Review

Mitochondrial fumarate reductase as a target of chemotherapy: From parasites to cancer cells

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A B S T R A C T

Recent research on respiratory chain of the parasitic helmint, Ascaris suum has shown that the mitochondrial NADH-fumarate reductase system (fumarate respiration), which is composed of complex I (NADH–rhodoquinone reductase), rhodoquinone and complex II (rhodoquinol–fumarate reductase) plays an important role in the anaerobic energy metabolism of adult parasites inhabiting hosts. The enzymes in these parasite-specific pathways are potential target for chemotherapy. We isolated a novel compound, nafuredin, from Aspergillus niger, which inhibits NADH-fumarate reductase in helminth mitochondria at nM order. It competes for the quinone-binding site in complex I and shows high selective toxicity to the helminth enzyme. Moreover, nafuredin exerts anthelmintic activity against Haemonchus contortus in vivo trials with sheep indicating that mitochondrial complex I is a promising target for chemotherapy. In addition to complex I, complex II is a good target because its catalytic direction is reverse of succinate–ubiquinone reductase in the host complex II. Furthermore, we found atepin and flutolanil strongly and specifically inhibit mitochondrial complex II. Interestingly, fumarate respiration was found not only in the parasites but also in some types of human cancer cells. Analysis of the mitochondria from the cancer cells identified an anthelmintic as a specific inhibitor of the fumarate respiration. Role of isoforms of human complex II in the hypoxic condition of cancer cells and fetal tissues is a challenge. This article is part of a Special Issue entitled Biochemistry of Mitochondria, Life and Intervention 2010.

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1. Introduction

In the general understanding of bioenergetics of higher eukaryotes, oxygen is a most important terminal electron acceptor of mitochondrial respiratory chain (Fig. 1). The major function of the aerobic respiratory chain is the electrogenic translocation of protons out of the mitochondrial or bacterial membrane to generate the proton motive force that drives ATP synthesis by F,F1-ATPase. This mechanism of oxidative phosphorylation is conserved basically from aerobic bacteria to human mitochondria. However, recent study on the respiratory chain of the lower eukaryotes which reside in micro-aerophilic environment has shown that the mitochondrial NADH-fumarate reductase system (fumarate respiration) plays an important role in the anaerobic energy metabolism [1]. This system is composed of complex I (NADH–quinone reductase), low potential quinone species and complex II (quinol–fumarate reductase: QFR). Fumarate respiration is well known electron transport chain in the anaerobic bacteria [2]. Reducing equivalent of NADH is transferred to low potential quinone such as naphthoquinone by complex I and finally is oxidized by fumarate by the fumarate reductase activity of complex II which is a reverse reaction of succinate–ubiquinone reductase (SQR) activity of complex II. By using this respiratory chain, bacteria are able to synthesize ATP even in the absence of oxygen. Recently our study of parasitic nematode, Ascaris suum, showed fumarate respiration also plays an important role in the anaerobic energy metabolism of adult worms, which reside in the host small intestine where oxygen tension is low [1]. Although fumarate reductase activities of bacterial and mitochondrial complex IIIs are the same reaction, evolutional positions of each enzyme are quite different. All four subunits of complex II in adult A. suum are more closely related to the bacterial and mitochondrial SQFR than to bacterial QFR [3–5].
Thus, mitochondrial QFR is a new enzyme evolved by “reverse evolution” of SQR rather than direct evolution from bacterial QFR [8].

Recently our study has revealed that fumarate respiration functions in some human cancer cells and supports a survival of cancer cells in low nutrition and low oxygen conditions [7,8]. Furthermore, we found complex II with high QFR activity produces reactive oxygen species (ROS) [9]. ROS has been reported to contribute to proliferation and metastasis of cancer cells via the stabilization of hypoxia-inducible factor-1 (HIF-1) [10]. In addition, succinate produced by fumarate respiration also stabilizes HIF-1 by the product inhibition of succinyl-CoA synthetase (SACS) [11]. Thus, the relationship between accumulation of succinate resulted from functional defect of human complex II by the mutation of the subunits and carcinogenesis has recently become a focus of research [8].

