論　　文

Mitochondria Toxicity of Silver Ion as the Molecular Mechanism for the Antifungal Activity

Kasumi Tsunoda[a], Satoshi Kozuka[b], Kiyoshi Kawai[b]

[a] Department of Food and Nutrition, Toyama College, Gankaiji, Mizuguchi 930-0193, Toyama, Japan

[b] Faculty of Wellness, Shigakkan University, Obu, 474-8651, Aichi, Japan

【Abstract】

The molecular mechanism for the antifungal activity of silver nitrate (Ag^+) against pathogenic fungi was investigated. Ag^+ inhibited oxygen uptake of *Saccharomyces cerevisiae* cells after a minute lag time, suggesting a toxic interaction of Ag^+ with fungal respiratory system. The effect of Ag^+ on mitochondrial respiratory system was then studied using fleshly isolated rat liver mitochondria, instead of fungal mitochondria. The rat liver mitochondrial respiratory system was disturbed by Ag^+, through an uncoupler-like effect (acceleration of state 4 respiration) at low concentration and respiratory chain inhibition(depression of state 3 and 4 respirations) at high concentration.

【Key words】

silver ion, antifungal activity, mitochondrial respiration, electron transport inhibition

1. Introduction

Silver nitrate is widely known to show the bacteriocidal effect and has been used as antiseptic agents for a variety of application such as dental work, catheters and burn wounds. Recently, silver nitrate and silver sulfazine cream have been used for the treatment cutaneous wounds (1). Silver nitrate have been demonstrated to be cytotoxic to human dermal fibroblasts (2). As mitochondria plays an essential role for the energy metabolism even in fungal cells, the dysfunction of mitochondria results in the ruin of fungal cells. Diversity sorts of antifungal agents, including azole compounds, have been shown to impair mitochondrial respiration, on which action mechanism have been obtained using isolated rat liver mitochondria (3). Heavy metal ions, including mercury,

induce drastic morphological change of mitochondria in consequence of the induction of ion-permeability transition in mitochondrial inner membrane mainly interacting with sulfhydryl residues of membrane protein(4). To know the bioactive mechanism of the antifungal activity of Ag^+, we investigated the injurious effect of silver nitrate on mitochondrial respiration in detail, using isolated rat liver mitochondria (RLM) and submitochondrial particles (SMP) which are reverted membrane vesicles of mitochondrial inner membrane to inside-out direction.

2. Materials and Methods

2.1 Reagents

ADP-2Na, NADH, bovine serum albumin (BSA, fraction Ⅴ), antimycin A were purchased from Sigma. Rotenone was a gift from Prof. King TE of New York State University. Silver nitrate (Sigma Ultra) was twice rechrystallized against hot ethanol and the water solution was kept in dark at below 4℃. Other reagents were of the purest grade commercially available. The pH of the mediums for preparing mitochondrial fraction and measuring mitochondrial respiration were adjacent by nitric acid instead of hydrogen chloride to prevent from the formation of insoluble silver chloride in the reaction system.

2.2 Preparations of *Saccharomyces cerevisiae* cells

Saccharomyces cerevisiae 40210 was obtained from IMF and was cultured for two days at 30℃ on the agar plate broth (1.5%) containing polypeptone (1%), yeast extract (0.5%) and glucose (3%). Cells collected were suspended in 0.15M KCl solution containing 20mM Tris-HCl (pH7.4) and was washed by twice centrifugations (1,500×g, for 5min) using the same solution.

2.3 Preparations of RLM and SMP

RLM were prepared by the usual method from liver homogenate of Wistar albino rats weighing 200-250g of body weight, using the ice-chilled isolation medium containing 0.25M sucrose, 10mM Tris-nitrate and 0.5mM EDTA (pH7.4)(5). SMP were prepared by the method as previously reported by Kawai et al (6). By the experiment using SMP, the direct interaction of Ag^+ with respiratory chain enzymes can be exclusively detected.

