

Surface Infrared Spectroscopic Study of ATP Synthesis in Mitochondria

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1. Introduction

Mitochondria play key roles in cell metabolism [1] and programmed cell death [2]. The essential function of mitochondria is the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate (Pi) through the oxidative phosphorylation (Fig. 1a). Recently, evaluation of drug-induced mitochondrial toxicity has become increasingly important, since it is revealed that mitochondrial dysfunction is implicated in numerous disease states as well as drug-induced toxicities and aging [3]. Various analytical methods have been employed to investigate ATP synthesis in mitochondria, including liquid chromatography [4,5] and bio-luminescence techniques [5,6]. However most of these methods require destruction of samples. Therefore, there is a great demand for non-destructive monitoring for ATP synthesis in mitochondria and its application to drug screening for mitochondrial toxicants.

In the present study, we have monitored ATP synthesis in isolated mitochondria by using IRAS in the multiple internal reflection (MIR) geometry. We have constructed a system for real-time monitoring of oxidative phosphorylation in mitochondria. ADP \leftrightarrow ATP conversion processes are monitored with MIR-IRAS, while dissolved oxygen level and solution pH were simultaneously monitored with O₂ and pH electrodes, respectively. It is demonstrated that ATP synthesis and hydrolysis in mitochondria can be monitored by the IR spectral changes of phosphate groups in adenine nucleotides and MIR-IRAS is useful for evaluating time-dependent drug effects of mitochondrial toxicants.

2. Experimental Section

Mitochondria were isolated from the liver of adult male Wistar (6-13 weeks old) or Wistar-ST (7 weeks old) rats as described previously [4]. Mitochondria were obtained as a suspension in 2 mM Tris-HCl buffer (pH 7.4). For the ATP synthesis, mitochondria were suspended in a solution con-

taining 200 mM sucrose, 20 mM KCl, 3 mM MgCl₂, 100 mM potassium phosphate, 0.68 μ g/ml rotenone and 100 mM succinate (pH7.4) (denoted as Pi-buffer).

A Si prism with an optical path length of 15 mm was fabricated and used as an MIR prism [7]. FZ Si (100) (5250-7050 Ω cm, double side polished, 450 μ m in thickness) wafer was anisotropically etched in tetramethylammonium hydroxide at 90 $^{\circ}$ C. Before each experiment, the prism was cleaned in 1:1 H₂O₂:H₂SO₄ solution for 5 min. During this process, the surface of Si prism was covered with a chemical oxide layer. Fig.1b shows a schematic of the MIR-IRAS measurement system. A Si prism was set on the side of the solution cell. Infrared light beam from an interferometer (Nicolet) was focused at normal incidence onto one of the two anisotropic etch pits of the Si prism, and penetrated through the Si prism. The light that exited the Si prism through the other etch pit was focused onto a liquid-nitrogen cooled mercury-cadmium-telluride (MCT) detector. Clark-type oxygen electrode (Yellow springs) was fixed at one side of the solution cell for the monitoring of dissolved oxygen in the mitochondrial suspension during MIR-IRAS measurement. To prevent oxygen depletion, oxygen gas was supplied to the chamber through silicone tubes. Time-dependent pH changes were also monitored with a pH electrode (Horiba).

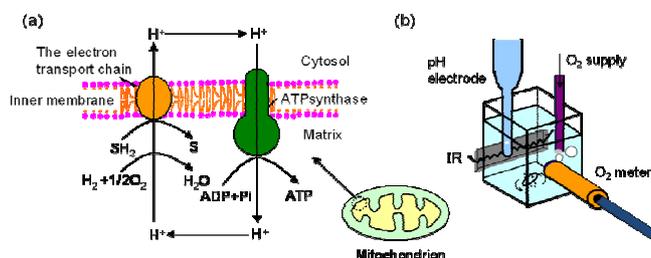


Fig. 1. (a) Mechanism of ATP synthesis by oxidative phosphorylation in a mitochondrion. (b) Schematic of the solution cells. A Si prism for MIR-IRAS measurements and an oxygen electrode are attached to the sides of the solution cell.