As fumarate respiration is essential for the growth and survival of the parasites and some cancer cells, it should be a promising target of chemotherapy for both parasitic diseases and cancer. In this review, we focus on recent advances in the study of parasite and human mitochondrial fumarate respiration and complex II which is an important component of the system [8].

2. Fumarate respiration of parasite mitochondria

2.1. Life cycle of A. suum and changes in respiratory chain

A. suum is the most widely known parasite, and has been studied as a representative of human and livestock parasites [12–14]. Because of its large size, A. suum is ideal for the study including biochemical analysis. Adult A. suum resides in the small intestine of mammals, and the female produces between 200,000 and 400,000 fertilized eggs per day (Fig. 2). Eggs are excreted with feces and become mature eggs containing infectious 3rd stage larvae (L3) in about 2–3 weeks at normal temperature. The eggs reach the small intestine and hatch, when orally ingested by a host. A hatched larva invades the intestinal wall, and migrates to the liver, lung, trachea, and pharyngeal region, and finally returns to the intestine via the esophagus and stomach, and becomes an adult worm. In humans, the larve of A. suum migrate to several organs including liver and lung and cause a wide variety of nonspecific symptoms such as general malaise, cough, liver dysfunction, hypereosinophilia with hepatomegaly and/or pneumonia. The oxygen concentration of the small intestine (~5%) is approximately 25% of that outside the body, and provides an environment of low oxygen tension in which the energy metabolism of the adult differs considerably from that of the larvae and the host (Fig. 3). The phosphoenolpyruvate carboxykinase (PEPCK)–succinate pathway, an anaerobic glycolytic pathway, operates in the adult worm, producing ATP under such a hypoxic conditions. This system is used by many other parasites such as Echinococcus multilocularis [15], and has also been observed in the adductor muscle of oysters and other bivalves that require energy conversion under anaerobic conditions. It is therefore considered to be a very common pathway for energy metabolism in adaptation to hypoxic environment [16,17].

The first half of the PEPCK–succinate pathway is the same glycolytic pathway found in mammals, in which phosphoenolpyruvate (PEP) is produced. In contrast to aerobic metabolism in mammals involving the conversion of PEP to pyruvate by pyruvate kinase, the A. suum adult fixes CO₂ with PEPCK to produce oxaloacetate (OAA). The OAA is converted to malate by the reverse reaction of malate dehydrogenase and transported into the mitochondria to produce pyruvate and fumarate. The NADH formed during production of pyruvate from malate is used in the reduction of fumarate to succinate. The NADH-fumarate reductase system, which is the anaerobic electron transport system characteristic of adult A. suum mitochondria, is the final step of this pathway. Unique property of this pathway is discussed in the next section.

In contrast to larvae which require oxygen for their development and possess the respiratory system to be almost the same as that of mammals, cytochrome c oxidase (complex IV) is not found in the respiratory chain of adult A. suum mitochondria, and the content of ubiquinol–cytochrome c reductase complex (complex III) is extremely low [18]. In addition to the enzymes, quinone species in the mitochondria also change during the life cycle of A. suum. In contrast to adult mitochondria, in which the low-potential rhodoquinone (RQ; Em’ = –63 mV) is the major quinone, ubiquinone (UQ; Em’ = +110 mV) is the major quinone of larvae (Fig. 4A) [19]. A combination of SQR and UQ, and that of QFR and a low-potential quinone, such as RQ or menaquinone (MK), is also observed in Escherichia coli and other bacteria during metabolic adaptation to changes in
oxygen supply [20,21]. Lower potential of RQ and MK is favorable for the electron transfer from NADH to fumarate (Fig. 4B). In this way, UQ participates in aerobic metabolism in *A. suum* larva, whereas RQ participates in anaerobic metabolism in adult *A. suum*.

