Raman Spectra of Isotope-substituted Mitochondria of Living Budding Yeast Cells: Possible Origin of the "Raman Spectroscopic Signature of Life"

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The ^{13}C and ^{2}H isotope substitution effects have been examined for the "Raman spectroscopic signature of life," a mitochondrial Raman band at 1602 cm^{-1} that sharply reflects the metabolic activity of living yeast cells. The band shifts to 1542 cm^{-1} with ^{13}C substitution and to 1599 cm^{-1} with ^{2}H substitution. Normal mode analysis based on a DFT calculation suggests that it originates from a C=C double bond having no hydrogen atoms attached to the carbon atoms. The in-phase C=C stretch mode of ubisemiquinone radicals (CoQ and/or $CoQH^{\bullet}$) emerges as a strong candidate for the origin of the 1602 cm^{-1} band.

In vivo, noninvasive and molecular-level monitoring of cellular metabolic activity is crucially important for the future development of quantitative physical chemistry looking at life. The "Raman spectroscopic signature of life," which is a strong and sharp Raman band at 1602 cm^{-1} measured from mitochondria of living yeast cells,[1,2] has great potential as a reliable molecular indicator of metabolic activity. This band has been shown to reflect cellular metabolic activity in many different ways, sharply responding to respiration inhibition,[2,3] starvation,[4] oxidative stress,[5] and anaerobic culture.[5] However, the origin of this 1602 cm^{-1} band is yet to be made clear. In the present study, we examine the Raman spectra of ^{13}C and ^{2}H isotope-substituted mitochondria in living yeast cells, in order to identify the origin of the "Raman spectroscopic signature of life." Isotope effects are observed as shifts of vibrational frequencies induced by the change of atomic mass. They provide a reliable experimental basis for band assignments of Raman and infrared spectra.

A tetraploid strain of budding yeast, a zygote of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*, was used in the present study. It was a gift from Suntory Co., Ltd. Cells were cultured at 30 °C in SC medium, which was a synthetic complete medium containing 2% D-glucose, 0.67% Bacto-yeast nitrogen base without amino acids, and 0.2% Drop-out mixture of amino acids. For isotope substitution experiments, ^{13}C-substituted D-glucose and ^{2}H-substituted D-glucose (Cambridge Isotope Laboratories, Inc.) were used. Raman spectroscopic measurements were carried out for yeast cells sampled in the stationary state after an overnight (24 h) culturing. In the isotope-substituted experiments, the overnight culturing was carried out twice. No changes of Raman spectra were found between these two consecutive culturings, indicating that the isotope substitution was already complete in the first overnight culturing. Raman spectra of mitochondria were recorded on a laboratory built confocal Raman microspectrometer. The details of the apparatus are described in our previous reports.[1–5] The excitation line was the 632.8 nm line of a He-Ne laser. The spatial resolution was 300 nm in the lateral direction and 2 μm in the axial. The 400–1800 cm^{-1} wavenumber region was measured with a spectral resolution of 3 cm^{-1}. Accumulation time was 300 s with excitation laser power of 6 mW at the sample point.

