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1．研究テーマ

線虫 Caenorhabditis elegans 短寿命変異株 mev-1 のコハク酸－ユビキノン還元酵素に関する生化学的解析

2．本年度の研究業績

(1) 学会・研究会等における発表 有 ・ 無 （学会名・演題）

無

(2) 学会誌等に発表した論文 有 ・ 無 （雑誌名・論文名）

無

3．今後の研究計画

As in *mev-1* complex II, SQR activity decreased and ROS production increased, I plan to concentrate to study the electron transferring system. I will try to know exactly at where electrons leak and produce ROS in the *mev-1* complex II. And, go on studying on how these produced ROS affect on complex II electron transferring system which results in *mev-1* ROS hypersensitivity and short life span.

As the mutation in CybL subunit in *mev-1* mutant, may cause perturbation in protein structure directly, I plan to confirm the existence and properties of heme in cytochrom *b* subunits. After that, I plan to purify cytochrome *b* and determine its structure and properties, especially its function in the way of electron transferring.

In addition, mutation in CybL subunit may not only cause cytochrome perturbation, but also in other two subunits Fp and Ip. I will go on the study on Fp and Ip subunits, and try to know the perturbation that may affect in electron transferring in function.

Then, next plan is try to elucidate the mechanism how the disorder in *mev-1* complex II leads to its short life span. The study will focus on ROS which cause oxidative stress in mitochondria. I will try to know exactly site of ROS production and how ROS affect metabolic system.

At last, in conclusion of my study, I expect to elucidate the mechanism of oxygen hypersensitivity and short life span in *mev-1* by biochemical methods. That will be very helpful to the study of the pathogenic mechanism of aging and age-related diseases.

4．指導責任者の意見

王さんはこれまで臨床医としての経験のもと，生化学や分子生物学的なアプローチによる研究ははじめての事がありました。
しかし非常な努力と元来の実力により，すでに私の研究室の大学院生の中心的存在となっております。
研究も寿命に関する線虫のミトコンドリア呼吸鎖の脱水素酵素の変異により活性酸素の発生が上昇し寿命が短縮している事を明らかにしました。
修士の評価も高く，大変に良い成績で今春より博士課程へ進学する予定です。
今後ともよろしく，ご支援のほど，お願い致します。

指導責任者氏名 土上 洋 ㊞

5．研 究 報 告 書

別紙報告書作成要領により、添付の用紙で研究報告書を作成して下さい。

研究発表中または研究中の本人のスナップ写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

※発表に当っては、*日中医学協会助成金*による旨を明記して下さい。

—日中医学協会助成事業—

線虫 *Caenorhabditis elegans* 短寿命変異株 *mev-1* の コハク酸—ユビキノン還元酵素に関する生化学的解析

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Abstract

To elucidate the mechanism of ageing, generation of reactive oxygen species (ROS) and the corresponding response to oxidative stress are considered as key factors in ageing.

The majority of intracellular ROS production is derived from mitochondria. Complex II is an important enzyme in inner membrane of mitochondria. *Caenorhabditis elegans (C. elegans)* is a good model animal for the study of ageing, because it possesses a system for oxidative metabolism which is quite similar to mammalian species. The *mev-1* (*kn1*) mutant is short life span mutant of *C. elegans* which is hypersensitive to oxygen. The mutation in *mev-1* encodes complex II CybL subunit and succinate-ubiquinone reductase (SQR) activity decreased.

Here, I focused on the study of the catalytic properties of *mev-1* complex II, and try to elucidate the mechanism of short life span in *mev-1* mutant.

E. coli strains and plates were compared to obtain a suitable culture condition of *C. elegans*. Several kinds of detergents were tested for the solubilzation of *C. elegans* complex II from mitochondria. By DEAE-Sepharose column, complex II with high SQR activity was partially purified and characterized biochemically. Kinetic parameters of *C. elegans* complex II were determined by ubiquinone-2 as electron acceptor. Then, ROS generation was measured using the chemiluminescent probe MCLA.

