3. Complex IIs of *A. suum* mitochondria

3.1 Multiple complex IIs 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 an FAD as a prosthetic group and contains the dicarboxylate-binding site. The Ip subunit generally contains three iron-sulfur clusters $[2Fe\text{-}2S]^{2+,1+}$, $[4Fe\text{-}4S]^{2+,1+}$, and $[3Fe\text{-}4S]^{1+,0}$. Subunits CybL and CybS, with heme *b* as the prosthetic group, form the anchor domain of the enzyme. This anchors the catalytic domain to 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 (LL3) *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^{Λ}) at a ratio of 1 : 0.56, and that most LL3 CybS subunits were of the adult form ($CybS^{\Lambda}$). 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) are much higher than that of the mammalian host (2~5 nmol/min/mg).

3.2 ROS production from complex II

Mitochondrial respiratory chain is a significant source of cellular ROS. Impairment of the respiratory chain complexes is known to increase the cellular ROS production [30]. In general, complexes I and III 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 ($O_2^{\cdot-}$) 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 $O_2^{\cdot-}$ and H_2O_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 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 pharmaceutical evidence to support this idea. However, as described in the previous section, progress in the study of the NADH-fumarate reductase 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* protoscoleces, was competitive and partially competitive against RQ and UQ, respectively [41].

The most potent inhibitor of complex II, Atpenin A5, was found during the screening of inhibitors for *A. suum* complex II [42]. To our regret, IC_{50} of Atpenin 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, the 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 anthelminthics. To enable rational drug optimization, a crystal of the *A. suum* QFR complexed with 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 1ZOY), *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 Ip subunit, Ile30, Trp35, Met39, Ser42, Ile43 and Arg46 from the CybL subunit, and Tyr91 from the CybS subunit. However, three residues, Trp35, Met39 and Ile53, are replaced by Pro65, Trp69 and Gly73, respectively, in *A. suum* QFR. The structures of the *A. suum* QFR together with those of QFRs from *Wolinella succinogenes* [24] and *E. coli* [23], and SQRs from *E. coli* [44], pig heart mitochondria [45], and avian heart mitochondria [46] should help clarify the structure-function relationship of complex II and provide useful information for the structure-based design of anthelminthics.

4. Fumarate respiration of human mitochondria

4.1 Human complex II

In human, many cases of diseases caused by mutations in subunits of complex II have been reported. Mutations found in the Ip, Cyb L or Cyb S are associated with the development of pheochromocytoma and paraganglioma [47-51]. The causes of tumorigenesis are suggested that ROS production from mutated complex II [52, 53]or accumulation of succinate as a result of SQR inhibition [11]. Accumulated succinate inhibits HIF-1α prolyl hydroxylases in the cytosol, leading to stabilization and activation of HIF-1α. Thus, succinate can increase expression of genes that facilitate angiogenesis, metastasis, and glycolysis, ultimately leading to tumor progression. On the other hand, no patient about mutation in Fp linked to tumorigenesis has been reported. There are two Fp isoforms in human, which will be discussed later, and this is probably the reason why mutations in Fp are not directly linked to tumorigenesis. Instead, mutations in Fp linked to severe metabolic disorders resulting from decreased activity of the TCA cycle and impairment of oxidative phosphorylation, although these are rare. These autosome-recessive disorders are manifested as childhood encephalopathy, myopathy, adult optic atrophy, and Leigh syndrome [54-57]. Recently, two new proteins, SDHAF1 (succinate dehydrogenase complex assembly factor 1) and SDHAF2, were found to be the first assembly factors of complex II [53, 58]. It was suggested that mutations found in SDHAF1 may result in the reduction of assembled complex II and cause infantile leukoencephalopathy [58]. SDHAF2 is suggested to be required for the 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]

4.2 Isoformes 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 to 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 (p*I*) of 6 - 7, whereas complex II with type II Fp shows its p*I* of 5 - 6. To explain the difference of p*I* 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 a hydrogen bond between Tyr 586 O_η and Glu 597 O_δ (3.13Å), the local structure around Tyr 586 as well as Tyr 500 phosphorylation status may be different between Fps of types 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 type I Fp (Sakai unpublished data). It may be possible that different phosphorylation status 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 isoformes 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 (HUV-EC-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 than that of type I Fp. Type I Fp seems to be essential for the ordinary function of complex II because 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 (pH8.0).

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 humen 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 play an important role in fumarate respiration in human mitochondria as terminal oxidase of the system. Further biochemical study on 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 evidences of fumarate respiration in human mitochondria 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.

Acknowledgements

This work was supported in part by Creative Scientific Research Grant 18GS0314 (to KK), Grant-in-aid for Scientific Research on Priority Areas 18073004 (to KK) from the Japanese Society for the Promotion of Science, and Targeted Proteins Research Program (to KK) from the Japanese Ministry of Education, Science, Culture, Sports and Technology (MEXT).

References

[1] K. Kita, K. Shiomi, and S. Õmura, Parasitology in Japan: Advances in drug discovery and biochemical studies, Trends in Parasitol. 23 (2007) 223-229.

[2] A. Kroger, V. Geisler, E. Lemma, F. Theis and R. Lenger, Bacterial fumarate respiration, Arch. Microbiol. 158 (1992) 311−314.

