding.

In an effort to clarify these differences, we have examined the RR spectra of the fully reduced CN^--bound adduct of cytochrome aa_3 from bovine heart and *Rb. sphaeroides* and the RR and FTIR spectra of the fully reduced CN^--bound adduct of cytochrome bo_3. Our data indicate that in the aa_3-type oxidases the frequency of the Fe−CN stretching mode is located at 468 cm^{-1} and the bending Fe−C−N vibration is located at 500 cm^{-1}. The fully reduced cytochrome bo_3−CN complex gives rise to a stretching vibration at 468 cm^{-1}, a bending vibration at 491 cm^{-1}, and a stretching C−N vibration at 2037 cm^{-1}. The observed differences between cytochromes aa_3 and bo_3 in the frequencies of the Fe−C−N group demonstrate a quantitative difference in the structure of the His−heme a_3^{2+}/Cu_B^{1+} and His−heme o_3^{2+}/Cu_B^{1+} binuclear pockets upon CN^- binding. The results are used to determine the details of cyanide ligation to fully reduced aa_3- and bo_3- type heme−copper oxidases.

Experimental Section

Cytochrome aa_3 oxidase from bovine[20] and *Rhodobacter sphaeroides*[21] was isolated as described elsewhere. Cytochrome bo_3 from *E. coli* 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 experiments were performed on a system described elsewhere.[20] The power incident on the oxidase samples was typically 4−6 mW. FTIR spectra were recorded from 180−250-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 cyanide samples were anaerobically loaded into a cell with CaF_2 windows and a 0.025-mm spacer. An average of 500 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−vis spectrophotometer. Isotopic CN^- ($^{13}C^{15}N^-$) was purchased from Isotec (Miamisburg, OH).

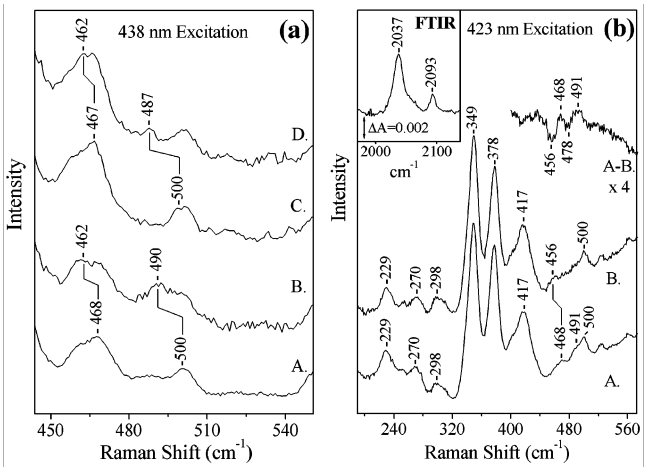

Figure 1. (a) Low-frequency resonance Raman spectra of CN^--bound aa_3-type heme−copper oxidases from bovine heart (A and B) and *Rb. sphaeroides* (C and D). The excitation wavelength is 438 nm. (A) $^{12}C^{14}N^-$; (B) $^{13}C^{15}N^-$; (C) $^{12}C^{14}N^-$; (D) $^{13}C^{15}N^-$. (b) Resonance Raman and FTIR spectra (inset) of the fully reduced CN-bound forms of cytochrome bo_3. The excitation wavelength is 423 nm. The $^{12}C^{14}N^-$ and the $^{13}C^{15}N^-$-bound forms are shown in A and B, respectively. The difference spectrum is (A − B) × 4. See Experimental section for details.

Results and Discussion

Figure 1a shows the RR spectra in the 450−550 cm^{-1} region of $^{12}C^{14}N^-$ (A) and $^{13}C^{15}N^-$ (B) bound bovine cytochrome aa_3 oxidase and of $^{12}C^{14}N^-$ (C) and $^{13}C^{15}N^-$ (D) bound cytochrome aa_3 oxidase from *Rb. sphaeroides*. The peak at 500 cm^{-1}, shown in spectrum A, is shifted to 490 cm^{-1} (spectrum B), and the peak at 468 (spectrum A) is shifted to 462 cm^{-1} for the bovine enzyme, in agreement with the reported results of Oertling and co-workers.[15] The FeC stretching vibration at 468 cm^{-1} displays a monotonic shift toward lower wavenumbers as the cyanide mass is increased, and the bending vibration at 500 cm^{-1} exhibits the increase−decrease−increase isotope dependence (zigzag) that is characteristic of a bending mode.[15] We observe similar RR spectra for the CN^- complex of *Rb. sphaeroides* aa_3-type oxidase (spectra C and D). This observation indicates the similarities between the proximal and distal environments in the heme a_3 pockets of the bovine and *Rb. sphaeroides* enzymes upon CN^- binding (see below). Similar conclusions have been reached from the ferrous−CO-bound complex and the photoproduct formed after CO photolysis studies in both enzymes.[22]