2.4 Measurement of mitochondrial respiration

Mitochondrial respiration was measured using Galbani-type oxygen electrode (Iijima Electronics MFG Co. Ltd, Japan) at 30℃. The reaction medium was composed of 0.25M sucrose, 5mM magnesium nitrate, 5mM inorganic phosphate, 10mM Tris-nitrate, 0.5mM EDTA in a final volume of 2.0ml (pH 7.4). The state 3 respiration (ADP-driven repiration) and state 4 respiration (a restricted respiration due to the lack of ADP), respiratory control (RC) ratio (a ratio of the state 3 respiration to the state 4 respiration) and ADP/O ratio (nmol ADP to natom oxygen consumed) were calculated from oxygraph data according to the method of Chance and Williams (7). Spectroscopic analysis of mitochondrial cytochromes was carried out using the recording spectrophotometer (Beckman DU-70). Protein was assayed by the method of Lowry et.al., using BSA as standard (8).

3. Results

3.1 Inhibitory effect of Ag^+ on oxygen uptake of *S. cerevisiae*

The effect of Ag^+ on the oxygen uptake of *S. cerevisiae* was examined using oxygen electrode (Fig.1). Ag^+ inhibited the cellular oxygen uptake by a dose dependent manner after a minute lag time, suggesting an interfering with mitochondrial function. On the base of this observation, the effect of Ag^+ on the respiratory system of isolated RLM was investigated to gain into the molecular mechanism for the toxic effect on fungal cells.

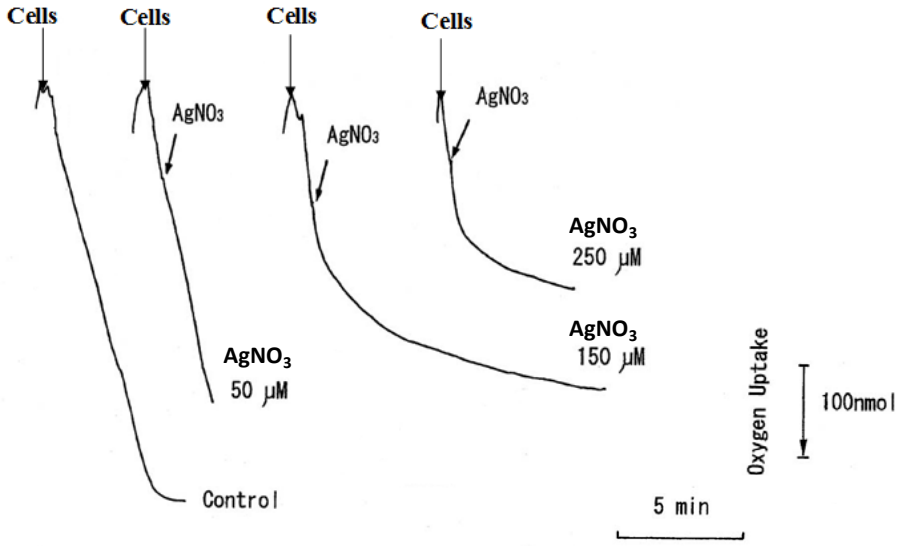

Fig.1 The effect of $AgNO_3$ on the oxygen uptake on *S.cerevisiae*
Oxygen uptake was measured by use of an oxygen electrode. The reaction medium was composed of 0.25M sucrose, 5mM magnesium nitrate, 5mM inorganic phosphate, 10mM Tris-nitrate, 0.5mM EDTA in a final volume of 2.0ml (pH 7.4). The reaction was performed at 30℃. Cells shows *S.cerevisiae*.

3.2 The impairing effect of Ag^+ on NAD- and FAD-linked respiration of isolated RLM

Oxygraph data of mitochondrial respiration with and without Ag^+ were displayed in Fig.2 and 3. Curve 1 in Fig.2 shows the control experiment of NAD-linked respiration oxidizing L-glutamate as the substrate without Ag^+. Fleshly isolated RLM showed a tightly coupled respiration showing high values of RC and ADP/O ratios. A low concentration of Ag^+ (3.4nmol) accelerated the state 4 respiration without ADP, which was thereafter gradually replaced by slow-speed respiration and state 3 respiration was no longer induced by ADP, At higher concentrations of Ag^+ (34 nmol), the respiration was immediately inhibited without showing initial acceleration of oxygen uptake (Curve2 in Fig.2) indicating the dual effects of Ag^+: the uncoupling effect at low concentrations and the repression of respiration at high concentrations. Same type of experiment was conducted for succinate-linked respiration (Fig.3). Curve 1 shows the control experiment of respiration oxidizing succinate as the substrate for the respiratory chain without Ag^+. The state 4 respiration was again accelerated by a low concentration of Ag^+ (6.8 nmol), which was again not accelerated by ADP (Curve 2), showing a complete uncoupling effect of the oxidative phosphorylation and repression of respiration. The accelerated respiration was inhibited by antimycin A, an electron transport inhibitor at

complex III of the respiratory chain (Curve 3). Higher concentrations of Ag^+ showed immediate inhibition without acceleration of the state 4 respiration. ADP did not induce the state 3 respiration (date not shown).

