Published in final edited form as:
Biochim Biophys Acta. 2013 May ; 1827(5): . doi:10.1016/j.bbabio.2012.12.007.

Physiological consequences of complex II inhibition for aging, disease, and the mK_{ATP} channel

Andrew P. Wojtovich[1], **C. Owen Smith**[2], **Cole M. Haynes**[3], **Keith W. Nehrke**[1], and **Paul S. Brookes**[4]

[1]Department of Medicine, University of Rochester Medical Center, Rochester, NY, USA

[2]Department of Biochemistry & Biophysics, University of Rochester Medical Center, Rochester, NY, USA

[3]Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

[4]Department of Anesthesiology, University of Rochester Medical Center, Rochester, NY, USA

Abstract

In recent years, it has become apparent that there exist several roles for respiratory complex II beyond metabolism. These include: (i) succinate signaling, (ii) reactive oxygen species (ROS) generation, (iii) ischemic preconditioning, (iv) various disease states and aging, (v) a role in the function of the mitochondrial ATP-sensitive K^+ (mK_{ATP}) channel. This review will address the involvement of complex II in each of these areas, with a focus on how complex II regulates or may be involved in the assembly of the mK_{ATP}.

Keywords

ATP sensitive potassium channel; mK_{ATP}; preconditioning; ischemia; succinate dehydrogenase; diazoxide; mitochondria

1. Introduction

Mitochondrial respiratory complex II/succinate dehydrogenase (SDH)/succinate ubiquinone oxidoreductase (SQR); EC 1.3.5.1 (referred to in this review as complex II) is a 124 kDa protein complex located on the matrix side of the mitochondrial inner membrane [1]. It is comprised of four subunits: SDHA, SDHB, SDHC and SDHD. SDHA, the flavin-adenine dinucleotide (FAD) containing subunit and SDHB, the iron-sulfur cluster containing subunit [2], are anchored to the membrane by the cytochrome *b* containing membrane proteins SDHC and SDHD. Complex II is a component of both the Krebs cycle and the respiratory chain [3]. Complex II oxidizes the Krebs cycle intermediate succinate, generating fumarate by passing electrons from succinate to FAD. The electrons are passed along the 2Fe-2S, 4Fe-4S and 3Fe-4S centers in SDHB, finally reducing one molecule of ubiquinone (co-enzyme Q_{10}) to ubiquinol (reviewed in [4,5]). A schematic is shown in Figure 1.

Unlike respiratory Complexes I or III, complex II does not pump protons across the inner membrane. However, complex II is capable of reducing co-enzyme Q_{10}, which can then be re-oxidized by complex III and thus participate in the proton pumping Q cycle of oxidative phosphorylation (Ox-Phos). It is generally believed that the primary function of complex II

*Corresponding author: Paul S. Brookes, PhD, Box 604, Anesthesiology, 601 Elmwood Avenue, Rochester, NY 14642, USA, Tel. 585-273-1626, paul_brookes@urmc.rochester.edu.

is to maintain the reduced state of the integral membrane Q pool [6]. Fully reduced co-enzyme Q_{10} has been shown to function as an antioxidant, protecting mitochondrial lipids and proteins from damage by constitutively produced reactive oxygen species (ROS) [7].

There exist several roles for complex II beyond metabolism, including succinate signaling, reactive oxygen species (ROS) generation, ischemic preconditioning, various disease states and aging, and in the function of the mitochondrial ATP-sensitive K^+ (mK_{ATP}) channel. This review will address the involvement of complex II in each of these areas.

2. Complex II inhibition and disease states

2.1 Cancer

Each of the four subunits of complex II is encoded by nuclear DNA. This is a unique feature of complex II compared to the other respiratory chain complexes, which contain additional mitochondrial DNA (mtDNA) encoded subunits. Mutations in each of the four complex II subunits in humans, although rare, have been identified (see Table 1), and result in two general phenotypic classes. Loss of function or nonsense mutations typically produce slow growing or benign (SDHC [8] and SDHD [9]) or highly aggressive (SDHB [10]) tumors of the carotid body, classified as hereditary paragangliomas or pheochromocytomas. In these cases there is typically no detectable SDH activity in tumors [11]. Mutations in the succinate dehydrogenase assembly factor 2 (SDHAF2) gene have also been identified, and produce tumors similar to those caused by SDH-B/C/D mutations [12]. Tumorous phenotypes only manifest after both copies of a particular SDH gene have been lost and thus are classified as tumor suppressor genes [13]. The link between complex II and tumor formation may involve the Warburg effect, wherein defects in Ox-Phos may play a role in driving metabolism more toward glycolysis. The shift in metabolism, which has been reported in SDH-derived pheochromocytomas, is thought to be a an important tumor survival mechanism [14].