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Table 1. Cyanide Stretching and Bending Frequencies of Ferrous Heme−Copper Oxidases

compound	ν(Fe−CN)	δ(Fe−C−N)	ν(CN)	ref
ba_3 (*T. thermophilus*)	512	485		14
aa_3 (bovine)	468	500	2058	15, 17, this work
aa_3 (*Rb. sphaeroides*)	467	500		this work
bo_3 (*E. coli*)	468	491	2037	this work

Figure 1b shows the low-frequency RR spectra and the FTIR spectrum (inset) of the fully reduced CN^--bound cytochrome bo_3. The RR spectrum of the $^{12}C^{14}N^-$-bound cytochrome bo_3 (spectrum A) obtained at neutral pH with 423-nm excitation and low laser irradiation (4−5 mW) exhibits peaks at 491 and 468 cm^{-1}, both of which display carbon and nitrogen isotopic sensitivity by shifting to 478 and 456 cm^{-1}, respectively, in the $^{13}C^{15}N^-$ derivative (spectrum B). The shifts are more evident in the difference spectrum of the $^{12}C^{14}N$-bound form (A) minus the $^{13}C^{15}N^-$-bound form (B). The RR spectrum of the $^{12}C^{15}N^-$-bound form (data not shown) shows two peaks at 491 and 465 cm^{-1} with intensities, however, that are 20−30% weaker than those of the $^{12}C^{14}N^-$-bound and $^{13}C^{15}N^-$ adducts, and thus, it is difficult to obtain a high-quality difference spectrum. On the basis of the assignment of the aa_3 oxidase and our observations, we assign the peak at 491 cm^{-1} as δ(Fe−C−N) and the peak at 468 cm^{-1} as ν(Fe−CN). It should be noted that in a linear Fe−C−N geometry the stretching vibration appears at a higher frequency than the bending vibration, whereas in a bent geometry, the opposite trend is observed.[15] In addition, when Fe−CN adopts a bent structure, ν(Fe−CN) and δ(Fe−C−N) are significantly mixed with each other, and both exhibit zigzag patterns depending on the extent of vibrational mixing.[13] We also assign the FTIR peak located at 2037 cm^{-1}, shown in the inset, to the C−N stretching mode. The other band located at 2093 cm^{-1} is due to HCN. Several measurements were made to determine if the spectra shown in Figure 1b originate from the fully reduced CN^--bound cytochrome bo_3 or a mixed-valence form. The absence of modes of the ferric form of the enzyme in our FTIR and RR spectra confirms the complete reduction of our samples. The formation of both the $^{12}C^{14}N^-$ and $^{13}C^{15}N^-$ fully reduced derivatives is further confirmed by the disappearance of the Fe−His stretching mode located at 208 cm^{-1} in the fully reduced ligand-free form of the enzyme.[23,24] In addition, the frequency of the C−N stretching mode (2037 cm^{-1}) observed is similar to that reported by Tsubaki et al.[18] and is assigned as originating from the fully reduced CN complex. The frequencies of the C−N stretching mode and the bending Fe−C−N mode are 21 and 9 cm^{-1}, respectively, lower than those observed in CcO. However, the frequency of the Fe−CN mode is very similar to that observed in CcO. The vibrational data from this resonance Raman and FTIR analysis are summarized in Table 1. The large frequency differences between the CN isotope-sensitive modes in the reduced CN^--bound forms of heme o_3 and heme a_3 indicate that the His−heme Fe−CN^-/Cu_B moiety of the bacterial enzyme is different from that of its mammalian counterpart.