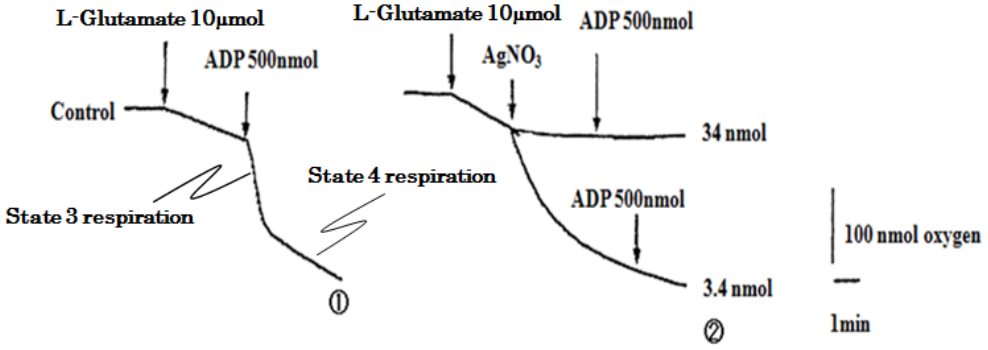

Fig.2 the effect of $AgNO_3$ on the NAD-linked respiration oxidizing L-glutamate.
Typical oxygraph data measured by use of an oxygen electrode. The reaction medium was composed of 0.25M sucrose, 5mM magnesium nitrate, 5mM inorganic phosphate, 10mM Tris-nitrate, 0.5mM EDTA, and ca 1mg mitochondrial protein in a final volume of 2.0ml (pH 7.4). The reaction was performed at 30℃ and was initiated by adding a substrate(L-glutamate).

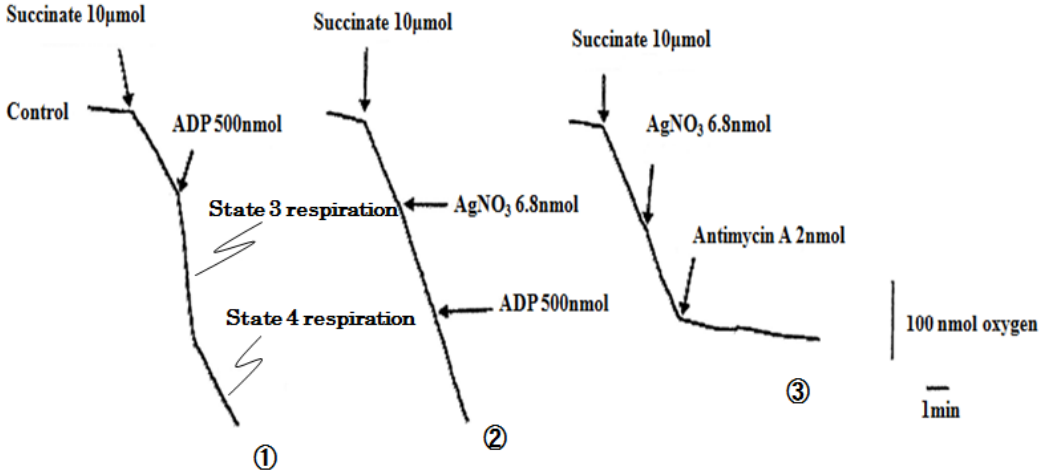

Fig.3 the effect of $AgNO_3$ on the FAD-linked respiration oxidizing succinate.
Typical oxygraph data measured by use of an oxygen electrode. The reaction medium was composed of 0.25M sucrose, 5mM magnesium nitrate, 5mM inorganic phosphate, 10mM Tris-nitrate, 0.5mM EDTA and ca 1mg mitochondrial protein in a final volume of 2.0ml (pH 7.4). The reaction was performed at 30℃ and was initiated by adding a substrate(succinate).