Complex II of N2 and *mev-1* show very similar properties except SQR activity. This conclusion comes from following data. First, N2 and *mev-1* complex II eluted at same salt concentration during ion-exchange chromatography. Second, both complexes have similar enzymatic activities of SDH. Although SQR activity in *mev-1* was lower than N2, their SDH activities were always comparable. The most important is their similar kinetic parameters of *Km* for ubiquinone which indicate their similarity in the spatial arrangement of the quinone binding site in cytochrome *b* subunits.

In ROS assay, the *mev-1* mutation results in high levels of ROS production at complex II. It suggests that mutation in CybL subunit could affect electron flow in the complex and electrons leaked from complex are transfered directly to molecular oxygen. From the result of enzymatic analysis, it seems there is no functional difference in quinone binding site between N2 and *mev-1*. That indicates perhaps quinone binding site is not the site where electrons transfer directly from complex II to molecular oxygen. Increased ROS generation in complex II ultimately results in *mev-1* short life span.

Key Words　*Caenorhabditis elegans, mev-1,* ageing, ROS, succinate-ubiquinone reductase

Introduction

The mechanism of ageing is likely to be a multifactorial process. Now, there is significant evidence implicating the generation of reactive oxygen species (ROS) and the corresponding response to oxidative stress as key factors in determining longevity (Ku, et al., 1993). ROS is metabolites of molecular oxygen. Mitochondria play a critical role in ageing because they are the major source and the most proximal target of ROS (Finkel, T. and Holbrook, N. J., 2000).

To investigate the molecular mechanisms of aging and etiology of oxidative damage, *Caenorhabditis elegans (C. elegans)* is a good model animal. The most important advantage is because *C. elegans* possess a system for oxidative metabolism which is quite similar to mammalian species (Murfitt, et al., 1976).

The *mev-1* (*kn1*) mutant is methyl viologen (paraquat)-sensitive mutant of *C. elegans.* It is hypersensitive to oxygen and has short life span than that of wild type (N2). The *mev-1* mutant is also hypersensitive to raised oxygen concentration. Its life span was increased and decreased under low and high concentrations of oxygen, respectively (Ishii, et al., 1990). The difference between *mev-1* and wild type is in gene *cyt-1* which encodes large subunit of cytochrome (CybL) in succinate-ubiquinone reductase (SQR, also reffered as complex II). At the DNA sequence, position 323, G is substituted by A, which resulting in glycine-to-glutamic acid substitution (Gly71Glu) in protein sequence (Ishii, et al., 1998). The ability of SQR to catalyse electron transport from succinate to ubiquinone is decreased in *mev-1* animal (80%)(Ishii, et al., 1998).

Complex II is an important enzyme complex in TCA cycle and the aerobic respiratory chain of mitochondria and prokaryotic organisms (Kita, et al., 1989). Under aerobic conditions, complex II catalyzes the oxidation of succinate to fumarate and transfers its reducing equivalent to ubiquinone (SQR) (Kita, et al., 2002) In cells, complex II appears to contribute to the basal production of ROS (McLennan, H. R. and Esposti, M. D., 2000). Those imply complex II has important function in oxidative stress damage on ageing.

Here, I focused on the study of the catalytic properties of *mev-1* complex II, and try to elucidate the mechanism of short life span in *mev-1* mutant. That will be very helpful to understand of the pathogenic mechanism of aging and age-related diseases.

Materials and Methods

Materials -- The wild type (N2) was Bristol strain. The *mev-1* mutant was *kn1*.

Optimum culture conditions -- *E. coli* strains and plates were compared. *E. coli* MK3 which lacks the *sdh* and *frd* operons (*sdhc:: Km*^{R}, *Δfrd ABCD*), NM522 *Δsdh::spc* which lacks the *sdh* operon, and OP50. *E. coli* were cultured overnight. Two types of LB plates and three types of NGA plates were compared. After incubated at 37^{0}C overnight, numbers of *E. coli* on the plates were counted. N2 and *mev-1* were grown at 20^{0}C on five types of plates with the supplement of three types of *E. coli.* Worms were harvested after 3-5 days when the quantity of adults reached to the peak population.