The mechanisms through which loss of SDH activity promotes tumor growth via the Warburg effect are debated, and are summarized in Figure 2. The most prominent theories pose that complex II deficiency results in accumulation of metabolic intermediates that signal activation of tumor-promoting pathways. The first theory is that loss of SDH activity leads to an increase in matrix succinate, which freely enters the cytosol through the dicarboxylic acid transporter. Succinate has been shown to act as a cytosolic signaling molecule in the hypoxia-response pathway [15–17], by acting as an inhibitor of prolyl hydroxylases (PHDs) [18]. In this signaling pathway, cells respond to environmental oxygen levels though hypoxia inducible factor (HIF)-1α. In the presence of molecular oxygen HIF-1α is hydroxylated by PHDs and targeted by the E3 ubiquitin ligase Von-Hippel-Lindau (VHL) protein for degradation [19]. In low oxygen, or if PHD is inhibited, HIF-1α migrates to the nucleus where it forms a heterodimeric transcription factor complex with HIF-1β, activating genes that lead to increased glycolysis, angiogenesis, motility and survival [20]. This signaling pathway is generally regarded as pro-tumor survival, enabling cancer cells to survive the low oxygen environment of the tumor. In support of this mechanism as a link between complex II mutations and cancer, increased cytoplasmic succinate levels have been confirmed in SDH–A [21], –B [22], -C and –D [11,23] mutants in association with an activated HIF pathway (see also[24,25]). For further reviews see: [6,26–28].

Another key player in cancer, which has been linked to complex II signaling, is the tumor suppressor p53. Mutant SDHB/D backgrounds are associated with decreased levels of p53 and reduced p53 binding to NADH ubiquinone oxidoreductase I [29], further strengthening the link between decreased Ox-Phos and tumorous phenotype. Of interest from a mitochondrial viewpoint, transcriptional targets of p53 include the glycolysis-inhibiting

gene, TP53-induced glycolysis regulator (TIGAR), and the Ox-Phos activating gene, synthesis of cytochrome *c* oxidase 2 (SCO2) [30,31]. Cellular p53 can be activated by increased reactive oxygen species (ROS). When p53 is not bound to the SCO2 gene, SCO2 is active and stimulates Ox-Phos, leading to increased generation of reactive oxygen species (ROS) as a byproduct. If ROS levels rise p53 is activated and translocates to the nucleus where it binds to the SCO2 and TIGAR genes, inactivating and activating transcription, respectively. The activated TIGAR leads to increased production of the ROS scavenger glutathione. Interestingly, both hyper-activation of HIF and reduced p53 activity have been observed in the rare autosomal-dominant conditions Cowden syndrome (CS) and Cowden-like syndrome (CSL). These syndromes are associated with increased risk of tumors and hamartomas. In addition, CS and CSL patients harboring SDHB and SDHD mutations have been identified [29]. This suggests complex II may play a role in other tumors in which both HIF and p53 are implicated.

Complex II has begun to be recognized as a source of ROS [27,32,33]. Thus a second mechanism by which complex II mutations may facilitate cancer is by the generation of damaging levels of ROS. Structural models of bacterial complex II first indicated that the flavin subunit SHDA was capable of generating ROS [34]. Other complex II subunits were later identified as a sources of ROS, specifically SDHB [35–37], -C and –D [38] in yeast, SDHC in *C. elegans* [39] and mice [40], and SDHB but not SDHA in human cells. In one study the increase in ROS induced by SDHB mutants was found to be capable of stimulating the hypoxia-response by inhibiting PHD [41]. However in another study ROS generation by SHDB was not observed with siRNA knockdown [42] or in human cancer cells exhibiting an SDHB mutation [23]. The concept that complex II is a biologically significant source of ROS generation is relatively new, and the production of ROS by various mutants of complex II subunits therefore requires further analysis for a consensus to be reached.