3.3 The effect of Ag^+ on NADH oxidases of SMP

The effect of Ag^+ on the respiratory chain enzymes (electron transfer system) was examined by using SMP to know the mechanism for the respiration-repressing effect (Fig.4). SMP are able to directly oxidize the NADH added to the reaction system because of their reverted membrane systems to the inside-out direction. The interaction of Ag^+ with respiratory chain enzymes can be, thereby, detected by using oxygen electrode. Curve 1 in Fig.4 shows the control experiment for NADH oxidase of SMP. Ag^+ completely inhibited the NADH oxidase at 34nmol (Curve 2 in Fig). The inhibition was eliminated by TMPD which generated an electron transport shunt over the inhibition site of rotenone in complex I and antimycin A in complex III(curve 3 in Fig), suggesting that the inhibition sites of Ag^+ are located at those of rotenone or/ and antimycin A. The succinate oxidase was not restored by TMPD, suggesting the interaction of Ag^+ with enzymes within complex II and/or complex III(date not shown).

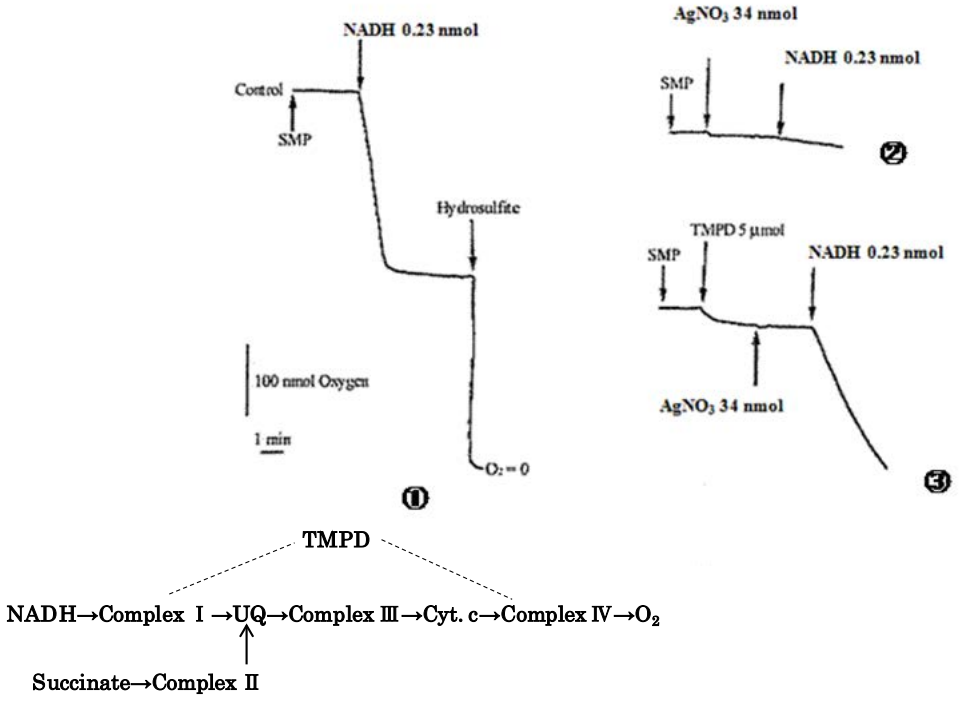

Fig.4 The effect of $AgNO_3$ on NADH oxidase in SMP
The reaction medium was the same as for Fig. 2 except for the absence of inorganic phosphate. At the bottom, the action site of TMPD(N,N,N',N', Tetramethyl-p-phenylenediamine on mitochondrial repiration.

3.4 Reduced-minus oxidized difference spectrum of NADH oxidase system

To know the further precise inhibition site of Ag^+, the reduced-minus oxidized difference spectra of SMP were measured in the presence and absence of Ag^+ (Fig.5). Curve1 shows the dithionite-reduced minus oxidized difference spectrum of SMP in the absence of Ag^+. Typical absorption peaks of cytochrome *c* with the absorption shoulder of cytochrome *b*, and cytochrome *aa*$_3$ were observed. Curve 3 shows the difference spectrum of SMP oxidizing succinate. None of cytochromes were reduced, indicating that the inhibition of electron transport at complex I and/or cytochrome *b*. Curve 2 shows the dithionite-reduced difference spectrum of SMP with Ag^+. The difference spectrum displayed two absorption peaks for cytochrome *c* and *aa*$_3$ except for the absorption shoulder of cytochrome *b*. This result indicates that cytochrome *b* was denatured by Ag^+ eliminating the ability of redox reaction of cytochrome *b*.

