論文の内容の要旨

論文題目

The cytochrome redox states and respiration activity of mitochondria studied by resonance Raman spectroscopy

(共鳴ラマン分光法を用いたシトクロム酸化還元状態とミトコンドリアの呼吸活性に関する研究)

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Introduction

Energy for maintaining life is produced by oxidative breakdown of food in mitochondria (cellular respiration). Mitochondrion possess an electron transport system in which electrons are successively transferred to molecules of higher redox potentials to generate a membrane potential that eventually facilitates the ATP production. Cytochromes of type *a*, *b* and *c* are key components of the electron transport system involved in respiration. The process of electron transfer leads to the formation of oxidized and reduced forms of cytochromes. Resonance Raman spectroscopy can detect these heme proteins in different redox states with high specificity. Raman spectroscopy generally has the advantage of being a non-destructive and non-staining technique that can be applied to living cells.

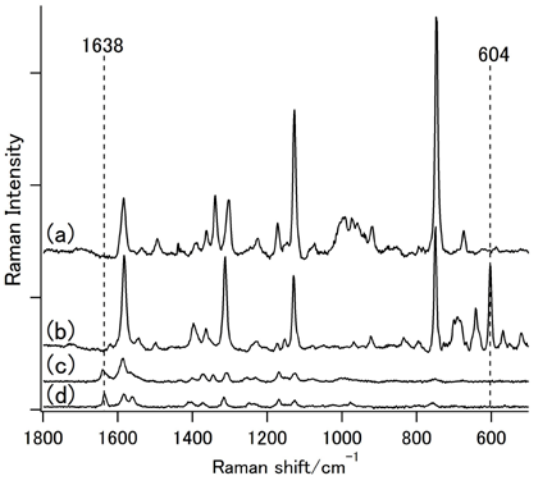

Figure 1 Standard resonance Raman spectra for (a): reduced cytochrome *b*, (b): reduced cytochrome *c*, (c): oxidized cytochrome *b* and (d): oxidized cytochrome *c* .

The present study demonstrates the possibility of simultaneous observation by resonance Raman spectroscopy of cytochrome *b* and *c* (cyt *b* and cyt *c*) from mitochondria in a single living yeast cell and also from isolated mitochondria *in vitro*. Figure 1 shows the Raman spectra of cyt *b* and cyt *c* in the reduced and oxidized forms in PBS (phosphate buffered saline (pH 7.2)) of the same concentration. These Raman spectra are obtained with the 532 nm excitation which selectively enhance the cytochrome band intensities. Two characteristic bands in Fig. 1 can be used for the identification and quantification of the cytochrome species. The 1638 cm^{-1} band is a marker of the oxidized

form of cyt *b* and cyt *c*, and the 604 cm^{-1} band is characteristic to the reduced form of cyt *c*. Using these marker bands, the redox states of cyt *b* and *c* have been quantitatively determined under different experimental conditions. In particular, the cytochrome redox states are shown to depend on the mitochondrial respiration activity. In addition, the spatial distribution of cyt *b* and *c* redox states in a living animal cell has been quantitatively visualized.

Mitochondrial respiration and the cytochrome redox states

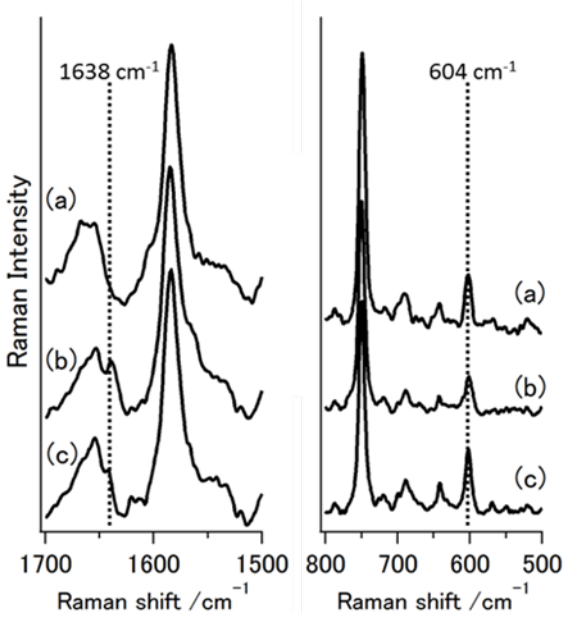

Figure 2 Raman spectra of (a): mitochondria in living yeast cells, (b): isolated mitochondria without ADP and succinate and (c): isolated mitochondria with ADP and succinate. Fluorescence backgrounds have been subtracted from the Raman spectra by polynomial fitting.