2.2 Diabetes

ROS and succinate signaling, implicated in cancer formation as a result of complex II mutations, have also been linked to diabetes. Diabetes is classically characterized by an inability to regulate blood glucose levels due to the absence of (type 1) or acquired resistance to (type 2) insulin. In diabetes, mitochondria are subjected to an increase in substrate delivery as a result of prolonged increases in blood glucose levels. This leads to an increased rate of succinate and ROS generation [43–45]. In addition to its role as a signaling molecule in the hypoxia response, succinate has been identified as a ligand for the orphan G protein coupled receptor GPCR91 [46]. This receptor is expressed in the kidney suggesting that increased succinate signaling could contribute to renal hypertension, a disease that is closely linked to diabetes.

A mechanism was recently proposed where succinate regulation/accumulation is a key mediator of insulin release by pancreatic β-cells [47]. Altered mitochondrial function as well as increased ROS production were both implicated as pathogenic products of the diabetic state [48,49]. ROS generation was shown to be increased in insulin deficient rats respiring on complex I or complex II substrates. The increased ROS was largely attributable to increased electron leak from the co-enzyme Q_{10} binding site of complex III [50], or reverse electron flow through complex II [51].

Recent work has shown that the flavoprotein subunit of complex II (SDHA) is capable of generating physiologically relevant levels of ROS in isolated mitochondria when concentrations of succinate were low (400μM) and the inner-membrane pool of co-enzyme Q_{10} was mostly in its reduced form (i.e. complex I and III were inhibited). The authors determined that complex II was capable of generating ROS either from forward flow of succinate to fumarate or from the reverse reaction from reduced co-enzyme Q_{10}. It has been

demonstrated that complex II was capable of reducing much smaller pools of co-enzyme Q_{10} than any of the other respiratory complexes, and that complex II was fully active upon reduction of the respiratory chain and in the presence of ATP [6,52]. This suggested that the main function of complex II is maintenance of the reduced co-enzyme Q_{10} pool. Fully reduced co-enzyme Q_{10} serves as an antioxidant and helps protect mitochondria from free radicals, whereas the semi-reduced form is a pro-oxidant and is actually a generator of free radicals [53]. Overall, these results suggest that both the succinate signaling and the ROS generating mechanisms, potentially linking complex II dysfunction and diabetes, are not mutually exclusive. For further review see: [5,32,54].

2.3 Neurodegenerative diseases

Rare instances exist where loss of function mutations in SDHA result in paragangliomas, however such mutations more commonly result in a neurodegenerative disease known as Leigh's syndrome [55]. Leigh's syndrome develops in early infancy and is characterized by centers of necrotic cells in the brain stem and other regions of the central nervous system. SDH activity has been shown to be reduced by 25–50% relative to normal tissue. Interestingly another common neurodegenerative disease, Huntington's, has also been linked to dysfunctional SDHA [56]. Huntington's Disease (HD) is an autosomal dominant neurodegenerative disease that affects the striatum and cerebral cortex. People affected with HD typically show behavior symptoms including loss of coordination, decline of cognitive faculties, dystonia, and the involuntary movement disorder chorea [57]. Patients with HD have shown reduced mitochondrial complex II and III activities [58,59], and decreases in the levels of SDHA and SDHB have been observed in the striatum [60]. Tissue from postmortem HD patents has increased oxidative damage indicative of increased ROS (reviewed in [61]). Additionally systematic treatment of animal models with complex II specific inhibitors 3-nitroproponic acid or malonate cause phenotypes similar to HD, further supporting a link between complex II and HD [62,63].

Finally, another neurological condition in which complex II may play a role is Down syndrome (trisomy 21). It is now recognized that at least some of the *robust* nature of Down syndrome individuals (e.g., resistance to trauma) may originate from their having higher levels of the cytoprotective gas hydrogen sulfide (H_2S), since the H_2S generating enzyme cystathionine β synthase is encoded on chromosome 21 [64]. Notably, SDH has been identified as a potential site of inhibition by H_2S [65], such that prolonged elevation of H_2S may lead to neurodegenerative effects [66] possibly mediated by complex II inhibition.

2.4 Aging

A commonly held theory is that oxidative damage caused in part by ROS from mitochondrial respiration contributes to aging, however this model has been questioned recently, as roles for ROS as protective signaling molecules have been documented [67]. Furthermore, recent evidence in the nematode *C. elegans* indicates that removal of ROS detoxification enzymes has little effect on worm lifespan [68,69]. Similar results have recently been reported in mammals [70].

