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Identification of Heme Propionate Vibrational Modes in the Resonance Raman Spectra of Cytochrome c Oxidase

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Abstract

The propionate groups of heme *a* and *a*₃ in cytochrome *c* oxidase (CcO), have been postulated to mediate both the electron and proton transfer within the enzyme. To establish structural markers for the propionate groups, their associated vibrational modes were identified in the resonance Raman spectra of CcO from bovine (bCcO) and *Rhodobacter sphaeroides* (RsCcO). The distinction between the modes from the propionates of heme *a* and heme *a*₃, as well as those from the propionates on the pyrrole rings A and D in each heme, was made on the basis of H₂O-D₂O isotope substitution experiments, combined with wavelength-selective resonance enhancement (for bCcO) or mutagenesis studies (for RsCcO).

Keywords

Cytochrome oxidase; Raman scattering; bioenergetics; heme proteins

In cytochrome *c* oxidase (CcO)¹, the propionate groups of heme *a* and *a*₃ have been postulated to mediate both the electron and proton transfer within the enzyme [1-3]. Hence, it is important to establish structural markers for the propionate groups in order to determine the changes in their conformational and chemical properties during the catalytic cycle of CcO. With Soret excitation into the aromatic heme π -system, the Raman modes of aliphatic peripheral groups are not expected to be resonance enhanced as their orbitals are not π -conjugated with those of the porphyrin core. However, as pointed out by Spiro and coworkers, these modes can be enhanced by inductive and hyperconjugative effects, in which the σ character of the C-C bond of the substituent mixes with the π^* orbitals of the porphyrin macrocycle [4, 5]. Accordingly, intense ethyl group modes in the resonance Raman spectrum of nickel octaethylporphyrins have been identified and assigned to a CH₂ twisting mode, $\gamma_t(\text{CH}_2)$, of the propionate chain in myoglobin at 1223 cm⁻¹ [4].

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¹Abbreviations: cytochrome *c* oxidase: CcO; bovine cytochrome *c* oxidase: bCcO; *Rhodobacter sphaeroides* cytochrome *c* oxidase: RsCcO; wild type: wt.

To identify the propionate structural marker lines in bovine CcO (bCcO), we have obtained resonance Raman spectra of the deoxy enzyme (a^{2+} , a_3^{2+}) in H₂O versus D₂O (ca. 85 % deuterium content) with 441.6 nm excitation from a He-Cd laser. To avoid protein conformational changes induced by long exposure of the protein to D₂O [6, 7], the resonance Raman spectra were measured within an hour following the exposure of the samples to the D₂O buffer. The absence of any changes induced in the heme macrocycles, was confirmed by examination of the high frequency modes (1350-1370 cm⁻¹) in which changes were seen only in the formyl C=O mode and can be attributed to the well-established H-bonding interactions between the formyl oxygen atom and Arg-38. The bCcO, isolated from bovine hearts (8), was prepared in 0.1 M Tris pH (pD) 8.5 with 0.1 % n-decyl- β -maltoside [8] and reduced by sodium dithionite in an argon atmosphere. For the Raman measurements, the bCcO samples were placed in a spinning cell; the scattered light from the samples was dispersed by a Spex 1.25 m polychromator and detected by a charge-coupled device detector [9].

As shown in Fig. 1A, in the H₂O-D₂O difference spectra several spectral features are apparent in the 1100 -1400 cm⁻¹ region, which we assigned to the CH₂ twisting modes of the propionate groups. It is important to note that as there are no exchangeable protons on the CH₂ groups, these isotope-dependent shifts are not the usual H/D isotope shifts; instead, they are a result of conformational changes originated from the alteration in the H-bonding network linked to the carboxylate groups of the propionates. To distinguish the contribution of each of the two hemes, we have measured the spectra of the CO-bound enzyme (a^{2+} , a_3^{2+} -CO) in H₂O and D₂O, as with 441.6 nm excitation in the CO-bound enzyme only the modes from heme *a* are resonance enhanced, whereas in the deoxy enzyme all the modes from both heme *a* and *a*₃ are enhanced[10]. On this basis, the modes at 1215/1251 and 1179/1233 cm⁻¹ are assigned to the propionate modes of heme *a* and *a*₃, respectively, as the former modes in the deoxy and the CO-bound forms are similar, whereas the intensities of the latter modes are greatly diminished in the CO-bound form as compared to the deoxy species, as manifested in the double difference spectrum shown at the bottom of Fig. 1A.