Although studies have shown a clear difference in energy metabolism between larval and adult *A. suum* mitochondria, little is known about changes in the properties of mitochondria during migration of *A. suum* larvae in the host. As described later, examination of the changes in enzymatic characteristics and subunit composition of *A. suum* larval complex II from lung stage L3 (LL3) larvae obtained from rabbits showed that properties of LL3 mitochondria differed from those of L3 and adult mitochondria [22]. Protein chemical analysis revealed that the change in complex II begins with the anchor subunit, and then occurs in the catalytic subunit. Thus, *A. suum* is able to adapt to changes in oxygen concentration in the environment during its life cycle by dynamic change of respiratory chain.

### 2.2. NADH-fumarate reductase system (fumarare respiration) of *A. suum* adult

The final step of the PEPCK–succinate pathway, which plays such an important role in the anaerobic energy metabolism of the *A. suum* adult, is catalyzed by the NADH-fumarate reductase system as
3. Complex IIs of A. suum mitochondria

3.1. Multiple complexes II in A. suum mitochondria

The complex II superfamily comprises succinate–quinone reductase (SQR) and quinol–fumarate reductase (QFR), which catalyze the interconversion of succinate and fumarate with quinone and quinol. SQR is a component of the aerobic respiratory chain as well as the tricarboxylic acid (TCA) cycle [26]. QFR is a component of the anaerobic respiratory chain in anaerobic and facultative anaerobic bacteria [27] and lower eukaryotes [6,28]. SQR and QFR complexes generally consist of four subunits referred to as the flavoprotein subunit (Fp), iron–sulfur subunit (Ip), cytochrome b large subunit (CybL), and cytochrome b small subunit (CybS). The Fp and Ip subunits comprise the catalytic domain of the enzyme. The Fp subunit has a FAD as a prosthetic group and contains the dicarboxylate-binding site. The Ip subunit generally contains three iron–sulfur clusters [2Fe–2S]$^{2+,1+}$, [4Fe–4S]$^{2+,1+}$, and [3Fe–4S]$^{1+,0}$, and the CybS subunits with heme b as the prosthetic group, form the anchor domain of the enzyme. Thus, the catalytic domain of the enzyme is the inner mitochondrial membrane and also serves as the quinone oxidation/reduction site [29].

Our previous study showed that A. suum mitochondria express stage-specific isoforms of complex II (SQR in larvae/QFR in adult) (Fig. 6). The Fp and CybS in adult complex II differ from those of infective third stage larval (L3) complex II. In contrast, there is no difference in the iron–sulfur cluster (Ip) and CybL between adult and L3 isoforms of complex II. However, recent analysis of the changes that occur in the respiratory chain of A. suum larvae during their migration in the host, we found that enzymatic activity, quinone content and complex II subunit composition in mitochondria of lung stage L3 (L3) A. suum larvae is different from those of L3 and adult [22]. Quantitative analysis of quinone content in LL3 mitochondria showed that ubiquinone is more abundant than rhodoquinone. Interestingly, the results of two-dimensional bule-native/sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses showed that LL3 mitochondria contained larval Fp (Fp$^L$) and adult Fp (Fp$^A$) at a ratio of 1:0.56, and that most LL3 CybS subunits were of the adult form (CybS$^A$). This result clearly indicates that the rearrangement of complex II begins with a change in the isoform of the anchor CybS subunit, followed by a similar change in the Fp subunit. At any event, the NADH–fumarate reductase activity of A. suum adult worms (~100 nmol/min/mg) is much higher than that of the mammalian host (2–5 nmol/min/mg).
increase the cellular ROS production [30]. In general, complexes I and II are considered as the two major sites of superoxide and hydrogen peroxide production in the respiratory chain [30–33]. Interestingly, our results show that complex II is the main site of ROS production in A. suum adult respiratory chain [9].

Analysis of submitochondrial particles for superoxide (O$_2^-$) production using superoxide dismutase inhibitable acetylated cytochrome c reduction, and hydrogen peroxide production using catalase inhibitable amplex red oxidation, in the presence and absence of respiratory chain inhibitors, showed the contribution from both the FAD site and quinone-binding site of complex II to produce O$_2^-$ and H$_2$O$_2$ when succinate is oxidized under aerobic conditions. Considering the conservation of amino acid residues critical for the enzyme reaction between A. suum complex II and mitochondrial SQR, our results show the ROS production from more than one site in mitochondrial complex II linked with subtle differences in the amino acid sequences of the enzyme complex.