A mild decrease in mitochondrial respiration can extend lifespan in many model organisms, including worms [71–73], and this effect is conserved in mice [74,75]. In particular, mutations in complex I and III subunits as well as in the *clk-1* gene which encodes a protein involved in ubiquinone synthesis confer longevity [76]. Interestingly, while the ubiquinone product of the CLK-1 protein can transfer electrons between complexes I–III or between complexes II–III, the I–III transfer is specifically inhibited in the mutant [77]. Not coincidentally, complexes I and III are classically thought to be the main sites of ROS

production in the respiratory chain, and ROS production is critical for lifespan extension in the mutants [78].

Furthermore, lifespan is sensitive to oxygen concentration and may be HIF dependent [79,80], though other signaling pathways appear to respond to mitochondrial dysfunction as well [78,81–84]. The interface between these pathways may ultimately determine the rate of aging. In concert these results argue that ROS produced by complexes I and III as a result of decreased respiration can act as a signal that promotes longevity.

In dramatic contrast to the life-extending effects of mutations in complex I and III subunits, the *mev-1* mutation in the SDHC subunit of *C. elegans* complex II also results in increased ROS production, but decreases lifespan [85]. This association between complex II dysfunction and reduced lifespan was strengthened by the finding that independent mutation of *sdhb-1* (SDHB) resulted in an phenotype that was indistinguishable from *mev-1*, including increased ROS production [86]. Many aspects of the *mev-1* mutant phenotype are recapitulated in *Drosophila* [87], and introducing the *mev-1* mutant into mouse SDHC results in excessive apoptosis, low birth weight and neonatal growth retardation due to ROS overproduction and mitochondrial stress [88]. Although more apoptosis occurs in the *mev-1* model through activation of the canonical pathway, there is also premature accumulation of aging biomarkers, suggesting that it is not merely inappropriately timed death occurring, but rather accelerated aging.

There are also unique metabolic signatures that differentiate complex II mutants from those in complexes I and III, and it has been suggested that long-lived *mit* mutants utilize a novel metabolism normally seen under hypoxic conditions [89]. However, mutations in *ucp-4* that decrease succinate-driven complex II respiration suppress shortened lifespan in *mev-1* mutants [90], suggesting that it is the physical act of respiring through the mutant complex II that is damaging. Taken together, these results support the idea that ROS production from complex II differs in either quantity or quality from that generated by complexes I and III. For example, one possibility is that the amount of superoxide produced by complex II is much greater than I or III, and merely overwhelms the detoxification processes. Another possibility is that the ROS from complex II is distributed in a way that it is less easily detoxified or more easily creates molecular damage. A third possibility is that the ROS arising from complex II function (or dysfunction) may not be able to elicit adaptive responses through similar signaling pathways as ROS from complexes I and III.

Regardless, there appears to be an evolutionary bias toward protecting complex II function. For example, *Drosophila* that have been adapted to live under otherwise lethal hypoxia exhibit nearly a two-fold decrease in complex II activity, with only mild changes to the other respiratory complexes and consequently less superoxide leakage occurring during mitochondrial respiration [91]. With respect to mitochondrial disease, for nearly all the other complexes (esp. complexes I and IV), there exist age-dependent correlations for mutations in the complex and in particular the mtDNA encoded subunits, which are acutely subject to mitochondrial ROS. However, complex II is entirely nuclear-encoded, which may be a kind of a safe-haven, removed from the ROS onslaught that the other mtDNA encoded complexes are bombarded with, and protected by nuclear DNA damage repair enzymes. It is intriguing to speculate that this is because the effects of complex II dysfunction are so detrimental.

A recent study on mitochondrial biochemistry in the developing mouse heart reported on a preference for complex II over complex I as the electron entry point in the respiratory chain at embryonic day 9.5, which switches to a preference for complex I by embryonic day 13.5 [92]. A second interesting finding of this paper is that cellular ROS levels rose at the time

complex II was the preferred electron entry point [92]. This work provides evidence that complex II is important in early development as well as aging.

3. Ischemia reperfusion (IR) injury and ischemic preconditioning (IPC)

Complex II and ROS are implicated in the acute pathology of ischemia reperfusion (IR) injury and the following sections are focused on the cardiac model. Key hallmarks of IR injury are a loss of oxygen and substrate supply, acidification, mitochondrial Ca^{2+} overload, loss of adenine nucleotides, permeability pore transition opening, and the overproduction of ROS [93]. One mechanism of protecting the heart from stress is ischemic preconditioning (IPC), whereby brief periods of ischemia prior to a longer ischemic insult provide protection against IR injury [94]. Mitochondria have emerged as a critical component of the signaling mechanisms involved in IPC [95–97]. The precise mechanism of IPC is elusive but involves an array of signaling cascades activated by a mild increase in ROS [98–100]. Many of these signaling events converge on the mitochondrion and the protection of IPC can be mimicked via the administration of pharmacological agents that act on the mitochondrion; in particular, complex II and the mitochondrial ATP-sensitive K^+ channel (mK_{ATP}) are targets of protective compounds [96].