Isolation of mitochondria -- Worms were collected in M9 buffer (Brenner, S. 1974). The sedimented worms were washed with M9 buffer until the buffer become clear. Worms were further purified by centrifugation. After polytron, worms were homogenized in MSE containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) using glass-glass homogenizer (Iwaki) with the inclusion of glass beads (B. Brown Melsungen AG, 0.10-0.11mm). The homogenates were then centrifuged at 960 x *g* for 10 min at 4^{0}C, twice. The supernatant was then centrifuged at 19,200 x

g for 10 min, and the precipitated mitochondrial fraction was resuspended in MSE and homogenized.

Extraction and separation of complex II – The detergents tried include 1%, 5% dodecyl maltoside (DOSIN); 1%, 5% Lubrol (NACALAI TESQUE INC.); 1%, 5% C12E9 (SIGMA); 1%, 5% C12E8 (TOKYO KASEI INC.) and 0.05%, 0.1%, 0.2%, 0.3%, 0.5% sucrose monolaurate (SML) (MITSUBISHI-KAGAKU FOODS Co.) (all in W/V).

All the steps were carried out at 0-4^{0}C. Mitochondria were solubilized with 0.2% (W/V) SML. The suspension was stirred at 0^{0}C (ice-water) for 30 min. After centrifugation at 200,000 x *g* for 60 min at 4^{0}C, the supernatant was applied to DEAE-Sepharose (Pharmacia) column (0.8 cm^{2} x 4 cm) equilibrated with 10 mM Tris-HCl pH 7.5, 5% sucrose, 1 mM malonate, 0.05% SML. The column was washed with the same buffer (15 ml) and then complex II was eluted with the same buffer containing a 60 ml gradient of 0-150 mM NaCl at a flow rate of 30 ml/h. Peak fractions were used for enzymatic analysis or stored at -80^{0}C. All conditions were the same for both N2 and *mev-1* complex II.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 2-15% gel (PAG mini DAIICHI)(Weber, 1969). The gels were stained with coomassie brilliant blue (CBB). For Western Blotting, 1D9/1A which is monoclone antibody against *A. suum* complex II Fp subunit and KM 2408 which is polyclonal antibody against *C. elegans* cyt-1 were used.

Enzyme assay – Succinate dehydrogenase (SDH) and SQR assay were determined by Spectrophotometric measurement was performed at 25 ^{0}C with a Shimadzu UV-3000 dual wavelength spectrophotometer.

ROS generation was measured using the chemiluminescent probe MCLA (Cypridina luciferin analog, 2-methl-6- (*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-alpha]pyrazin-3-one). Chemiluminescence assays were conducted at 37^{0}C on a Hamamatsu C1230 photo counter with a H-R 550 photomultiplier (Hamamatsu photonics). The quantity of N2 and *mev-1* complex II was normalized by total SDH activity.

Results

Optimum culture conditions of N2 and mev-1 – To elucidate accurate SDH and SQR activity in *C. elegans* and determine its *K*m value, I wanted to eliminate *E. coli* SDH and SQR activity. MK3 should be the best choice because it has neither SDH nor SQR activities itself. Finally, the optimum conditions for the mass culture of N2 and *mev-1* has been found as follows. As food source, MK3 is grown on LB medium with 0.2% (W/V) glucose and is spread on to the plates. Then, worms are cultured on NGA plate (pH 7.0) with 8-fold Bacto-peptone and 0.2% (W/V) glucose; and 50 μ g/ml of kanamycine.

Isolation of mitochondria from N2 and mev-1 – To elucidate the enzymatic properties and get purified complex II, I prepared quantities of N2 and *mev-1* mitochondria.

Extraction and separation of complex II from N2 and mev-1 mitochondria – For purification, complex II which is membrane-bound proteins, should be solubilized by detergent in unidispersed form. From the results, 0.2% (W/V) SML is the best detergent for solubilizing complex II. It can solubilize almost all complex II in the membrane according to its highest specific activity.

