Photobleaching of the "Raman Spectroscopic Signature of Life" and Mitochondrial Activity in Rho− Budding Yeast Cells

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Raman spectra of mitochondria in single living budding yeast (zygote of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*) cells have been measured and compared for two types of tetraploid strains, the wild-type strain and the rho− strain. The "Raman spectroscopic signature of life", a strong Raman band at 1602 cm^{-1} that sharply reflects the mitochondrial activity, has been found in both of the two strains with similar intensities. This signature showed photobleaching under irradiation of the 632.8 nm line of a He−Ne laser, only for the rho− strain. The photobleaching recovered by keeping the cell in the dark for two hours without laser irradiation. These findings indicate that the molecular species that gives rise to the 1602 cm^{-1} band is an intermediate formed and accumulated during the metabolic cycle and that it absorbs at 632.8 nm to undergo photodecomposition. We also found a number of weak Raman bands in the 400−1200 cm^{-1} region that show the same temporal behaviors as that of the 1602 cm^{-1} band by an SVD analysis of time-resolved Raman spectra. Several new pieces of information on the origin of the "Raman spectroscopic signature of life" have thus been obtained.

Introduction

The "Raman spectroscopic signature of life" is a strong and sharp Raman band at 1602 cm^{-1} that has been observed from the mitochondria in living yeast cells.[1−5] It has recently been found in two other living cells, tobacco BY-2 cell[6] and rat liver cell.[7] We have already shown that the intensity of this band sharply reflects the metabolic activity of living cells. Addition of KCN to a living fission yeast cell as a respiration inhibitor induces a rapid decrease and eventual disappearance of this band.[2,3] During the spontaneous death process of a starving budding yeast cell, the 1602 cm^{-1} band disappears much earlier than the collapse of cell organelle structures. It has been argued that the 1602 cm^{-1} band can be an excellent molecular indicator for monitoring the cell bioactivity.[4] In order to make clearer the relationship between the "Raman spectroscopic signature of life" and the metabolic activity in mitochondria, we have carried out several time- and space-resolved Raman experiments under various nutrient, stress and atmospheric conditions.[5] We have found that the 1602 cm^{-1} band is much weaker for fission yeast cells in water than for those in culture and that its intensity increases markedly by the addition of YE medium as nutrients. It is strong under good nutritious conditions but is weak if nutrients are deficient. It is depleted rapidly if an oxidative stress inducer, H_2O_2, is added to the solution. It is totally absent in fission yeast cells cultured under an anaerobic condition in N_2 atmosphere. All these observations show that the 1602 cm^{-1} band is strongly correlated with the aerobic respiration and hence with the metabolic activity in mitochondria. We have also cultured fission yeast cells under an $^{18}O_2$ atmosphere to see whether the "Raman spectroscopic signature of life" shows an isotope shift. If the signature originates from an unknown oxygen compound existing in the process of respiration and if the oxygen atom is taken in from the environment, it should show a shift with the replacement of $^{16}O_2$ with $^{18}O_2$. We have found no obvious shift of the 1602 cm^{-1} band.

In the present study, we examine and compare the Raman spectra of two different types of tetraploid strains of budding yeast (zygote of *Saccharomyces cerevisiae, S. cerevisiae*, and *Saccharomyces bayanus, S. bayanus*), the wild-type strain and the rho− strain, in order to obtain more insight into the relationship between the "Raman spectroscopic signature of life" and mitochondrial activity. The rho− strain lacks in mitochondrial DNA and does not have aerobic respiration activity in mitochondria. Therefore, different behaviors of the signature are expected for these two different strains having different respiration efficiencies. We have found that both of the strains show the 1602 cm^{-1} band in mitochondria with similar intensities but that only the rho− strain shows photobleaching of the band under irradiation of the 632.8 nm line of a He−Ne laser. We have also found that the photobleaching recovers if the laser irradiation is stopped. This photobleaching process has been analyzed with the singular value decomposition (SVD) method to extract the photobleaching component of the Raman spectra, which behaves in the same way as the "Raman spectroscopic signature of life" does.