It is well established that the heme is a π acceptor and the proximal histidine is a good σ and π donor to the heme Fe^{2+}, whereas cyanide is a good σ donor and a weak π acceptor.[15] Although the Fe^{2+}−CN adduct is isoelectronic with Fe^{2+}−CO, back bonding from Fe^{2+} is weaker for CN^- than that for CO, as reflected in the higher *XY* stretching frequency at 2050 cm^{-1} for CN^- versus 1950 cm^{-1} for CO. Therefore, linear and tilted geometries are more likely to occur in Fe^{2+}−CO than in Fe^{2+}−CN^- complexes because of the strong Fe−ligand π bonding. In the case of CN^-, π bonding is weaker, and the Fe−C−N moiety is more easily bent. Vibrational analysis predicts that strengthening of the trans-axial ligand bond weakens the Fe−C bond of the bound cyanide, and the weakening of the trans-axial ligand bond is expected to cause a stronger σ donation from the CN^- via a trans effect.[25,26]

In the five-coordinate heme Fe^{2+} species, the ν(Fe^{2+}−His) of heme o_3 is located at 208 cm^{-1} and is 6 cm^{-1} lower than that of heme a_3.[22−24] Despite the observed differences in the strength of the proximal Fe−His bond between the five-coordinate heme a_3 and heme o_3 species, the net result is that the frequency of the Fe−CN mode in the six-coordinate His−Fe−CN species is the same in both enzymes. The 21-cm^{-1} downshift in the ν(CN) mode in heme o_3, as compared to that in heme a_3, can be interpreted as a consequence of the variation of the proximal histidine H-bonding interaction and thus the strength of the Fe−His bond. In this way, a stronger π donation from the proximal histidine can influence the strength of the CN stretch, causing stronger Fe−CN d_π back bonding and, consequently, a decrease in ν(CN). This argument is consistent with RR data that indicate that the bent Fe−C−N form is sensitive to the strength of the trans iron ligand and is further supported by reports that indicate that a weaker σ donation from CN^- decreases ν(CN).[25,26] The 9-cm^{-1} downshift in δ(Fe^{2+}−C−N) in cytochrome bo_3 indicates that the bound cyanide is more prone to distortion. The larger isotopic shift of the Fe−CN stretching in cytochrome bo_3 (12 vs 6 cm^{-1} in aa_3) indicates a more bent structure. It should be noted that ν(CN) is also sensitive to the imposed electric fields of neighboring amino acids and/or water molecules within the heme pocket. In this way, the negative CN attracts the positive sides of the water dipoles and repulses the negative sides. Accordingly, water molecules can produce an electric field that attracts extra electron density to the heme-bound CN, producing a weaker CN bond. The structural interpretation outlined above specifies a H_2O molecule as the source of the 21-cm^{-1} downshift of ν(CN^-) in cytochrome bo_3. This argument is supported by the observed 23-cm^{-1} downshift of ν(CN^-) in microperoxidase, as compared to that of ferrous myoglobin−CN, due to the presence of H_2O molecules near the bound CN.[27]

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On the basis of the spectra of model compounds, it is expected that the Fe−C stretch increases upon reduction of the heme Fe.[28] However, the Fe−C mode in the aa_3-type oxidases is decreased by 20 cm^{-1} in the ferric-to-ferrous CN transition, indicating that upon reduction of the binuclear center structural changes occur to facilitate the change in the ligand geometry from linear to bent. The FTIR spectrum of the CN-bound oxidized cytochrome bo_3 obtained at neutral pH exhibits a ^{13}CN-sensitive mode at 2146 cm^{-1} that is characteristic of an $Fe^{3+}-C\equiv N-Cu_B^{2+}$ bridging structure.[18] The frequency of this mode is 6 cm^{-1} lower than that found in CcO, reflecting small changes in the bonding/geometry of the $Fe^{3+}-C\equiv N-Cu_B^{2+}$ bridging cyanide in the two types of heme−copper oxidases. The observed differences in the ferric CN complexes between cytochrome aa_3 and bo_3 oxidases are further extended in the reduced CN forms. The analysis above indicates that there are significant differences in the structures and local interactions of the His−heme a_3^{2+}/Cu_B^{1+} and His−heme o_3^{2+}/Cu_B^{1+} binuclear pockets upon CN^- binding. The present study provides the first direct observation of variations in the distal environment, adjacent to the heme-bound ligand (in this case, CN^-), of the binuclear center in heme−copper oxidases.

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