[3] T. Kuramochi, H. Hirawake, S. Kojima, S. Takamiya, R. Furushima T. Aoki, R. Komuniecki, and K. Kita, Sequence comparison between the flavoprotein subunit of the fumarate reductase (complex II)of the anaerobic parasitic nematode, *Ascaris suum* and the succinate dehydrogenase of theaerobic, free-living nematode, *Caenorhabditis elegans*. Mol. Biochem. Parasitol. 68 (1994) 177-87.

[4] F. Saruta, H. Hirawake, S. Takamiya, Y-C. Ma, T. Aoki, K. Sekimizu, S. Kojima and K. Kita, Cloning of a cDNA encoding the small subunit of cytochrome b_{558} (cybS) of mitochondrial fumarate reductase (complex II) from adult *Ascaris suum*, Biochim. Biophys. Acta 1276 (1996) 1−5.

[5] H. Amino, H. Wang, H. Hirawake, F. Saruta, D. Mizuchi, R. Mineki, N. Shindo, K. Murayama, S. Takamiya, T. Aoki, S. Kojima and K. Kita, Stage-specific isoforms of *Ascaris suum* complex II: the fumarate reductase of the parasitic adult and the succinate dehydrogenase of free-living larvae share a common iron-sulfur subunit, Mol. Biochem. Parasitol. 106 (2000), 63−76.

[6] K. Kita, S. Takamiya, Electron-transfer complexes in *Ascaris* mitochondria, Adv Parasitol. 51 (2002) 95-131.

[7] E. Tomitsuka, K. Kita, H. Esumi, Regulation of succinate-ubiquinone reductase and fumarate reductase activities in human complex II by phosphorylation of its flavoprotein subunit, Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 85 (2009) 258-65.

[8] E. Tomitsuka, K. Kita H. Esumi, The NADH-fumarate reductase system, a novel mitochondrial energy metabolism, is a new target for anticancer therapy in tumor microenvironments, Ann. N. Y. Acad. Sci. 201 (2011) 44-9.

[9] M.P. Paranagama, K. Sakamoto, H. Amino, M. Awano, H. Miyoshi, K. Kita, Contribution of the FAD and quinone binding sites to the production of reactive oxygen species from *Ascaris suum* mitochondrial complex II, Mitochondrion 10 (2010) 158-65.

[10] R. D. Guzy, B. Sharma, E. Bell, N. S. Chandel, P. T. Schumacker, Loss of the SdhB, but Not the SdhA, subunit of complex II triggers reactive oxygen species-dependent hypoxia-inducible factor activation and tumorigenesis, Mol Cell Biol. 28 (2008) 718-731.

[11] M.A. Selak, S.M. MacKenzie, H. Boulahbel, D.G. Watson, K.D. Mansfield, Y. Pan, M.C. Simon, C.B. Thompson, E. Gottlieb, Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase, Cancer Cell 7 (2005) 77-85.

[12] R. Komuniecki, B.G. Harris, J. Marr, M. Mueller, (Eds.), Biochemistry and Molecular Biology of Parasites,

Academic Press, London, 1995, pp. 49-66.

[13] A.G.M. Tielens, J. Van Hellemond, The electron transport chain in anaerobically functioning eukaryotes, Biochim. Biophys. Acta 1365 (1998) 71-78.

[14] K. Kita, H. Hirawake, S. Takamiya, Cytochromes in the respiratory chain of helminth mitochondria, Int. J. Parasitol. 27 (1997) 617-630.

[15] J. Matsumoto, K. Sakamoto, N. Shinjyo, Y. Kido, N. Yamamoto, K. Yagi, H. Miyoshi, N. Nonaka, K. Katakura, K. Kita and Y. Oku, Anaerobic NADH-Fumarate Reductase System Is Predominant in the Respiratory Chain of *Echinococcus multilocularis*, Providing a Novel Target for the Chemotherapy of Alveolar Echinococcosis, Antimicrob. Agents. Chemother. 52 (2008) 164-170.

[16] P. Kohler, R. Bachmann, Mechanisms of respiration and phosphorylation in *Ascaris* muscle mitochondria, Mol Biochem Parasitol. 1 (1980) 75-90.

[17] H. Oya, K. Kita, in: E. Bennet, C. Behm, C. Bryant (Eds.), Comparative Biochemistry of Parasitic Helminths, Chapman and Hall, London, 1988, pp. 35-53.

[18] S. Takamiya, R. Furushima, R.H. Oya, Electron transfer complexes of *Ascaris suum* muscle mitochondria: I. Characterization of NADH-cytochrome *c* reductase (complex I-III), with special reference to cytochrome localization, Mol. Biochem. Parasitol. 13 (1984) 121-134.

[19] S. Takamiya, K. Kita, H. Wang, P.P. Weinstein, A. Hiraishi, H. Oya, T. Aoki Developmental changes in the respiratory chain of *Ascaris* mitochondria, Biochim. Biophys. Acta 1141 (1993) 65-74.

[20] S.T. Cole, C. Condon, B.D. Lemire, J.H. Weiner, Molecular biology, biochemistry and bioenergetics of fumarate reductase, a complex membrane-bound iron-sulfur flavoenzyme of *Escherichia coli*, Biochim. Biophys. Acta 811 (1985) 381-403.