3.1 complex II and IPC

IPC is known to inhibit mitochondrial electron transport and not surprisingly mimicking this reduction in activity with mitochondrial respiratory inhibitors is cardioprotective [97]. IPC reversibly inhibits complex II [101,102]; however, the mechanism remains unclear. One potential mechanism involves formation of endogenous complex II inhibitors. ROS are an important component in IPC signaling, and under conditions of oxidative stress the complex II inhibitor malonate can be generated [102] via the non-enzymatic decarboxylation reaction between H_2O_2 and oxaloacetate (OAA) [103]. The competitive inhibitor malonate is also cardioprotective [102,104].

While both malonate and OAA are inhibitors of complex II, malonate-mediated inhibition is more readily reversed. Thus, a malonate-occupied complex II would be desirable at reperfusion [97]. However, the removal of malonate from complex II requires ATP [105], which is not immediately available in early reperfusion. This suggests that malonate removal and reversal of complex II inhibition following ischemia may be a gradual process. As such, malonate may be considered an appropriate inhibitor, since it is easy to remove at the right time. Furthermore, malonate formation from OAA may offer additional benefit since a decrease in the concentration of OAA would prevent rapid re-introduction of acetyl-CoA into the Krebs cycle at reperfusion. Effectively, malonate formation supports a “gradual wake-up” of the respiratory chain from ischemia/reperfusion, thereby providing cardioprotection [97].

In addition to an ROS-mediated mechanism of complex II inhibition during IPC, reactive nitrogen species (RNS) may also participate. For example, nitro-linoleate is generated during IPC, is cardioprotective [106,107] and inhibits complex II [108]. Also, nitroxyl (HNO) inhibits complex II [108,109] and is cardioprotective [108,110]. These compounds react with important thiols in complex II and suggest that complex II may act as a redox sensor. Notably, complex II can also be glutathionylated on the SDHA subunit, resulting in an increase in activity [111]. In IR injury, SDHA becomes deglutathionylated, resulting in impaired activity [111]. It is currently unknown if protective interventions such as IPC are capable of modulating the degree of complex II de-gluathionylation that occurs in subsequent IR injury. If so, this would provide another mechanism of control over complex II during the critical early reperfusion phase.

Finally, while the modulation of complex II by endogenous inhibitors may provide protection, it has recently become apparent that more potent and specific complex II inhibitors such as 3-nitropropionic acid [112,113] and atpenin A5, [104,114] are cardioprotective, opening the door for complex II as a therapeutic target in IR injury.

3.2 complex II and the mitochondrial ATP-sensitive K^+ channel (mK_{ATP})

The mechanism of complex II inhibition and how it is protective remains unclear, but is thought to involve the mitochondrial ATP-sensitive potassium channel (mK_{ATP}). The mK_{ATP} is a central component of IPC-mediated protection, such that channel openers can mimic IPC [115,116] and channel blockers block IPC [117–119]. The mitochondrial K^+ cycle plays an established role in regulating mitochondrial volume and function [120,121]. The precise mechanism of protection elicited by the mK_{ATP} remains elusive but may involve the regulation of mitochondrial matrix volume and adenine nucleotide status, as well as the prevention of mitochondrial Ca^{2+} overload and the overproduction of ROS [122,123].

Despite the central role of the mK_{ATP} in protecting the heart, the molecular composition of the channel remains debated (see Figure 3). Similarities in pharmacologic agents and channel characteristics suggest that the mK_{ATP} may be related to the well-defined surface K_{ATP} channel. However, mK_{ATP} specific agents (e.g., diazoxide and 5-HD) which are used to distinguish the channel from the surface K_{ATP} [115] suggest a variation in structure. Furthermore, the mK_{ATP} specific opener diazoxide inhibits complex II activity, and other complex II inhibitors open mK_{ATP} [102,104,104,108,113]. While the relationship between complex II inhibitors and the mK_{ATP} has been alluded to, this relationship remains to be fully characterized.