3. Results & Discussion

Fig. 2a shows IR absorption spectra of ADP added to a mitochondrial suspension. Reference was the spectrum collected just before the addition of ADP. At first, the IR spectrum was almost same as the IR spectrum of ADP obtained in the Pi-buffer without mitochondria. A broad peak around 1200 cm^{-1} was attributed to the antisymmetric stretching vibration of $\alpha\text{-PO}_2^-$ [8]. Then, the shape of IR spectrum gradually changed with time. After ~ 35 min, the spectrum became similar to that of ATP, whose peak around 1230 cm^{-1} are attributed to the antisymmetric stretching vibrations of $\alpha\text{-}$ and $\beta\text{-PO}_2^-$ [8]. The difference spectrum between the lines 2.6 min and 35.5 min [Fig. 2b, line (i)] showed a significant peak around 1240 cm^{-1} . Such spectral changes were not observed without mitochondria [line (ii)]. When the same volume of the Pi-buffer was added to the mitochondrial suspension, no significant changes in IR spectra were observed around 1240 cm^{-1} [line (iii)]. These results indicate that the ADP \rightarrow ATP conversion was successfully monitored with MIR-IRAS.

Then we stopped oxygen supply and added an uncoupler SF6847 to the mitochondrial suspension. Uncouplers inhibit ATP synthesis in mitochondria by dissipating the H^+ -electrochemical gradient across the inner membrane, which is a driving force for ATP synthesis [9]. Addition of an uncoupler to mitochondria is known to cause hydrolysis of ATP to maintain a proton-electrochemical gradient. After the addition of the uncoupler SF6847, the spectrum showed similar profiles to those of the spectrum measured just after the addition of ADP. The spectral changes before and after addition of SF6847 exhibited a significant dip around 1244 cm^{-1} , where significant peak was observed during ATP synthesis. The average decrease in the peak intensity at 1244 cm^{-1} was $52\pm 5\%$ ($n=8$, the mean \pm standard error of the mean). The addition of SF6847 to the mitochondria in the absence of ADP induced no significant changes in IR spectra around 1244 cm^{-1} . When the same volume of ethanol, which was the solvent of SF6847, was added to the mitochondria in the presence of ADP, slight reduction ($17\pm 4\%$, $n=3$) in the peak intensity at 1244 cm^{-1} was observed. These results indicate that the ATP hydrolysis induced by an uncoupler was successfully monitored with MIR-IRAS. The possible reason for the peak reduction induced by the addition of ethanol was due to the background proton leakage in the inner membrane of mitochondria. Thus it is demonstrated that the reversible process of ATP \leftrightarrow ADP conversion can be *in situ* monitored with MIR-IRAS by analyzing the stretching modes of $\alpha\text{-}$ and $\beta\text{-PO}_2^-$ in adenine nucleotides.

3. Conclusions

We have developed a real-time method for monitoring ATP synthesis and hydrolysis in mitochondria by using MIR-IRAS. It was demonstrated that the ATP \leftrightarrow ADP conversion process in mitochondria can be monitored *in situ* by analyzing stretching modes of $\alpha\text{-}$ and $\beta\text{-PO}_2^-$ in adenine nucleotides. Our method has potential to evaluate mito-

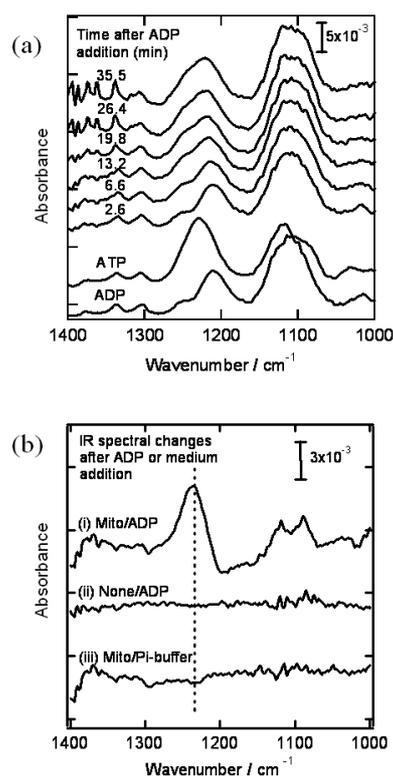


Fig. 2. (a) IR absorption spectral changes after addition of ADP to mitochondrial suspension. Spectra of ATP and ADP dissolved in the Pi-buffer are also shown. (b) Difference spectra of mitochondrial suspension before and after addition of (i) (ii) ADP and (iii) Pi-buffer. (i), (iii) In the presence of mitochondria, and (ii) in the absence of mitochondria.

chondrial toxicity in terms of mitochondrial activity of ATP synthesis and hydrolysis.

Acknowledgements

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