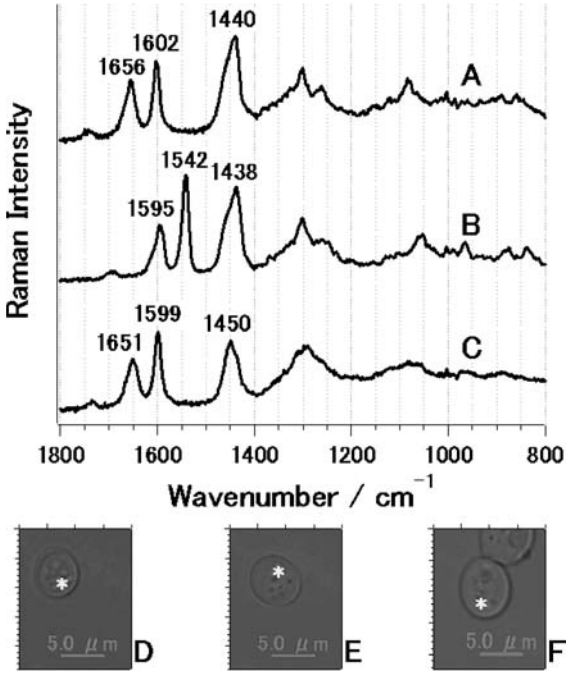

Figure 1. Space-resolved Raman spectra of budding yeast cell in (A) unsubstituted medium, (B) ^{13}C-substituted D-glucose medium, (C) deuterium-substituted D-glucose medium. The optical microscopic images, D, E, and F indicate the points of the laser spot (*) where A, B, and C were measured.

Space-resolved Raman spectra of mitochondria in single living budding yeast cells are shown in Figure 1. Figure 1A shows the Raman spectrum of a cell cultured in ordinary SC medium, Figure 1B shows that in SC medium containing ^{13}C-substituted D-glucose, and Figure 1C with ^{2}H-substituted D-glucose. The optical microscopic images, D, E, and F indicate the points of the laser spot where Raman spectra A, B, and C were measured, respectively.

The spectrum A shows a number of Raman bands that are already assigned to phospholipids.[3] The band at 1440 cm^{-1} is assigned to the C–H bend modes of methyl and methylene groups. The 1656 cm^{-1} band is due to the *cis*-C=C stretch mode of unsaturated chains of phospholipids. Between these two prominent bands of phospholipids, the "Raman spectroscopic signature of

life" is observed at 1602 cm^{-1}. As seen from Figures 1B and 1C, these three bands show clear isotope shifts. The 1656 cm^{-1} band (*cis*-C=C stretch) in Figure 1A completely disappears in Figure 1B, indicating that all carbon atoms of phospholipids are replaced under culturing with ^{13}C-substituted D-glucose. The band at 1595 cm^{-1} in Figure 1B is assigned to the *cis*-^{13}C=^{13}C stretch, for the shift of 61 cm^{-1} (1656 − 1595 = 61 cm^{-1}) is exactly what is expected for a C=C stretch mode (See the following discussion based on a DFT calculation). Then, it is straightforward to assign the strong 1542 cm^{-1} band in Figure 1B to the ^{13}C-substituted "Raman spectroscopic signature of life." Note that ^{13}C substitution does not change much the normal modes and the electronic structure of molecules so that Raman spectral patterns are generally well preserved. The shift of 60 cm^{-1} (1602 − 1542 = 60 cm^{-1}) suggests that the 1602 cm^{-1} band also originates from a C=C double bond.

The effect of ^{2}H substitution is much less substantial than that of ^{13}C substitution. The *cis*-C=C stretch band shifts down by 5 cm^{-1} (1656 to 1651 cm^{-1}) and the 1602 cm^{-1} band shifts down only by 3 to 1599 cm^{-1}. These small ^{2}H shifts are not due to incomplete ^{2}H substitution in the experiment, as already proven above. The 10 cm^{-1} upshift of the 1440 cm^{-1} band to 1450 cm^{-1} indicates that most of the CH bend bands shift down to ca. 1100 cm^{-1} region, and the remaining CH-bend bands, which are mostly protein CH bend bands, have higher frequencies around 1450 cm^{-1}. Note that proteins are mostly synthesized from amino acids and that the hydrogen atoms in proteins may not be substituted under ^{2}H D-glucose culturing.