The combination of a lack of a consensus structure and ‘off-target’ effects of channel modulators has led to the hypothesis that the mK_{ATP} may be composed of known mitochondrial components such as complex II, rather than canonical K_{ATP} subunits. The evidence for each hypothesis is provided in the following sections, with current models for mK_{ATP} shown in Figure 3.

3.3 Molecular composition of the mK_{ATP}

3.3.1 Canonical Kir6.x/SUR composition?—A canonical surface K_{ATP} channel is made of a pore-forming inwardly rectifying K^+ channel subunit (Kir6.1 or Kir6.2) coupled with an auxiliary sulfonylurea receptor (SUR1, SUR2A or SUR2B) subunit [124], in an octomeric conformation (Figure 3). Agents which inhibit (e.g., glyburide) or activate (e.g., pinacidil and nicorandil) the surface K_{ATP} also affect the mK_{ATP}, and following the discovery of ATP-sensitive K^+ currents in mitochondria [125] efforts were focused on ascribing a Kir6.x/SURx composition to the channel. However, Kir6.x/SURx are not found in mitochondrial proteome databases [126] and they lack canonical mitochondrial targeting sequences [127]. It has therefore been proposed that other mechanisms that involve structural features such as protein folding [128] or post-translational modification [129] may target these proteins to the mitochondrion.

Previous approaches to identify the mK_{ATP} have relied heavily on immunologic techniques and yield a complicated outlook, with reports of Kir6.1 [130–132], Kir6.2 [133], both [134–136] or neither [137] in mitochondria. Moreover two widely used anti-Kir6.1 antibodies have been shown to recognize mitochondrial proteins not related to a Kir subunit [138]. Trafficking of a Kir6.x subunit to the cell surface requires its association with a SURx and suggests that if a Kir6.x subunit exists in the mitochondrion, it would be associated with SURx. Recently, a splice variant of SUR2 was found to localize to mitochondria, however it did not contribute to mK_{ATP} activity [139].

The mK_{ATP} is central to IPC-mediated protection, and while knockout mice for Kir6.1, Kir6.2, SUR1 and SUR2 [140–142] exist, confounding vascular effects preclude definitive evidence for the role of Kir6.x/SURx in IPC [140,143–145]. A key example is that many of these K_{ATP} channel mice exhibit diabetes, which is known to diminish the response to IPC [144]. Previous measurements of mK_{ATP} activity relied on flavoprotein fluorescence as an indicator of channel activity and found that the loss of Kir6.1 or Kir6.2 in mice does not affect flavoprotein fluorescence [146,147].

More recent studies suggest an alternative mK_{ATP} composition, hypothesizing that a variant of Kir1.1 (the canonical renal outer medullary K^+ channel, ROMK) may be the pore-forming subunit of the mK_{ATP} [148]. However, reconciliation of several inconsistencies would help to validate this claim. For example, the ATP sensitivity of ROMK is much higher ($K_{1/2}$ = 2.3 mM) [149] than that measured for the mK_{ATP} (1–25 μM) [150,151]. Furthermore, the tricyclic antidepressant drug fluoxetine (Prozac) is known to inhibit both the mK_{ATP} (IC_{50} 2.4 μM) and IPC [151], but Kir1.1 is insensitive to fluoxetine [152,153]. Direct measurements of mK_{ATP} activity from genetic models (e.g., ROMK knockout mice), which are currently lacking, may be necessary to designate mK_{ATP} channel status to Kir1.1. Therefore, while Kir1.1 may indeed be present in mitochondria, its role in IPC remains to be fully elucidated.

The mK_{ATP} channel activity is conserved across a range of species and cross-species comparisons may help to identify or rule out certain candidate genes. For example, while the mammalian genome codes for 15 Kir isoforms, the *C. elegans* Kir family contains only three genes, *irk-1*, *irk-2*, and *irk-3*. Using the power of worm genetics, we recently showed that a triple knockout *irk-1/2/3* worm exhibited perfectly normal mK_{ATP} activity and preconditioning [154]. These data demonstrate that at least in *C. elegans*, the mK_{ATP} is not derived from the Kir family. While more work is needed to fully elucidate the role of canonical Kir/SUR subunits in the mammalian mitochondrion, these results suggest that non-Kir proteins should also be considered as candidates for the mK_{ATP}. In this regard, the lack of consensus regarding off-target effects of K_{ATP} agents (described below) has led to the hypothesis that the mK_{ATP} is composed of known mitochondrial proteins, including complex II.