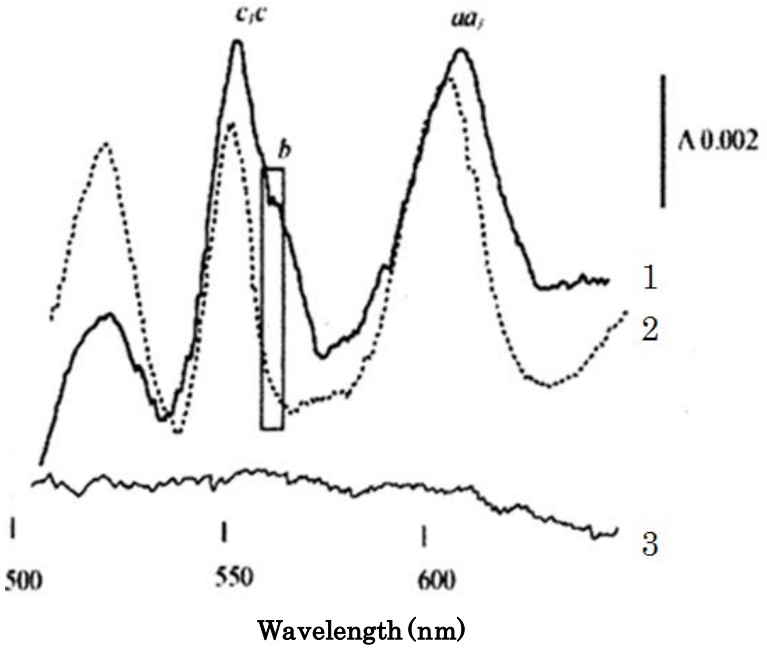

Fig.5 Reduced-minus oxidized difference spectrum measured using SMP in absence and presence of $AgNO_3$.
The reaction medium contained 0.15M KNO_3, 5mM $Mg(NO_3)_2$, 20mM Tris, 0.5 mM EDTA, 0.5%chloate and ca. 3mg protein of SMP in a final volume of 2.5ml(pH7.4).Curve1 shows the control spectrum in the absence of Ag^+. Curve 2 shows the difference spectrum in the presence of Ag^+. Curve 3 shows the succinate-reduced minus oxidized difference spectrum.
This figure shows a representative experiment. Results were obtained from 3 experiments.

4. Discussion

Heavy metal ions, such as Hg^{2+}, Cu^{2+} and Cd^{2+} have been known to impair the mitochondrial reaction due to the induction of permeability transition in the inner membranes (9). Ag^+ has been recently found to generate a cyclosporine A-insensitive permeability pore in the inner membranes (10), which might be involved in the uncoupling effect of Ag^+. To know the precise mechanism for the bioactivity of Ag^+, the effects of Ag^+ on the oxygen uptake of *S. cerevisiae*. Ag^+ inhibited the oxygen uptake of *S. cerevisiae* probably due to the inhibition of mitochondrial respiration in the present study. The effect of Ag^+ on mitochondrial respiration was therefore investigated using isolated RLM and SMP to clarify the molecular mechanism for the antifungal activity. Ag^+ exerted rotenone- and antimycin A-sensitive uncoupling effect of oxidative phosphorylation at low concentrations. The accelerated respiration was followed by the