A. suum adult complex II is a good model to study the mechanism of ROS production from mitochondrial complex II, since amino acid residues conserved among the catalytic domains in mitochondrial SQR enzymes are well conserved in this enzyme and it produces high levels of ROS. Absence of complex III and IV activities in its respiratory chain is an additional advantage of this model. These studies will provide further insight into the possibility of high levels of ROS production from both the FAD site and the Q site in the complex II of A. suum adult worm and help to understand the role of mutations in human complex II for carcinogenesis.

### 3.3. Specific inhibitors of complex II

The differences between parasite and host mitochondria described in this review hold great promise as targets for chemotherapy. For example, the anti-malarial drug Atovaquone, which was recently developed, acts on the mitochondrial respiratory chain [34]. Atovaquone is effective against chloroquine-resistant strains [35]. The specific target is thought to be complex III, and biochemical analysis has shown that it acts on the ubiquinone oxidation site in the cytochrome b of complex III [36,37]. Such a chemotherapeutic approach is also applicable to the helminthes. It has been proposed that the fumarate respiration is the target of such drugs as bithionol and thiabendazole [38,39], but there is no clear biochemical or pharmacological evidence to support this idea. However, as described in the previous section, progress in the study of the NADH-fumarate reduction pathway permits screening of new anthelmintic compound.

Nafuredin, selectively inhibits helminth complex I at concentrations in the order of nanomoles [40] (Fig. 7). Kinetic analysis revealed that the inhibition by nafuredin is competitive against RQ (Fig. 5). These findings, coupled with the fact that helminth complex I uses both RQ and UQ as an electron acceptor, suggest that the structural features of the quinone reduction site of helminth complex I may differ from that of mammalian complex I. In fact, the inhibitory mechanism of quinazolines, which effectively kill the E. multilocularis protozoa, was competitive and partially competitive against RQ and UQ, respectively [41].

The most potent inhibitor of complex II, Atopenin A5, was found during the screening of inhibitors for A. suum complex II [42]. To our regret, IC$_{50}$ of Atopenin A5 for bovine complex II (3.6 nM) was lower than that for A. suum complex II (12 nM for QFR and 32 nM for SQR). However, further screening of inhibitors showed that flutolanil, a commercially available fungicide, specifically inhibits A. suum SQR [43] (Fig. 7). The IC$_{50}$ of flutolanil against A. suum and bovine SQR was 0.081 and 16 μM, respectively, indicating that flutolanil is a promising lead compound for anthelmintics. To enable rational drug optimization, a crystal of the A. suum QFR complexed with

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**Fig. 6.** Schematic representation of A. suum complexes II from larval type and adult type. The mitochondrial metabolic pathway of the parasitic nematode A. suum changes dramatically during its life cycle, to adapt to changes in the environmental oxygen concentration. A. suum mitochondria express stage-specific isoforms of complex II. While there is no difference in the isoforms of the Fp and cyb subunits of complex II between L3 larvae and adult A. suum, they have different isoforms of complex II subunits Fp (larval, Fp$^L$; adult, Fp$^A$) and cyb (larval, cyb$L$; adult, cyb$^A$) in A. suum adult respiratory chain. Complex II produces high amounts of ROS [29].

**Fig. 7.** Chemical structure of inhibitors of complex II. A. Nafuresin, a competitive inhibitor for the rhodoquinone binding site of A. suum complex II; B. Atopenin A5, a competitive inhibitor for the quinone binding site of complex II of many species; C. Flutolanil, a competitive inhibitor for the quinone binding site of A. suum complex II.
flutolanil was prepared by soaking, and X-ray structure analysis has been performed. The current structural model of the flutolanil bound form of the *A. suum* QFR (Harada, unpublished observation) indicates that flutolanil is bound to the same site as those of the quinone binding observed in complex IIs from pig heart mitochondria (pdb code 1Z0Y), *E. coli* (1NEK and 1L0V) and avian (1YQ4). The site of the pig enzyme, for example, is composed of ten residues highly conserved across amino acid sequences of these complex IIs; Pro169, Trp173 and Ile218 from the z subunit, Ile30, Trp35, Met39, and Ile53, are replaced by Pro65, Trp69 and Gly73, respectively, in CybS subunit. However, three residues, Trp35, Met39 and Ile53, are replaced by Pro169, Trp173 and Ile218 from the Ip subunit, Ile30, Trp35, Met39, and Ile53 are well conserved among mammals' Fps and type II Fp is well conserved among mammals. Fps and type II Fp is found only in human complex II (Fig. 8). Type I Fp gene has an exon–intron structure, while the structure of type II Fp gene has not been determined. The type II Fp gene is not found in the NCBI database and the location has not been clarified yet while type I Fp gene is located on chromosome 5p15 [59,60].