Experimental Methods

The Confocal Raman Microspectrometer. We used a confocal Raman microspectrometer for measurements of the Raman spectra of living budding yeast cells under a microscope. The details of the apparatus were described in our previous papers.[1−5] Raman scattering was excited by the 632.8 nm line of a He−Ne laser with the laser power of 5 mW at the sample point. The laser beam was focused by a 100× oil immersion

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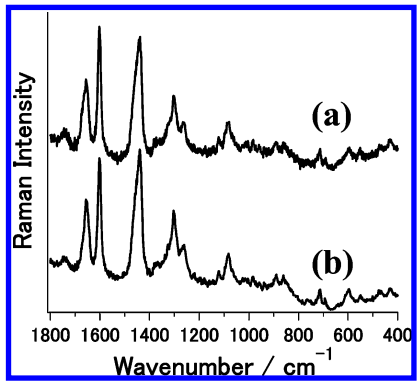

Figure 1. Space-resolved Raman spectra of mitochondria in wild-type strain (a) and rho− strain (b).

objective lens (UPlanFl) on the sample stage of a microscope (Olympus IX 50). The laser spot was about 1 μm in diameter. The backward Raman scattering was collected by the same objective and detected by a cooled (−65 °C) charge-coupled device (CCD, Andor DU420-BV). A 100 μm pinhole was used for confocal detection. Space resolution was ∼0.3 μm in the lateral direction and ∼2 μm in the axial direction. All Raman spectra were obtained with integration time of 100 s.

Cell Culture. The wild-type budding yeast strain tetraploid zygote used in this study was AJL3062 provided by Alfred Jorgensen Laboratory. The rho− strain, which lacks in mitochondrial DNA, was made by a treatment with ethidium bromide following the literature.[8] The absence of mitochondrial respiration activity was checked by the TTC (2,3,5-triphenyltetrazolium chloride) reduction test.[9] Both the wild-type and the rho− strains of tetraploid zygotes were gifts from Suntory Co., Ltd. Cells were grown overnight at 30 °C in YPD medium (2% w/v glucose, 2% w/v peptone, 1% w/v yeast extract). The growing cells were placed between a slide glass and a cover glass and sealed with Vaseline for the Raman measurements. The measurements were carried out at room temperature (∼20 °C).

Results and Discussion

Yeast cells from the tetraploid strain are generally bigger than those from the diploid. Experiences in the field of beer brewing tell that the tetraploid ferments more actively than the diploid. We compare in Figure 1 the Raman spectra of mitochondria from the two kinds of living budding yeast cells, the tetraploid strain of the wild-type (Figure 1a) and the rho− (Figure 1b). Contrary to our expectation, the rho− strain shows a strong "Raman spectroscopic signature of life", as strong as that of the wild-type strain does. According to the TTC reduction test, the rho− strain showed no aerobic respiration activity in mitochondria. If respiration activity is completely lost in mitochondria, the "Raman spectroscopic signature of life" should disappear. We consider that the 1602 cm^{-1} band observed for the rho− strain represents a weak respiration activity that the biochemical TTC test fails to detect (see the discussion below).

During the measurement of a cell from the rho− strain, we have found an interesting effect of laser irradiation. Figure 2a shows the time-resolved Raman spectra of mitochondria in a cell from the rho− strain. The spectrum for 0−100 s shows a strong band at 1602 cm^{-1}, but the spectra at the later times show rapid decrease of the intensity of this band. The band totally disappears at 400−500 s. On the other hand, such spectral changes do not take place for a cell from the wild-type strain (Figure 2b); the intensity of the 1602 cm^{-1} band relative to 1440