Elution from a DEAE-Sepharose column further increased SDH and SQR specific activities. The specific activity of SDH was similar in both N2 and *mev-1*, though SQR was lower in *mev-1*.

This result is consistant with low SQR activity of *mev-1* mitochondrial membrane reported previously (Ishii, et al., 1998). Salt concentrations (101 mM) of the peak fraction were same in N2 and *mev-1*. The stability of N2 and *mev-1* complex II was kept at least 80% when left in –80°C freezer.

To know the purity of the purified enzyme, SDS-PAGE analysis was performed and shows complex II from DEAE-Sepharose column was great increase.

To investigate the subunit composition, Western Blotting analysis was performed as shown in Antibody against *A. suum* Fp subunits recognized the corresponding subunits of *C. elegans* complex II. One protein band with molecular weight about 14.8 kDa was observed corresponding to the CybL subunit of *C. elegans* complex II.

Enzymatic properties of N2 and mev-1 complex II -- SQR kinetic values (*K*m and *V*max) of purified enzymes were measured using ubiquinone-2 as electron acceptor, and enzymatic properties of mitochondria and complex II from N2 and *mev-1* were compared. Complex II samples were the peak fraction in chromatography. The *K*m of purified enzymes for ubiquinone-2 was 10.2 μM (N2) and 8.77 μM (*mev-1*) which were similar to those of mitochondria, 6.25 μM in N2 and 8.5 μM in *mev-1*.

ROS assay of N2 and mev-1 complex II -- To examine the effects of the *mev-1* mutation on complex II ROS production, complex II purified from *mev-1* and wild type were analyzed. The levels of ROS production were found to be approximately 4.6 fold higher in purified complex II from *mev-1* as compared with wild type (Fig.1). This result suggests that *mev-1* mutant in complex II contributes to ROS production. Succinate stimulated ROS production greatly and *mev-1* was 1.6 fold higher than wild type. It may indicate that electrons cannot be transferred to ubiquinone and are leaked from the complex. This elevates ROS level in *mev-1* mutant.

Discussion

Purification of complex IIs from N2 and mev-1 mitochondria

In this study, *E. coli* MK3 was used as food source for *C. elegans* mass culture because it has neither SDH nor SQR activity. There was no influence of *E. coli* SDH and SQR in *C. elegans* mitochondria samples, because MK3 lacks both SDH and SQR activity.

My data showed that SML is a very useful detergent for solubilizing integral membrane proteins, especially complex II in *C. elegans*. Almost total complex II in inner membrane were solubilized and kept high activity during the purification by column chromatography. Finally, a highly active SQR preparation has been purified for the first time from *C. elegans* mitochondria.

Complex II in N2 and *mev-1* eluted at same salt concentration during ion exchange chromatography. This indicates that there is no significant difference in net charge of complex IIs from wild type and *mev-1* mutant.

Comparison of enzymatic properties of N2 and mev-1 complex II

Comparing SQR *K*m and *V*max value between N2 and *mev-1*, I found that *K*m for ubiquinone was not apparently different from that of the wild type, while its *V*max was far lower than wild type. Therefore, in *mev-1* complex II, affinity between complex II and ubiquinone has no significant difference with the wild type, although rate of electron transfer from the enzyme to ubiquinone is greatly reduced. My work indicates for the first time that the mutation in *mev-1* does not affect quinone-binding site. Substitution of Gly-71 to Glu in CybL may disturb electron transfer from Ip subunit to ubiquinone in the complex, although this does not affect electron transfer from

succinate to artifical electron acceptor such as PMS.

The data presented here clearly demonstrate complex II in N2 and *mev-1* has very similar protein structures. This conclusion is based upon three definitive observations. First, N2 and *mev-1* complex II eluted at same salt concentration during ion-exchange chromatography. Second, both complexes have similar enzymatic activities of SDH. Although SQR activity in *mev-1* was lower than N2, their SDH activities were always comparable. The most important is their similar kinetic parameters of *K*m for ubiquinone which indicate they are similar in the spatial arrangement of the quinone binding site in cytochrome *b* subunits.