3.3.2. Alternative hypothesis: a complex II composition for mK_{ATP}?—The notion that the mK_{ATP} resembles the surface K_{ATP} was largely built on the use of small molecules. Inhibitors and activators of the defined surface K_{ATP} had expected effects on isolated mitochondria K^+ fluxes. However, most of these molecules have off-target effects on mitochondrial function (reviewed in [155]). For example, glyburide is a sulfonylurea used to treat type 2 diabetes. Via its interaction with the SUR (140–180 kDa), glyburide inhibits K_{ATP} channels [156]. Glyburide also prevents the protective effects of mK_{ATP} activation. Studies using labeled glyburide found that it binds to a 28 kDa protein in heart [157] and a 64 kDa in brain mitochondria [158]. These weights are significantly smaller than the apparent molecular weight of the surface K_{ATP} channel, and they may represent a mitochondrial targeted splice variant [139] or a SUR-independent target. In this regard, glyburide at high doses inhibits respiration [159,160] and molecular modeling demonstrates that it may interact with the adenine nucleotide translocator (ANT) [161]. Work in our laboratory (unpublished) has found that a BODIPY conjugate of glyburide binds to intact complexes I and V on clear-native electrophoresis gels.

Perhaps the most widely used tool related to mK_{ATP} studies is diazoxide. While diazoxide is a general K_{ATP} opener, it is more potent at opening the mK_{ATP} and is considered a mK_{ATP} specific agonist [115]. However, diazoxide has off-target mitochondrial effects, such as mitochondrial uncoupling at high concentrations, and inhibition of complex II. It is

important to note that while diazoxide does implicate a relationship between complex II and the mK_{ATP}, other channel openers such as cromakalim do not affect complex II activity. This suggests that complex II is not the sole component of the channel and may represent an important regulator. Nonetheless, these effects of diazoxide on complex II are interesting when viewed alongside another characteristic of complex II – namely the fact that ATP allosterically activates complex II activity [102,162]. This suggests an inverse relationship between complex II and mK_{ATP} activities.

Rather than serving as the mK_{ATP} channel alone, it has been proposed that complex II may form a complex with other known mitochondrial proteins, to elicit mK_{ATP} channel activity [137]. This complex includes the phosphate carrier, mitochondrial ATP-binding cassette protein-1, the ANT, and respiratory complex V [137]. These proteins were purified as a fraction from mitochondria, and while the fraction contained over twenty proteins identifiable by silver staining, these 5 proteins were the majority components [137]. The purification and reconstitution of these proteins into proteoliposomes reproduced the pharmacologic characteristics of the mK_{ATP} channel [137], including the ability of complex II inhibitors to stimulate K^+ flux. Thus, it was suggested that these proteins may exist in a super-complex comprising the mK_{ATP} channel. There is a precedence for super-complexes in the respiratory chain [163] and evidence for coupling between complex II and complex V deficiencies in humans [164]. It has also been suggested that diazoxide interacts with complex V, facilitating the binding of its endogenous inhibitor to the F(1) subunit [165] and reversibly inhibiting ATP hydrolysis.

A shortcoming of this proposed mK_{ATP} super-complex is the lack of an actual K^+ transporting protein in its composition [137]. All known K^+ channels contain the consensus sequence GYG in their pore region, providing a selectivity filter for potassium. There is some evidence that the ANT can transport K^+ [166,167], and molecular modeling suggests that the K_{ATP} blockers glyburide and 5-HD can interact with the ANT [168], but ANT does not have a GYG motif. Thus the possibility remains that a low abundance [169] bona fide K^+ channel subunit may be present in the super-complex, to confer mK_{ATP} activity.

The functional and pharmacologic evidence supporting the hypothesis that complex II is a component or regulator of the mK_{ATP} channel is summarized as follows: 1) complex II inhibition is observed in IPC [101,102], 2) mK_{ATP} inhibitors stimulate complex II [102,162,170], 3) mK_{ATP} openers inhibit complex II [102,104,170,171], 4) complex II inhibitors open mK_{ATP} and are cardioprotective in a manner sensitive to mK_{ATP} blockade [104,108,113]. The array of structurally distinct complex II inhibitors capable of protecting by opening mK_{ATP} range from endogenously generated (e.g., malonate) to potent and specific (e.g., atpenin A5). These inhibitors interact with complex II at different sites and all elicit mK_{ATP} activity.