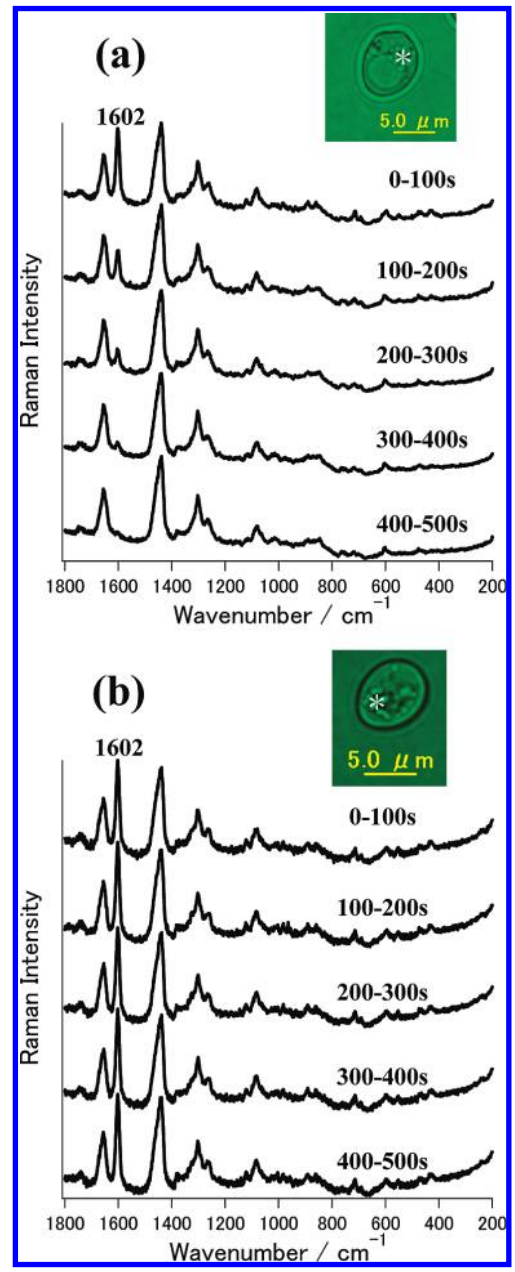

Figure 2. Time-resolved Raman spectra of mitochondira in rho− strain (a) and wild-type strain (b). Inset image is optical microscope image with the position of the laser spot marked by an asterisk.

cm^{-1} stays constant for the time period from 0−100 s through 400−500 s. It is then of great interest to see what happens to the once decreased 1602 cm^{-1} band if laser irradiation is stopped. For this sake, we have measured the time-resolved Raman spectra of another budding yeast cell from the rho− strain. We first confirmed the decrease of the 1602 cm^{-1} band and then observed the changes after stopping laser irradiation keeping the cell in the dark. The results are shown in Figure 3. After 500 s laser irradiation, the relative intensity of the 1602 cm^{-1} band to 1440 cm^{-1} decreases to ∼15% of the original value at 0−100 s (Figure 4, ▲). Two hours after the stopping of laser irradiation, the intensity of the 1602 cm^{-1} band is restored to about 60% (Figure 4, ■). This recovery of the 1602 cm^{-1} band intensity indicates that, without laser irradiation, the number of the molecular species that gives rise to the 1602 cm^{-1} band increases during the period of two hours in the dark. We have also checked that the decrease of the 1602 cm^{-1} band intensity occurs even for the wild-type strain, if the laser power

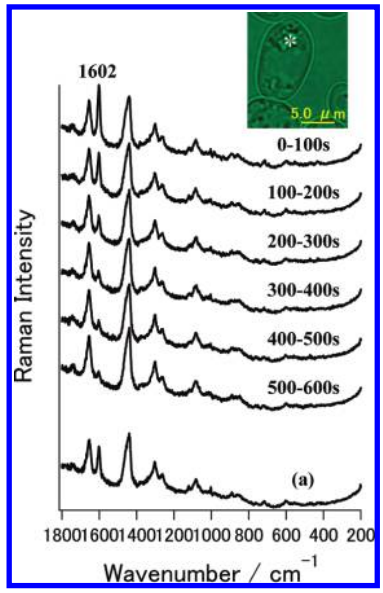

Figure 3. Time-resolved Raman spectra of mitochondria in rho− strain. Spectrum (a) was recorded two hours after the laser line shut. Inset image is optical microscope image with the position of the laser spot marked by an asterisk.