[21] A. Hiraishi, Fumarate reduction systems in members of the family *Rhodospirillaceae* with different quinone types, Arch. Microbiol. 150 (1988) 56-60.

[22] F. Iwata, N. Shinjyo, H. Amino, K. Sakamoto, M.K. Islam, N. Tsuji, K. Kita, Change of subunit composition of mitochondrial complex II (succinate-ubiquinone reductase/quinol-fumarate reductase) in *Ascaris suum* during the migration in the experimental host, Parasitol. Int. 57 (2008) 54-61.

[23] T.M. Iverson, C. Luna-Chavez, G. Cecchini, D.C. Rees, Structure of the *Escherichia coli* fumarate reductase respiratory complex, Science 284 (1999) 1961-1966.

[24] C.R. Lancaster, A. Kröger, M. Auer, H. Michel, Structure of fumarate reductase from *Wolinella succinogenes* at 2.2 A resolution, Nature 402 (1999) 377-85.

[25] K. Kita, C. Vibat, S. Meinhardt, J. Guest, R. Gennis, One-step purification from *Escherichia coli* of complex II (succinate: ubiquinone oxidoreductase) associated with succinate-reducible cytochrome b_{556}, J. Biol. Chem. 264 (1989) 2672-2677.

[26] G. Cecchini, I. Schroder, R.P. Gunsalus, E. Maklashina, Succinate dehydrogenase and fumarate reductase from *Escherichia coli*, Biochim. Biophys. Acta. 1553 (2002) 140−157.

[27] C.R.D. Lancaster, Structure and function of succinate: quinone oxidoreductases and the role of quinol: fumarate reductases in fumarate respiration. In: Zannoni, D. (Eds.), Respiration in Archaea and Bacteria: Diversity of Prokaryotic Electron Transport Carriers. Kluwer Academic Publishers, The Netherlands, 2004, pp. 57-85.

[28] J.J. Van Hellmond, A. van der Klei, S.W.H. van Weelden, A.G.M. Tielens, Biochemical and evolutionary aspects of anaerobically functioning bacteria, Phil. Trans. R. Soc. B. 358 (2003) 205-215.

[29] K. Kita, H. Hirawake, H. Miyadera, H. Amino and S. Takeo, Role of complex II in anaerobic respiration of the parasite mitochondria from *Ascaris suum* and *Plasmodium falciparum*, Biochim. Biophys. Acta, 1553 (2002) 123-139

[30] H.P. Indo, M. Davidson, H.C. Yen, S. Suenaga, K. Tomita, T. Nishii, M. Higuchi, Y. Koga, T. Ozawa, H.J. Majima, Evidence of ROS generation by mitochondria in cells with impaired electron transport chain and mitochondrial DNA damage, Mitochondrion 7, (2007) 106-118.

[31] P. Jezek, L. Hlavata, Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism, Int. J. Biochem. Cell Biol. 37 (2005) 2478-2503.

[32] M.P. Murphy, How mitochondria produce reactive oxygen species, Biochem. J. 417, (2009) 1−13.

[33] J. St-Pierre, J.A. Buckingham, S.J. Roebuck, M.D. Brand, Topology of superoxide production from different sites in the mitochondrial electron transport chain, J. Biol. Chem. 277 (2002) 44784-44790.

[34] I.K. Srivastava, H. Rottenberg, A.B. Vaidya, Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite, J. Biol. Chem. 272 (1997) 3961-3966.

[35] S. Looareesuwan, C. Viravan, H.K. Webster, D.E. Kyle, D.B. Hutchinson, C.J. Canęeld, Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand, Am. J. Trop. Med. Hyg. 54 (1996) 62-66.

[36] D. Syafruddin, J.E. Siregar, S. Marzuki, Mutations in the cytochrome *b* gene of *Plasmodium berghei* conferring resistance to atovaquone, Mol. Biochem. Parasitol. 104 (1999) 185-194.

[37] I. K. Srivastava, J. M. Morrisey, E. Darrouzet, F. Daldal, A. B. Vaidya, Resistance mutations reveal the atovaquone-binding domain of cytochrome *b* in malaria parasites, Mol. Microbiol. 33 (1999) 704-711.

[38] P. Kohler, R. Bachmann, The effects of the antiparasitic drugs levamisole, thiabendazole, praziquantel, and chloroquine on mitochondrial electron transport in muscle tissue from *Ascaris suum*, Mol. Biochem. Parasitol. 14 (1978)155-163.

[39] A. Armson, W.B. Grubb, A.H.W. Mendis, The effect of electron transport (ET) inhibitors and thiabendazole on the fumarate reductase (FR) and succinate dehydrogenase (SDH) of *Strongyloides ratti* infective (L3) larvae, Int. J. Parasitol. 25 (1995) 261-263.