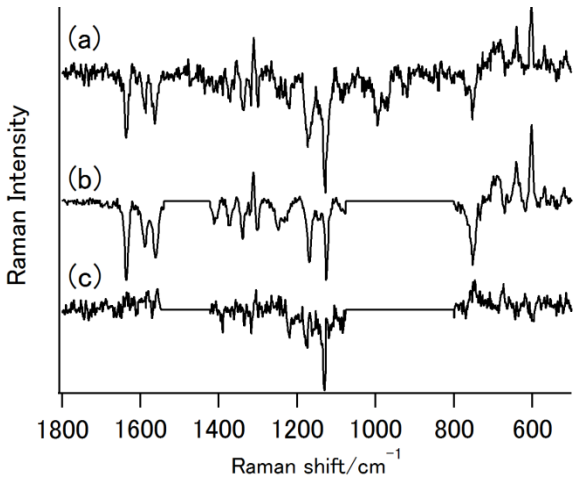

Figure 3 (a): Difference Raman spectrum (Fig. 2(c) - (b)) (b): best fit linear combination of the four authentic cytochrome Raman spectra in Fig. 1 and (c): the residual spectrum. Flat parts were not used for fitting.

The relationship between the cytochrome redox states and the mitochondrial respiration activity has been studied for living cells as well as for isolated mitochondria. Precultured budding yeast (*S. cerevisiae*) cells in the mid-log phase were used. Budding yeast cells were cultured in lactate medium for 13 hours and mitochondria were isolated by two different speed centrifugation and density gradient combination.

A representative Raman spectrum from mitochondria in living *S. cerevisiae* cells is shown in Fig. 2 in comparison with that of isolated mitochondria suspended in a non-nutrient buffer. The living cell spectrum (Fig. 2(a)) corresponds well with that of the isolated mitochondria spectrum (Fig. 2(b)). However, the oxidation marker band at 1638 cm^{-1} is observed only in isolated mitochondria and not in living cells. The oxidized form of cyt *b* and/or cyt *c* exists only in the isolated mitochondria. Respiration in isolated mitochondria can be induced by adding ADP and succinate to the medium. The isolated mitochondrial spectrum after addition of ADP and succinate is shown in Fig. 2(c). Small but significant changes are observed at 1638 cm^{-1} and 604 cm^{-1} on going from Fig. 2(b) to 2 (c). In order to clarify these small changes, the difference spectrum between the respiration inactive (Fig. 2 (b)) and active (Fig. 2(c)) mitochondria spectra is calculated (Fig. 3(a)). The difference spectrum shows more clearly that the intensity of the 1638 cm^{-1} marker band for oxidized cytochromes decreases while the 604 cm^{-1} marker band specific for reduced cyt *c* increases.

A more quantitative analysis of these changes is carried out by fitting this difference spectrum with a linear combination of the four Raman spectra of cyt *b* and *c* in the oxidized and reduced forms. The best least-squares fitted spectrum (Fig.3 (b)) is obtained with the following coefficients, +10 for cyt *b* oxidized, -8 for cyt *b* reduced, -71 for cyt *c* oxidized and +13 for cyt *c* reduced. These coefficients indicate that mitochondrial respiration activity induced by ADP and succinate promotes the oxidation of cyt *b* (+10 for the oxidized form and -8 for the reduced) and the reduction of cyt *c* (-71 for the oxidized form and +13 for the reduced).

Although these coefficients need further quantitative confirmation, the change of the cytochrome redox state is successfully correlated with the mitochondrial respiration activity.

Spatial distribution of cyt *b* and *c* redox states in a living animal cell

Spatial distribution of cyt *b* and *c* redox states has been studied in a living L929 (NCNC) cell. L929 cells were cultured in DMEM (Dulbeco's modified eagle medium) supplemented with 10% fetal bovine serum for 4 days on a glass bottom dish. Figure 4 (a) shows a representative Raman spectrum obtained from the mitochondria in living L929 cells. In order to extract the spectral component derived from cytochromes, I have performed the following spectral analysis. The Raman spectrum of bovine albumin (Fig.4 (b)) as a standard protein spectrum is subtracted from the raw spectrum in order to eliminate the spectral contribution from non-heme proteins. The Raman band at 1003 cm^{-1} due to the phenylalanine residue is used as an intensity standard. Figure 4 (c) shows the spectrum obtained after subtracting the albumin spectrum and the fluorescence background. The Raman band at 1638 cm^{-1}, which is a marker band of oxidized cytochromes, is clearly observed. This is the first detection of oxidized cytochromes in a living cell by Raman microspectroscopy.