In the crystallographic structure of bCcO (Fig. 1B), the propionate groups attached to the D-rings of hemes *a* and *a*₃ form strong H-bonds with Arg-438. We hypothesize that the H₂O/D₂O associated changes in the CH₂ twisting modes of the propionate groups shown in Fig. 1A are a result of the perturbation in this H-bonding network. To evaluate this hypothesis and to differentiate the propionate group on the D-ring from that on the A-ring, we examined the resonance Raman spectra of the wild-type (wt) and R481H mutant of *Rhodobacter sphaeroides* CcO (RsCcO). As the R481 residue in RsCcO is equivalent to the R438 in bCcO (Fig. 1 B-C); the R481H mutation is expected to eliminate the H-bonding interaction. The wt and R481H proteins were prepared as previously described [11] and handled in the same fashion as the aforementioned protocol applied to the bCcO samples. The H₂O-D₂O difference spectrum for the wt deoxy RsCcO exhibits the same spectral pattern in the 1160-1280 cm⁻¹ region (Fig. 2A, bottom trace) as that observed in bCcO (Fig. 1A, trace c). Hence the propionate modes were assigned in the same manner as those in bCcO, in which the 1218/1248 and 1178/1233 cm⁻¹ lines are attributed to heme *a* and *a*₃, respectively.

One of the two propionates modes from heme *a*₃, that at 1233 cm⁻¹, disappeared completely in the R481H mutant (Fig. 2B, bottom trace). It is therefore assigned to the D-ring propionate mode of heme *a*₃, whereas the remaining line at 1178 cm⁻¹ is assigned to an A-ring propionate mode of heme *a*₃. On the other hand, when the deoxy spectrum of the R481H mutant (in H₂O) was compared with that of the wt protein, significant line broadening was evident at 1248 cm⁻¹ (Fig. 2C). As the R481H mutation is expected to only affect the D-ring propionates and the 1233 cm⁻¹ line has been assigned to the D-ring

propionate mode of heme a_3 , the 1248 cm^{-1} is assigned to the D-ring propionate of heme a . It is important to note that, although in the mutant the H-bonding interactions with R481 are not present, the $\text{H}_2\text{O}/\text{D}_2\text{O}$ -sensitivity of the 1248 cm^{-1} line is plausibly a result of a new H-bonding interactions between the D-ring propionate of heme a and a nearby residue, such as R482 (Fig. 1C). The remaining mode at 1218 cm^{-1} is assigned to the A-ring propionate of the heme a .

In summary, we detected and assigned resonance Raman lines of the four propionate groups of heme a and heme a_3 in CcO. As the propionates have been postulated to play critical roles in both the electron transfer and proton translocation events [1-3], the establishment of these well-defined marker lines reporting the structural information of each of the four propionates of heme a and heme a_3 forms an important basis for the future studies of the catalytic process of CcO.

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Figure 1.

Resonance Raman spectra of the deoxy and CO-bound forms of bovine CcO in H₂O and D₂O with 441.6 nm excitation (A) and the X-ray crystallographic structures of the hemes *a* and *a*₃ of bovine CcO (B) and *R. sphaeroides* CcO (C). In (A), the dotted and solid traces in (a) and (b) are those obtained in H₂O and D₂O, respectively; traces (c-e) are the various difference spectra as indicated. All the difference spectra are scaled-up by 6-fold with respect to the parent spectra. The dotted lines in (B-C) show H-bonding interactions between R438 (R481) and the propionate side chains. The PDB codes for the crystallographic data are 2OCC (bovine) and 1M56 (*R. sphaeroides*).

Figure 2.

Resonance Raman spectra of the wt and R481H mutant of deoxy *R. sphaeroides* CcO in H₂O and D₂O with 441.6 nm excitation. In (A-B), the dotted and solid traces on the top are those obtained in H₂O and D₂O, respectively; the bottom traces are the associated H₂O-D₂O difference spectra. In (C), the dotted and solid traces on the top are those associated with the wt and the R481H mutant, respectively; the bottom trace is the associated wt-R481H difference spectrum. The difference spectra in (A-B) are scaled-up by 4-fold as compared to the parent spectra.