[40] S.Omura, H. Miyadera, H. Ui, K. Shiomi, Y. Yamaguchi, R. Masuma, T. Nagamitsu, D. Takano, T. Sunazuka, A. Harder, H. Kölbl, M. Namikoshi, H. Miyoshi, K. Sakamoto and K. Kita, An anthelmintic compound, nafuredin, shows selective inhibition of complex I in helminth mitochondria, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 60-62

[41] T. Yamashita, T. Ino, H. Miyoshi, K. Sakamoto, A. Osanai, E. Nakamaru-Ogiso and K. Kita, Rhodoquinone reaction site of mitochondrial complex I, in parasitic helminth, *Ascaris suum*, Biochim. Biophys. Acta 1608 (2004) 97-103

[42] H. Miyadera, K. Shiomi, H. Ui, Y. Yamaguchi, R. Masuma, H. Tomoda, H. Miyoshi, A. Osanai, K. Kita, S. Omura, Atpenins, potent and specific inhibitors of mitochondrial complex II (succinate-ubiquinone oxidoreductase), Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 473-477.

[43] A. Osanai, S. Harada, K. Sakamoto, H. Shimizu, D.K. Inaoka, K. Kita, Crystallization of mitochondrial rhodoquinol-fumarate reductase from the parasitic nematode *Ascaris suum* with the specific inhibitor flutolanil, Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 65 (2009) 941-954.

[44] V. Yankovskaya, R. Horsefield, S. Törnroth, C. Luna-Chavez, H. Miyoshi, C. Léger, B. Byrne, G. Cecchini, S. Iwata, Architecture of succinate dehydrogenase and reactive oxygen species generation, Science 299 (2003) 700-704.

[45] F. Sun, X. Huo, Y. Zhai, A. Wang, J. Xu, D, Su, M, Bartlam, Z, Rao, Crystal structure of mitochondrial respiratory membrane protein complex II, Cell 121 (2005) 1043-1057.

[46] L.S. Huang, G. Sun, D. Cobessi, A.C. Wang, J.T. Shen, E.Y. Tung, V.E. Anderson, E.A. Berry, 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme, J. Biol. Chem. 281 (2006) 5965-5972.

[47] J.P. Bayley, P. Devilee and E.M.P. Taschner, The SDH mutation database: an online resource for succinate dehydrogenase sequence variants involved in pheochromocytoma, paraganglioma and mitochondrial complex II deficiency, BMC. Med.Genet. 6 (2005) 39.

[48] B.E. Baysal, On the association of succinate dehydrogenase mutations with hereditary paraganglioma, Trends Endocrinol. Metab. 14 (2003) 453-459.

[49] C. Eng, M. Kiuru, M.J. Fernandez and L.A. Aaltonen, A role for hypoxic mitochondrial enzymes in inherited neoplasia and beyond, Nat. Rev. Cancer 3 (2003) 193-202

[50] P.J. Pollard, N.C. Wortham and I.P. Tomlinson, The TCA cycle and tumorigenesis: The examples of fumarate hydratase and succinate dehydrogenase, Ann. Med. 35 (2003) 632-639.

[51] P. Rustin and A. Rotig, Inborn errors of complex II–unusual human mitochondrial diseases, Biochim. Biophys. Acta 1553 (2002) 117-122.

[52] N. Ishii, T. Ishii, P.S. Hartman, The role of the electron transport SDHC gene on lifespan and cancer, Mitochondrion 7 (2007) 24-38.

[53] H.X. Hao, O. Khalimonchuk, M. Schraders, N. Dephoure, J.P. Bayley, H. Kunst, P. Devilee, C.W. Cremers, J.D.

Schiffman, B.G. Bentz, S.P. Gygi, D.R. Winge, H. Kremer, J. Rutter, SDH5, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma, Science 325 (2009) 1139-1142.

[54] M.A. Birch-Machin, R.W. Taylor, B. Cochran, B.A. Ackrell, D.M. Turnbull, Late-onset optic atrophy, ataxia, and myopathy associated with a mutation of a complex II gene, Ann. Neurol. 48 (2000) 330-335.

[55] T. Bourgeron, P. Rustin, D. Chretien, M. Birch-Machin, M. Bourgeois, E. Viegas-Pequignot, A. Munnich and A. Rotig, Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency, Nat. Genet. 11 (1995) 144-149.

[56] B. Parfait, D. Chretien, A. Rötig, C. Marsac, A. Munnich, P. Rustin, Compound heterozygous mutations in the flavoprotein gene of the respiratory chain complex II in a patient with Leigh syndrome, Hum. Genet. 106 (2000) 236-243.

[57] R. Van Coster, S. Seneca, J. Smet, R. Van Hecke, E. Gerlo, B. Devreese, J. Van Beeumen, J.G. Leroy, L. De Meirleir, W. Lissens, Homozygous Gly555Glu mutation in the nuclear-encoded 70 kDa flavoprotein gene causes instability of the respiratory chain complex II, Am. J. Med. Genet. A. 120 (2003) 13-18

[58] D. Ghezzi, P. Goffrini, G. Uziel, R.Horvath, T. Klopstock, H. Lochmüller, P. D'Adamo, P. Gasparini, T.M. Strom, H. Prokisch, F. Invernizzi, I. Ferrero, M. Zeviani, SDHAF1, encoding a LYR complex-II specific assembly factor, is mutated in SDH-defective infantile leukoencephalopathy, Nature Genetics 41 (2009) 654-656.