The spectrum in Fig. 4 (c) is again fitted with a linear combination of the standard Raman spectra of cytochrome *b*/*c* reduced/oxidized forms. The best fit model spectrum (Fig. 4 (d)) is obtained with coefficients 14 for reduced cyt *b*, 39 for oxidized cyt *b*, 27 for reduced cyt *c* and 20 for oxidized cyt *c*. Since the concentrations of the four cytochrome standard solutions are the same, the determined coefficients correspond to the relative molecular abundance of the four cytochrome species. Fig. 4 (e) shows the residue spectrum (Fig. 4 (c) – Fig. 4 (d)). It shows no spectral features corresponding to cytochrome species and is likely to be due to lipids. The fitting analysis seems to be performed adequately.

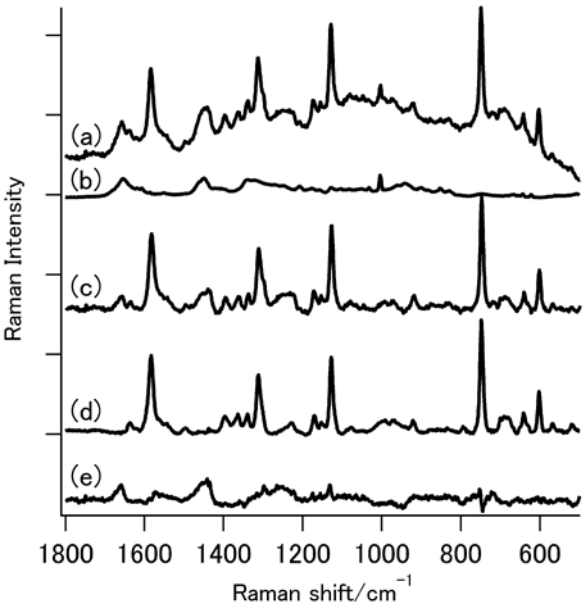

Figure 4 (a): typical Raman spectrum from a living cell, (b): Raman spectrum of albumin, (c): albumin and background subtracted spectrum, (d): model spectrum made by standard cytochrome spectra, (e): residual spectrum.

All the 100 x 100 points Raman spectra from the whole cell are analyzed in the same way as shown above. Figures 5 (a)~(d) show the obtained distributions of reduced cyt *b*, reduced cyt *c*, oxidized cyt *b* and oxidized cyt *c*, respectively. These four distributions are similar to one another, indicating that they grossly correspond to the distribution of mitochondria, which contain both cyt *b* and *c* abundantly. In the case of cyt *c*, the reduced form is more abundant than the oxidized form all over the cell. On the other hand, in the case of cyt *b*, the concentrations of both the oxidized and reduced forms are only slightly different from each other. As far as the author is aware, such

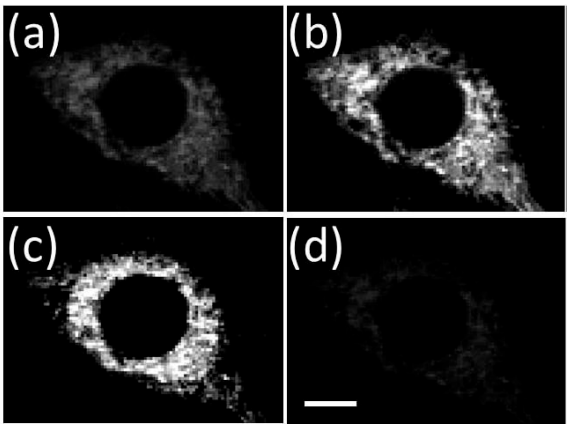

Figure 5 Raman images of (a): reduced cyt *b*, (b): reduced cyt *c*, (c): oxidized cyt *b* and (d): oxidized cyt *c*. The bar indicates 10 μm.

quantitative information about the redox states of cytochromes in a living cell has been obtained for the first time.

Finally, the cytochrome redox state changes have been observed after 2 hours under a non-nourishment condition. Spectral changes similar to Fig. 3 (c) are observed. Although further experiments under various culture conditions are required to dissect out the spectral changes due solely to respiration, the present resonance Raman approach has an unparalleled potential for accessing the mitochondrial respiration activity *in vivo* and label free.

Conclusion

In summary, this thesis demonstrates that resonance Raman spectroscopy can assess the mitochondrial respiration activity by quantifying the redox states of cytochrome *b* and *c* simultaneously. This new method is applicable *in vivo*, *in vitro* and *in situ* without using any labeling or genetic manipulation and is therefore promising for many biological applications.