4. Isoforms of human complex II

In 2003, we found two isoforms of human Fp, type I and type II [59,60] (Fig. 8). These isoforms differ from each other only in two amino acid residues. Tyr 586 and Val 614 of type I Fp are replaced by Phe 586 and Ile 614 in type II Fp, respectively. Tyr 586 and Val 614 are well conserved among mammals’ Fps and type II Fp is found only in human complex II (Fig. 9). Type I Fp gene has an exon–intron structure, while the structure of type II Fp gene has not been determined. The type II Fp gene is not found in the NCBI database and the location has not been clarified yet while type I Fp gene is located on chromosome 5p15 [59,60].

Complex II with type I Fp has isoelectric point (pI) of 6–7, whereas complex II with type II Fp shows its pI of 5–6. To explain the difference of pI values, several reports suggested the phosphorylation of amino acid residues in Fp subunit [7,61]. One of these residues, Tyr 500, is located close to Tyr 586, which is replaced by Phe in type II Fp (Fig. 10). Since the Tyr 586 Phe substitution will certainly destroy the hydrogen bond between Tyr 586 O and Glu 597 O (3.13 Å), the local structure around Tyr 586 as well as Tyr 500 phosphorylation status may be different between Fps of type I and II.

The result of biochemical analysis of complex II with each isoform, complex II with each Fp was found to have almost the same SQR specific activities. However, type II Fp has lower optimal pH than type I Fp and at optimal pH of type II Fp, $K_m$ value for succinate of type II Fp is lower than that of type I Fp (Sakai unpublished data). It may be possible that different phosphorylation statuses of complex II with each isoform cause biochemical differences.

4.3. Expression of human complex II containing type II Fp

Our previous study on the expression of isoforms showed that both types were expressed in all the organs tested (liver, heart, skeletal muscle, brain and kidney) and expression of type I Fp was higher than that of type II Fp [59,60]. This tendency was also found in the cultured cells such as Fibroblast, Myoblast, Human Umbilical Vein Endothelial Cells (HUVEC-C), colon cancer cells (HT-29) and lung cancer cells (A549). However, colorectal adenocarcinoma cells (DLD-1), breast cancer cells (MCF-7) and lymphoma cells (Raji) showed higher expression of type II Fp than that of type I Fp. Type I Fp seems to be essential for the ordinary function of complex II because incorporation of the flavin adenine dinucleotide cofactor (flavination) of SDHA (succinate dehydrogenase complex, subunit A, flavoprotein), and it is also necessary for complex II assembly and function [53]. Furthermore, the mutation found in SDHAF2 has been suggested to link to familial paraganglioma [53].

Fig. 8. Fp isoform gene structure. Type I and II Fps differ from each other in six bases in DNA sequences and in two amino acid residues in proteins. Type I Fp gene has an exon–intron structure, while type II Fp gene is suggested to be intron-less. Although type I Fp gene is located on chromosome 5p15, the type II Fp gene is not found in the NCBI database and the location has not been clarified yet [59,60].
all the examined tissues and many of the cultured cells showed abundant expression of type I Fp and optimum pH for this isoform is around physiological mitochondrial matrix pH (pH 8.0).