[59] E. Tomitsuka, H. Hirawake, Y. Goto, M. Taniwaki, S. Harada, K. Kita, Direct evidence for two distinct forms of the flavoprotein subunit of human mitochondrial complex II (succinate-ubiquinone reductase), J. Biochem. 134 (2003) 191-195

[60] E. Tomitsuka, Y. Goto, M. Taniwaki, K. Kita, Direct evidence for expression of type II flavoprotein subunit in human complex II (succinate-ubiquinone reductase), Biochem Biophys. Res. Commun. 311 (2003) 74-79

[61] M. Salvi, N. Morrice, A. Brunati, A. Toninello, Identification of the flavoprotein of succinate dehydrogenase and aconitase as in vitro mitochondrial substrates of Fgr tyrosine kinase, FEBS Letters, 581 (2007) 5579-5585

[62] H. Esumi H, J. Lu, Y. Kurashima, T. Hanaoka, Antitumor activity of pyrvinium pamoate, 6-(dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1H-pyrrol-3-yl)ethenyl]-1-me thyl-quinolinium pamoate salt, showing preferential cytotoxicity during glucose starvation, Cancer Sci. 95 (2004) 685-690.

[63] B. E. Baysal, R. E. Ferrell, J. E. Willett-Brozick, E. C. Lawrence, D. Myssiorek, A. Bosch, A., A. van der Mey, P. E. Taschner, W. S. Rubinstein, E. N. Myers, C. W. Richard, C. W., C. J. Cornelisse, P. Devilee, B. Devlin, Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma, Science 287 (2000) 848-851.

[64] J. M. Weinberg, M. A. Venkatachalam, N. F. Roeser, I. Nissim, Mitochondrial dysfunction during hypoxia/reoxygenation and its correction by anaerobic metabolism of citric acid cycle intermediates, Proc. Natl. Acad. Sci. U. S. A .97 (2000) 2826-2831.

[65] A. Hirayama, K. Kami, M. Sugimoto, M. Sugawara, N. Toki, H. Onozuka, T. Kinoshita, N. Saito, A.

Ochiai, M. Tomita, H. Esumi, and T. Soga, Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry, Cancer Res. 69 (2009) 4918-4925.

Legend to Figures

Figure 1. Complex II is the member of TCA cycle and respiratory chain

Complex II catalyzes the oxidation of succinate to fumarate in the TCA cycle and transports the electron generated by this oxidation to ubiquinone in the respiratory chain. Generally, complex II consists of four subunits. Flavoprotein (Fp) subunit contains a flavin adenine dinucleotide prosthetic group and iron sulfur protein (Ip) subunit contains three iron-sulfur clusters. There are two hydrophobic cytochrome*b* (Cyb L, Cyb S) subunits. The succinate binding site is located in Fp subunit, while the quinone binding site is formed by three subunits, Ip, Cyb L and CybS. Complex II also catalyzes the reduction of fumarate, a reverse-reaction of succinate dehydrogenase, in the respiratory chain of mitochondria from anaerobic animals, such as *Ascaris suum*, as well as anaerobic bacteria.

Figure 2. Life cycle of *Ascaris suum*

Fertilized eggs grow to be infective L3 under aerobic environment. Infective L3 larvae are ingested by the host, reach the small intestine and hatch there. Afterwards, larvae migrate into host body (liver, heart, lung, pharynx), and finally migrate back to the small intestine and become adults. In the host small intestine, the oxygen concentration is low (pO_2=2.5 ~ 5%) compared with the exogenous environment (pO_2=20%). The metabolic pathway of *A. suum* changes dramatically during its life cycle, to adapt to changes in the environmental oxygen concentration [6].

Figure 3. Glucose metabolism of *A. suum* larval and adult mitochondria

The metabolic pathway of *A. suum* adult has a unique anaerobic electron transport system, NADH-fumarate reductase system. In the phospho*enol*pyruvate carboxykinase (PEPCK)-succinate pathway, phospho*enol*pyruvate (PEP) produced by a glycolytic process is carboxylated to form oxaloacetate and is then reduced to malate. The cytosolic malate is transported into the mitochondria, where it is first reduced to fumarate, and finally to succinate by the rhodoquinol-fumarate reductase activity of complex II. The terminal step is catalyzed by the NADH-fumarate reductase system (Boxed in broken lines) comprised of complex I, rhodoquinone (RQ), and complex II. PEP, phosphoenolpyuvate; PEPCK, phoshoenolpyruvate carboxykinase; OAA, oxaloacete [6].

Figure 4. Chemical structure and redox potentials of the quinones. A. Chemical structures of UQ and RQ. n, numbers of isoprenyl groups in side-chain. B. Redox potentials of quinones and substrates.

Figure 5. NADH-fumarate reductase System of *A. suum* as a target of chemotherapy

The differences in energy metabolisms between host and helminths is an attractive therapeutic targets for

helminthiasis. NADH-fumarate reductase is a part of a unique respiratory system in parasitic helminths and is the terminal step of the phospho*enol*pyruvate carboxykinase-succinate pathway, which is found in many anaerobic organisms. NADH-Fumarate Reductase System is a potential target for chemotherapy. Nafuredin was found to be competitive inhibitor for rhodoquinone binding site of *A. suum* complex II [1].