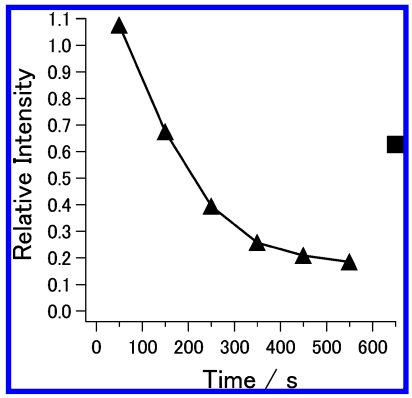

Figure 4. Time variations of the intensity of the 1602 cm^{-1} band in Figure 2 normalized by the 1440 cm^{-1} band. Triangles (▲) show the time variation of 0−600 s, and the square (■) shows the relative intensity of the 1602 cm^{-1} band recorded after a two hour rest.

is increased (data not shown). From these observations, we conclude that the "Raman spectroscopic signature of life" can be photobleached and that the photobleaching rate is much larger in the rho− than in the wild-type.

The photobleaching and the recovery in the dark of the "Raman spectroscopic signature of life" point to the following two considerations on its origin. First, the molecular species giving rise to this signature absorbs the 632.8 nm light. This species must have an electronic absorption band around 632.8 nm, and therefore the intensity of the 1602 cm^{-1} band must be enhanced by the resonance Raman effect. This possibility has already been suggested in our earlier papers.[3] It should be noted that, on account of the resonance Raman effect, a molecular species with very low concentration can give rise to a strong Raman band comparable to those of phospholipids, which are highly abundant in mitochondria. Second, the once disappeared intensity of the 1602 cm^{-1} band recovers if the laser irradiation is stopped. This fact means that the yeast cell continuously produces and accumulates the molecular species responsible for the 1602 cm^{-1} band. Considering the fact that only living yeast cells show the "Raman spectroscopic signature of life", the molecular species is produced if and only if yeasts cells are living. We consider that this molecular species is a reaction intermediate that is produced in the metabolic cycle in mitochondria. With this hypothesis, we can explain the observed different behaviors of the "Raman spectroscopic signature of life" between the wild-type strain and the rho− strain. The depletion of the 1602 cm^{-1} band occurs if the photodecomposition rate is comparable to the production rate of the intermediate. In the case of the wild-type strain, which shows strong respiration activity, the production rate of the intermediate is much higher than the photobleaching rate and therefore the intensity of the 1602 cm^{-1} band does not change appreciably under laser irradiation. If the laser power is increased so that the photodecomposition rate is comparable with the intermediate production rate, even cells from the wild-type strain show photobleaching. On the other hand, in the case of the rho− strain, the metabolic activity is very weak. The photodecomposition rate is larger than the production rate, leading to the rapid photobleaching of the "Raman spectroscopic signature of life". The presence of the 1602 cm^{-1} band indicates that the respiration activity is not completely lost in the rho− strain. The photobleaching rate of the 1602 cm^{-1} band is thought to be more significant than the intensity itself, for the sake of monitoring the respiration/metabolic activity of yeast cells.

We have already noted that the 632.8 nm laser irradiation during Raman measurements tends to slow down the cell cycle of fission yeast (*Schizosaccharomyces pombe*) significantly.[3] We discussed this finding in terms of possible temperature rise in the cell due to laser irradiation. We now point out that this slowing down of the cell cycle might have been caused by the photobleaching of the reaction intermediate, which gives rise to the "Raman spectroscopic signature of life", of the metabolic cycle in mitochondria. If this is the case, we have a means to photoregulate the mitochondrial metabolic activity. It is reported in the literature that low-level irradiation with red light causes a variety of biomedical effects including wound healing and tissue regeneration (low level light therapy, LLLT) and that the primary photoreceptor in these effects is likely to be a mitochondrion.[10] The light levels used in LLLT are much lower (typically 10 mW cm^{-2}) than that used in the present experiments (10^8 mW cm^{-2}), and therefore photobleaching of the intermediate is not likely the primary cause of the LLLT effects.