The very small ^{2}H shifts in the 1656 and 1602 cm^{-1} bands are intriguing. Here we focus on the 1602 cm^{-1} band, whose assignment is the primary interest in this letter; the 1656 cm^{-1} band will be discussed in a separate paper. We know empirically that ^{2}H shifts of the C=C stretch modes of olefins are much larger than 3 cm^{-1}.[6] This trend is confirmed by a DFT calculation. Table 1 shows the ^{13}C and ^{2}H shifts for the C=C stretch modes of *cis*- and *trans*-2-butene and 2-methyl-2-butene calculated at the B3LYP/6-31+G(2df,p) level. The calculated ^{13}C shifts are all very close to 60 cm^{-1} as expected from the ^{13}C/^{12}C mass ratio. They accord very well with the observed shift of the 1602 cm^{-1} band as well. The ^{2}H shifts are 26.2 and 32.8 cm^{-1} for *cis*- and *trans*-2-butene and 13.0 cm^{-1} for 2-methyl-2-butene. The smallest shift, 13.0 cm^{-1} for 2-methyl-2-butene, is still much larger than 3 cm^{-1}. We, therefore, argue that the 1602 cm^{-1} band originates from a C=C bond with no hydrogen atoms attached to the carbon atoms.

The C=C double bond in a quinoid ring is an obvious candidate for such a C=C bond without any hydrogen attached to it. Here we consider the ubisemiquinone radical anion ($CoQ^{-\bullet}$, Figure 2) as possible origin of the 1602 cm^{-1} band. According to a pulse radiolysis experiment,[7] the transient Raman spectrum of the 2,5-dichloro-*p*-benzosemiquinone radical anion has a strong and sharp band at 1602 cm^{-1}, which is assigned to the in-phase C=C stretch mode of the quinoid ring. The spectrum shows a number of extra Raman bands in the wavenumber region lower than 1600 cm^{-1}. However, the intensities of these bands are much lower than that of the 1602 cm^{-1} band, and, therefore, they may well be invisible in the Raman spectra of mitochondria. Resonance Raman spectra of electrolyzed CoQ10 also show strong isolated bands around 1600 cm^{-1}, which are assigned to the quinoid ring C=C stretch modes.[8] We have yet to observe the ^{13}C and ^{2}H shift of the 1602 cm^{-1} band of this radical but all the vibrational data presented here are consistent with the assignment of the "Raman spectroscopic signature of life" to $CoQ^{-\bullet}$.

Table 1. The ^{13}C and ^{2}H isotope shifts of the C=C stretch frequency of *cis*-2-butene, *trans*-2-butene, and 2-methyl-2-butene calculated at the B3LYP/6-31+G(2df,p) level

	^{13}C shift	^{2}H shift
cis-2-butene	−60.0	−26.2
trans-2-butene	−60.0	−32.8
2-methyl-2-butene	−60.7	−13.0

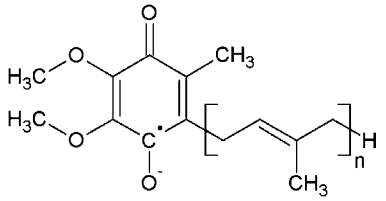

Figure 2. Molecular structure of the ubisemiquinone radical anion ($CoQ^{-\bullet}$). The number of *n* takes values from 6 to 10. Fission yeast cell has $n = 10$ and budding yeast cell has $n = 6$.

Ubiquinone (CoQ) plays an essential role as an electron carrier between Complex I, II, and III in the electron-transport chain in mitochondria. In the electron-transport process, the $CoQ^{-\bullet}$ and/or its protonated form ($CoQH^{\bullet}$) appear as intermediates between CoQ and the reduced form ubiquinol ($CoQH_2$). The higher the metabolic activity in mitochondria is, the higher the concentrations of these intermediates. Thus, if the 1602 cm^{-1} band originates from $CoQ^{-\bullet}$ and/or $CoQH^{\bullet}$, its sensitivity to the cellular metabolic activity is very well accounted for.

In vivo Raman measurements of isotope-substituted yeast cells have provided us with crucial information on the identity of the so far unassigned band at 1602 cm^{-1}, the "Raman spectroscopic signature of life." With the help of a normal mode analysis, we have come to a thought that $CoQ^{-\bullet}$ and/or $CoQH^{\bullet}$ is the origin of this intriguing Raman signature. Further studies are now in progress to establish this assignment by the isotope substitution of $CoQ^{-\bullet}$ and $CoQH^{\bullet}$.

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