Figure 6. Schematic representation of *A. suum* complex IIs from larva 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 Ip and cybL 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 cybS (larval, $cybS^{L}$; adult, $cybS^{A}$) in *A. suum* adult respiratory chain, complex II produces high amount ROS [29].

Figure 7. Chemical structure of inhibitors of complex II

A. Nafuresin, a competitive inhibitor for the rhodoquinone binding site of *A. suum* complex II; B. Atpenin A5, a competitive inhibitor for the quinone binding site of complex II of many species; C. Fulutolanil, a competitive inhibitor for the quinone binding site of *A. siuum* complex II.

Figure 8. Fp isoform gene structure

Type I and II Fps differ to 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 issuggested 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].

Figure 9. Alignment of Amino acid sequences of Mammalian Fp subunits

Two amino acid desidues 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 human have type II Fp [59].

Figure 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.

Table 1 mRNA expression of Fp isoforms in human cultured cells and tissues.

The expression ratio of the two Fp isoforms was analyzed by RT-PCR-RFLP (restriction fragment length polymorphism with AvaII).Total RNAs were obtained from NIPPON GENE (Japan) for normal liver, heart, skeletal muscle, brain, kidney and breast tumor, colon tumor, stomach tumor and uterus tumor. Wako (Japan) for normal pancreas and fetal tissues. Invitrogen (USA) for normal testes and breast tumor, liver tumor, kidney tumor, colon tumor, pancreas tumor, cervix tumor, ovary tumor, prostate tumor. Cells ; Fibroblast and Myoblast : kind gift from Dr. Yu-ichi Goto (National Institute of Neuroscience, Japan) A549, DLD-1 and MCF-7 : kind gift from Mr. Yasuyuki Yamazaki (Taiho pharma_ceutical, Japan) Panc-1 : kind gift from Dr. Yasuhiro Esumi (National Cancer Institute, Japan) Raji : kind gift from Dr. Kazurou Shiomi (Kitasato university, Japan) HT-29, HU-VEC-C, MDA-M-231, BT-20 and T-47D : ATCC (USA). Pancreatic epithelial and stromal cells : DS pharma (Japan).

		Race	Gender	Age	I (%) / II (%)
Tissue (Normal)	Liver*	Caucasoid	Female	15	70 / 30
	Heart*	Caucasoid	Pool of 7 donors		61 / 39
	Skeletal muscle*	—	Male	23	80 / 20
	Brain*	Caucasoid	Male	50	84 / 16
	Kidney*	Caucasoid	Pool of 8 donors		62 / 38
	Pancreas	—	Male	44	30 / 70
	Testes	Caucasoid	Male	19	100 / 0
Cell (Normal)	Fibroblast*	Mongoloid	—	—	94 / 6
	Myoblast*	Mongoloid	—	—	87 / 13
	HUV-EC-C*	—	—	—	88 / 12
	Pancreatic epithelial	—	—	—	100 / 0
	Pancreatic stromal	—	—	—	100 / 0
Tissue (Fetal)	Brain	—	Female	22 weeks	100 / 0
	Brain	—	Male	41 weeks	38 / 62
	Skeletal muscle	—	Male	22 weeks	0 / 100
	Skeletal muscle	—	Female	19 weeks	100 / 0
Tissue (Cancer)	Breast	—	Female	55	100 / 0
	Breast	Mongoloid	Female	Pool of 6 donors	0 / 100
	Liver	Caucasoid	Male	60	0 / 100
	Kidney	Caucasioid	Female	54	23 / 77
	Colon	Caucasoid	Male	75	100 / 0
	Colon	—	—	—	100 / 0
	Pancreas	Mongoloid	Male	32	100 / 0
	Stomach	—	—	—	100 / 0
	Uterus	—	Female	—	100 / 0
	Cervix	Caucasoid	Female	59	23 / 77
	Ovary	Caucasoid	Female	32	100 / 0
	Prostate	—	Male	—	100 / 0
Cell (Cancer)	HT-29*	Caucasoid	Female	44	92 / 8
	A549*	Caucasoid	Male	58	96 / 4
	DLD-1*	—	Male	—	25 / 75
	MCF-7*	Caucasoid	Female	69	23 / 77
	Raji*	Neglod	Male	11	17 / 83
	Panc-1	Caucasoid	Male	56	12 / 88
	MDA-M-231	Caucasoid	Female	51	100 / 0
	BT-20	Caucasoid	Female	78	78 / 22
	T-47D	Caucasoid	Female	54	53 / 47