In the time-resolved Raman spectra of the rho− strain (Figure 2a), we see small but meaningful spectral changes in the low wavenumber region, in addition to the depletion of the 1602 cm^{-1} band. In order to separate out these spectral changes in the low wavenumber region, we use a singular value decomposition (SVD) analysis,[11] which has given two spectral components with different temporal behaviors. One is the constant spectral component (Figure 5a) that remains constant throughout the observation time (Figure 6, ■). The other is the depleting component (Figure 5b) whose amplitude decreases rapidly with time (Figure 6, ▲). The depleting component shows a number of weak Raman bands in the wavenumber range 400−1200 cm^{-1}, in addition to the strong band at 1602 cm^{-1}. The intensities of these weak bands decrease at the same rate as that of the 1602 cm^{-1} band. Two possibilities are conceivable. First, these weak bands originate from the same intermediate species as the 1602 cm^{-1} band. In this case, the intermediate must be a large molecule with many degrees of freedom of molecular vibrations. Second, these bands are from other molecular species that exist downstream of the metabolic cycle and are produced from the 1602 cm^{-1} species. We have tried to identify these small peaks referring to the known vibrational frequencies of mitochondria located biomolecules but in vain.

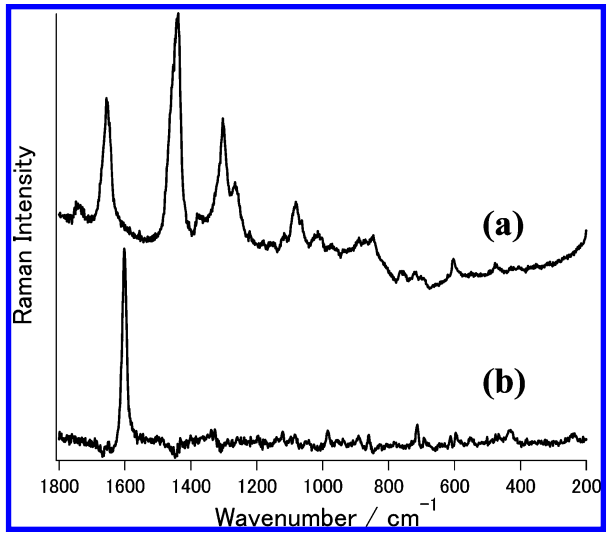

Figure 5. Spectra components obtained by an SVD analysis: (a) constant component spectrum; (b) depleting component spectrum.

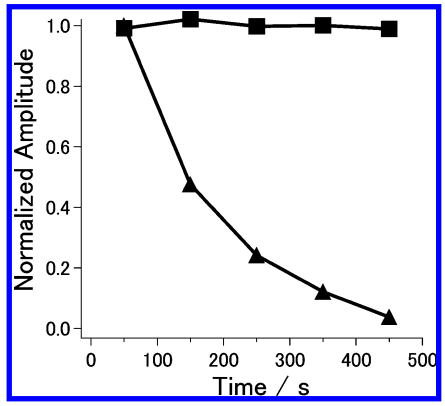

Figure 6. Time variation of constant component (■) and depleting component (▲).

In any case, we have obtained new vibrational spectroscopic information which is most probably relevant to the metabolic activity in mitochondria.

Conclusion

We have compared the Raman spectra of two different types of tetraploid strains of budding yeast, the wild-type strain and the rho− strain, and obtained a few pieces of new information about the origin of the band at 1602 cm^{-1}, the "Raman spectroscopic signature of life". The rapid photobleaching of this signature under laser irradiation indicates that the species responsible for this band absorbs the 632.8 nm light and that the band intensity is enhanced by the resonance Raman effect. Observed recovery of the 1602 cm^{-1} band after stopping laser irradiation has led us to a working hypothesis that the molecular species giving rise to the 1602 cm^{-1} band is a reaction intermediate in the metabolic cycle in mitochondria. An SVD analysis of time-resolved Raman spectra of the rho− strain indicates a number of new bands in the low wavenumber region that show the same temporal behaviors as that of the 1602 cm^{-1} band.

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