depression of oxygen uptake, suggesting the interference of electron transport. The duration of accelerated respiration was shortened corresponding to the increase of Ag^+ concentrations. Ag^+ might uncouple the oxidative phosphorylation not by the H^+-conductivity of silver itself but by canceling the H^+ gradient across the inner membrane, resulting from the generation of H^+ permeable pore in the inner membranes. The experiments using SMP revealed that Ag^+ directly interacted with the respiratory chain enzymes. Our research reached nearly the same results of Terada et al (10). They emphasized the inhibition of Ag^+ to the phosphate carrier in the inner membranes. We newly found that Ag^+ exerted the dual impairing effect on mitochondrial respiration; the uncoupling effect at low concentration and electron transport inhibition at high concentration. The respiration accelerated by the uncoupling effect was gradually replaced by the depression in consequence of the interaction of Ag^+ to the respiratory chain enzymes. By the spectroscopic study measuring the reduced-minus oxidized difference spectrum, major inhibition site of Ag^+ was prospected to be located at the cytochrome bc_1 complex which possessed iron sulfur centers as the electron transfer components. Therefore, Ag^+ might interact with sulfhydryl-and/or disulfide containing proteins, resulting in the inhibition of electron transport from succinate or ubihydroquinone to cytochrome c_1. The finding that Ag^+ inhibited the respiration of *S. cerevisiae*, suggests that Ag^+ might permeate across cell walls and plasma membranes into cytoplasma. The mitochondrial permeability transition was involved in the collapse of membrane potential, resulting in a stop of ATP generation in mitochondria. This respiration-disturbing reaction might be occurred in fungal cells. It was concluded that Ag^+ exhibited the antifungal activity by strongly disturbing mitochondrial respiratory functions.

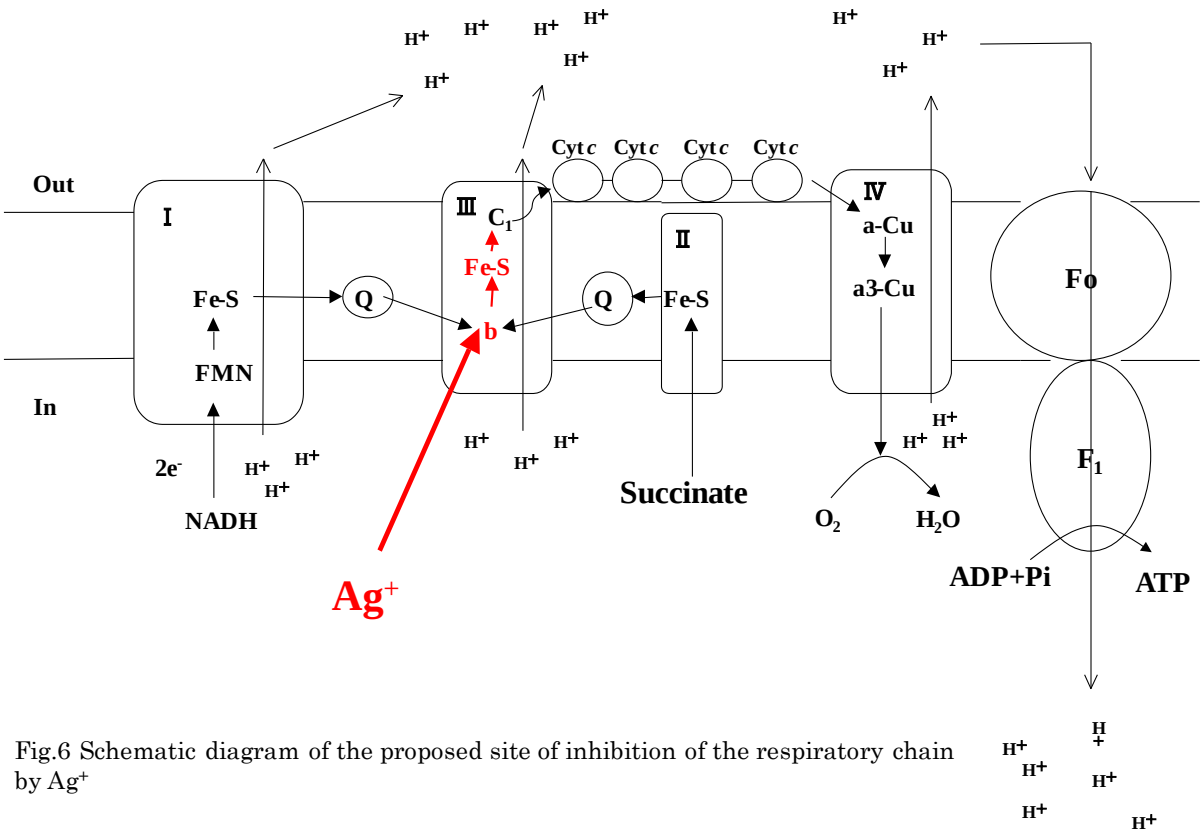

Fig.6 Schematic diagram of the proposed site of inhibition of the respiratory chain by Ag^+

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