ROS assay of N2 and mev-1 complex II

It has been known that oxidants and oxidative stress are connected to ageing. ROS production and corresponding responses to oxidative stress act as key factors in determining longevity (Finkel, T. and Holbrook, N. J. 2000). Mitochondria are the major source of ROS, and are also the first compartment in the cell that is damaged by these ROS. It has been wildly known that complex I and III produce ROS in mitochondria (Finkel, T. and Holbrook, N. J. 2000). There is still no evidence in the literature to suggest that complex II contributes directly to ROS production.

The *mev-1* mutant is hypersensitive to exogenous oxygen and short life span. The levels of superoxide anion production were found higher in *mev-1* intact mitochondria than wild type. Furthermore, cell damage and precocious ageing are caused by endogenous generation of ROS in mitochondria rather than by the direct toxicity of exogenous oxygen (Matsuda, et al., 2001). But, the mechanism of a complex II deficiency causes oxygen hypersensitivity and short life span is still unknown.

In this work, I demonstrated that ROS production was significantly higher in *mev-1* than wild type. My data provide the biochemical connection that links the molecular defect in complex II to ROS hypersensitivity and short life span of the *mev-1* mutant. More importantly, my data may be helpful to study the mechanism of where and how ROS is generated.

From my data, the *mev-1* mutation results in high levels of ROS production at complex II. It suggests that the complex II defect could affect electron flow, that electrons probably transfer directly from complex II to molecular oxygen. And ROS overproduction in *mev-1* complex II might be major reason for its short life span.

The mechanism of ROS production in *mev-1* complex II is still unclear. Kinetic data shows there is no difference in quinone-binding site between N2 and *mev-1*. That indicates perhaps quinone-binding site is not the position where electrons transfer directly from complex II to molecular oxygen.

It seems that some ROS generation may occur at complex II even in wild type. In fact, with the addition of succinate, a complex II substrate, ROS production is increased in wild type complex II. This may suggest that complex II may be a secondary source of ROS generation in the mitochondrial respiratory chain.

In summary, I have shown that *mev-1* complex II has similar quinone-binding site structure with N2 and produces increased level of ROS from complex II. This ROS ultimately results in *mev-1* short life span.

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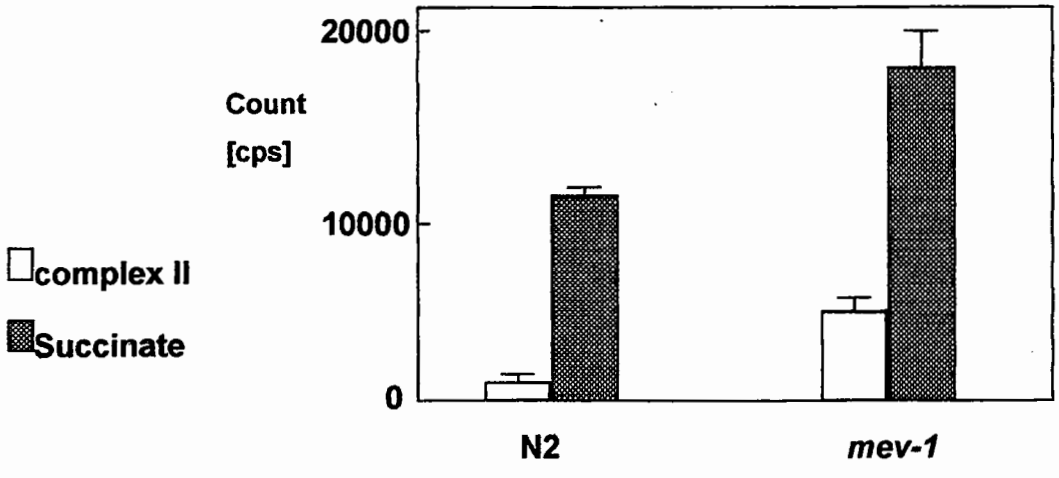

Fig. 1 ROS production in purified N2 and *mev-1* complex II

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