Since type II Fp was expressed in some cancer cells, this isoform may play an important role in the metabolism of tumor tissue. To investigate the link between type II Fp and tumor tissue in detail, we analyzed mRNA expression ratio of Fp isoforms in several tissues including tumor tissues and cultured cells. Since some tumor marker genes are expressed in fetal tissues, we included the fetal tissues in this analysis.

As shown in Table 1, in cultured cells, all the normal cells tested showed mainly type I Fp expression as reported previously [59, 60]. In tissues, expression of type I Fp was higher than that of type II Fp in all the organs tested including normal testes tissue. Interestingly, normal pancreatic tissue showed higher expression of type II Fp. In addition, several tumor tissues expressed predominantly type II Fp such as breast tumor, liver tumor, kidney tumor and cervix tumor. Among fetal tissues, brain and skeletal muscle showed higher expression of type II Fp than type I Fp.

4.4. Fumarate respiration of human cancer cells

Several observations suggested the presence of a reverse reaction of complex II, fumarate reductase (FRD), in mammalian cells, although no direct evidence of FRD activity in mammalian complex II has been available until recently [62, 63]. The accumulation of succinate under hypoxic conditions has been reported, and complex II has been suggested to function as FRD in mammalian cells [64]. Metabolome analysis of the cancer cells supports this idea, because succinate, fumarate and malate were present at higher levels in cancer tissues than normal tissues [65]. FRD inhibitor pyrvinium pamoate, an anthelmintic, has also been reported to act as an anticancer compound in human cancer cells [62]. Furthermore, recent biochemical studies showed fumarate respiration in human mitochondria clearly [7, 8]. Mitochondria isolated from DLD-1 cells showed FRD activity with 3 nmol/min/mg protein, although this number is quite lower than that of the A. suum mitochondria (200 nmol/min/mg). Interestingly, the cancer cells had higher FRD/SQR ratio than the normal cells. For example, FRD/SQR ratio in Panc-1 cells is 0.066 ± 0.010, while that in Human Dermal Fibroblast cells is 0.011 ± 0.002. In addition, FRD/SQR ratio increased when the cancer cells were cultured under hypoxic and glucose limited condition [7]. Effect of a treatment by phosphatase and protein kinase on the direction of enzyme activity of human complex II suggests the changes from SQR to QFR by phosphorylation of Fp.

Different from A. suum, which has at least two distinct complex IIs as mentioned previously, only one gene is found for each subunit of human complex II except Fp. In this connection, it is of interest to speculate that complex II with type II Fp has higher QFR activity and plays an important role in fumarate respiration in human mitochondria as terminal oxidase of the system. Further biochemical study on

Fig. 9. Alignment of amino acid sequences of Mammalian Fp subunits. Two amino acid residues in the red box are different in human Fp isoforms. Tyr 586 and Val 614 in type I Fp are changed to Phe 586 and Ile 614 in type II Fp, respectively. Tyr 586 and Val 614 are well conserved among mammals and no animals but humans have type II Fp [59].

Fig. 10. Positions of Tyr 586 and Val 614 in the structure of porcine complex II. Two amino acid residues different in human isoforms, Y586F and V614I, shown in the cartoon representation of the porcine complex II structure (left) and the close-up view of the region including Y586F and V614I (right). V614I is surrounded mainly by hydrophobic residues, whereas Y586F by both hydrophilic and hydrophobic residues. Y586 and E598 are in the hydrogen bond distance (3.15 Å) to each other. UQ shows ubiquinone. The numbers of amino acid residues in the box represent the human amino acid sequences and the others are the porcine amino acid sequences.
the difference between type I and type II Fp will bring final conclusion on this attractive idea.

5. Conclusions

The recent findings described in this review indicate that the respiratory chain plays an important role in responses to changes in the amount of oxygen in the environment. Complex II functions as a fumarate reductase during adaptation to a hypoxic condition to ensure the maintenance of oxygen homeostasis. In this connection, the reports indicating that complex II functions as an oxygen sensor are of great interest [63].

In addition, direct evidence of fumarate respiration in human mitochondrial iron are quite important in the study of energy metabolism in hypoxic condition including cancer cells. Differences in energy metabolism between hosts and parasites and/or cancer cells are attractive therapeutic targets.

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