* Tomitsuka, E., *et al.*, 2003

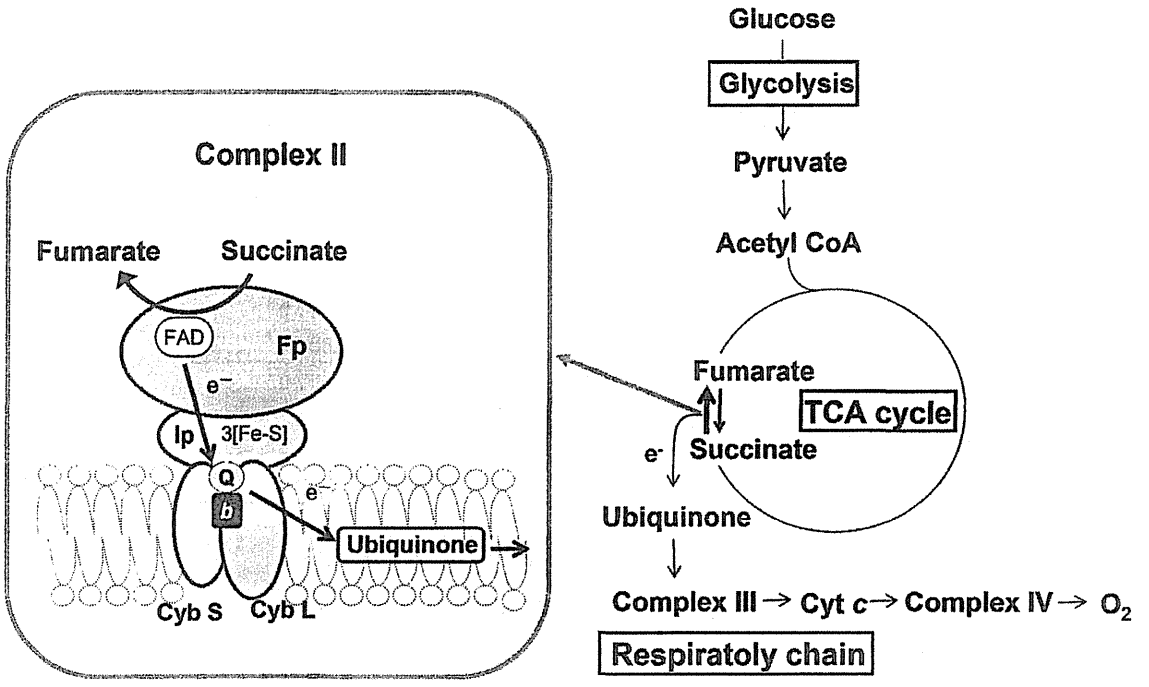
Complex II
Fumarate
Succinate
FAD
Fp
e⁻
Ip
3[Fe-S]
Q
b
e⁻
Ubiquinone
Cyb S
Cyb L
Glucose
Glycolysis
Pyruvate
Acetyl CoA
Fumarate
TCA cycle
e⁻
Succinate
Ubiquinone
Complex III → Cyt c → Complex IV → O2
Respiratoly chain

Figure 1

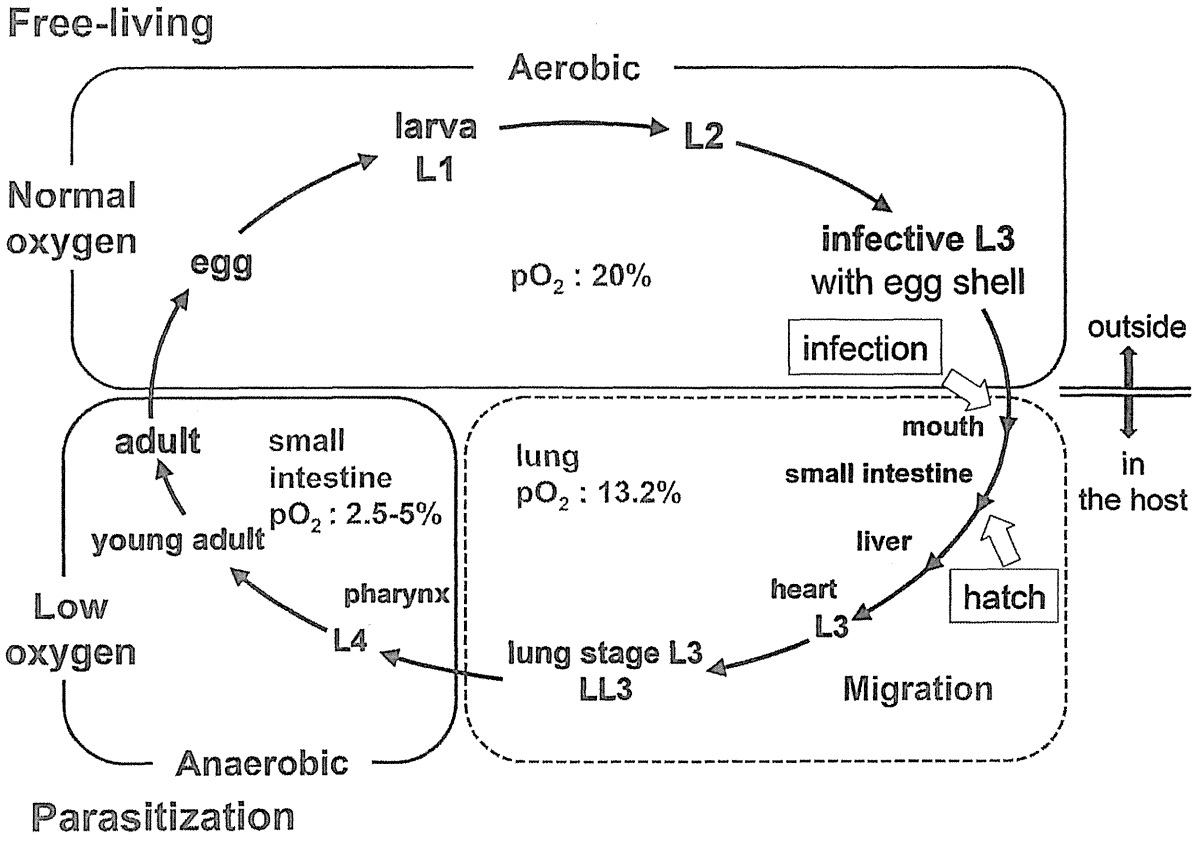
Free-living
Aerobic
larva
L1
L2
Normal
oxygen
egg
infective L3
with egg shell
pO2 : 20%
infection
outside
in
the host
mouth
small intestine
liver
heart
hatch
L3
lung
pO2 : 13.2%
lung stage L3
LL3
Migration
adult
small
intestine
pO2 : 2.5-5%
young adult
pharynx
L4
Low
oxygen
Anaerobic
Parasitization

Figure 2

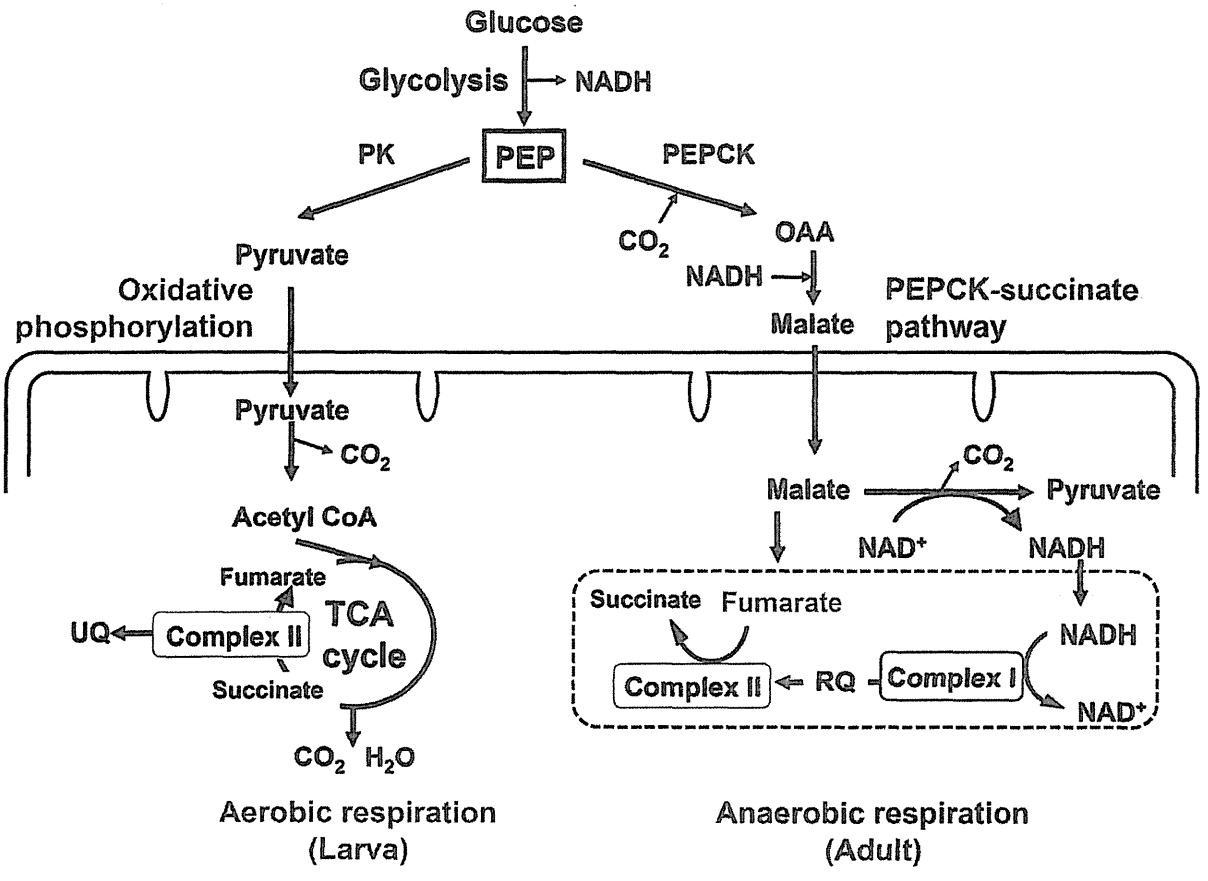
Glucose
Glycolysis
NADH
PK
PEP
PEPCK
CO2
OAA
NADH
Malate
PEPCK-succinate pathway
Pyruvate
Oxidative phosphorylation
Pyruvate
CO2
Acetyl CoA
Fumarate
UQ
Complex II
TCA cycle
Succinate
CO2 H2O
Malate
CO2
Pyruvate
NAD+
NADH
Succinate
Fumarate
NADH
Complex II
RQ
Complex I
NAD+
Aerobic respiration (Larva)
Anaerobic respiration (Adult)

Figure 3