One unique aspect that has arisen from these studies is that, like diazoxide, the amount of inhibitor required for maximal mK_{ATP} activity is much lower than that required for the enzymatic inhibition of complex II. For example, atpenin A5 has an IC_{50} for complex II activity of 10 nM, while 1 nM yields maximal mK_{ATP} activity. Taking advantage of the potency of atpenin A5, the relationship between complex II and mK_{ATP} activity was quantified, with the finding that only 0.4% of complex II molecules were necessary to elicit maximal mK_{ATP} activity [169]. Not only does this account for the low abundance of the channel [172] it also demonstrates that bulk complex II activity is not affected by the presence of the inhibitor. These results imply that the specificity of diazoxide for the mK_{ATP} vs. the surface K_{ATP} may reside at the level of complex II [169]. Finally, it is notable that all known complex II inhibitors can activate the mK_{ATP} channel even when mitochondria are

respiring on complex I linked substrates (e.g. pyruvate plus malate). This suggests that complex II does not need to be enzymatically active to participate in the mK_{ATP}.

4. What is the endogenous physiologic role of the complex II – mK_{ATP} channel interaction?

As discussed in the preceding sections, a role for both complex II and mK_{ATP} channels in IPC has been well established. However, these proteins clearly did not evolve for this purpose. Therefore, how do they interact under normal conditions, and what is their baseline/endogenous physiologic role in the absence of a stress signal such as IPC?

4.1 A ROS signal?

A key question in the mK_{ATP} field is how complex II transmits a signal to induce mitochondrial K^+ uptake, and the physiologic relevance of this phenomenon. One proposed mechanism is that, independent from direct complex II ROS generation [173], complex II inhibition may trigger ROS formation by a different part of the respiratory chain (complex III), and this is responsible for subsequent protective signaling [174]. In brief, the reduction state of the co-enzyme Q_{10} pool can influence complex III ROS production, especially when complex III is inhibited with antimycin [32]. When electrons are supplied via complex II, inhibitors of complex II can stimulate antimycin-induced ROS production in a bell-shaped response, as a consequence of the redox state of the co-enzyme Q_{10} pool [175,176] (recently reviewed in [177]). Since mild ROS generation is a critical component of IPC, this mechanism was hypothesized to mediate the protective effect of complex II inhibitors and diazoxide, completely independent of any role for the mK_{ATP} channel [174]. However, several pieces of evidence suggest this ROS phenomenon is independent of complex II's role in the mK_{ATP} or in IPC. These will now be discussed in detail:

- **i.** The increase in ROS is dependent on succinate: The increase in ROS with complex II inhibitors was only observed when mitochondria respired on succinate and not present when complex I or complex III were used as electron sources [174,178]. On the contrary, complex II inhibitors can open the mK_{ATP} even when mitochondria respire on complex I or complex IV linked substrates [102,104,104,169], or when channel components are reconstituted in proteoliposomes or lipid bilayers [137] (reviewed in [179]).
- **ii.** ROS generation requires the presence of antimycin A: In the absence of antimycin, complex II inhibitors did not increase ROS formation, rather the addition of inhibitors resulted in a decrease in ROS [174,176]. Another study demonstrated a decrease in ROS formation with complex II inhibitors in the presence of succinate, rotenone and myxothiazol [173]. Furthermore, modulation of the co-enzyme Q_{10} pool redox state by cyanide [176] or loss of cytochrome *c* does not increase complex III ROS [180]. Thus, the increase in ROS induced by complex II inhibitors appears unique to antimycin. Contrastingly, studies demonstrating mitochondrial K^+ flux induced by complex II inhibitors did not have antimycin present [102,104,108,137,151,169,181].
- **iii.** ROS generation is independent of K^+, or mK_{ATP} inhibitors: A hallmark of mK_{ATP} activity is its sensitivity to K^+. In this regard, both mitochondrial K^+ fluxes and the protective effects of complex II inhibition are K^+ sensitive [102,104,108,113,137,151], while antimycin induced ROS generation was insensitive to loss of K^+ [174,178]. Similarly, both mitochondrial K^+ fluxes and the protective effects of complex II inhibition are sensitive to mK_{ATP} channel inhibitors [102,104,108,113,137,151,182], while antimycin-induced ROS

generation is not [174,178]. These findings highlight that ROS generation in response to complex II modulation is completely independent from mK_{ATP} and mitochondrial K^+ flux.

iv. ROS generation via this mechanism requires higher concentrations of complex II inhibitors than required to activate the mK_{ATP} channel [174]: Diazoxide at high concentrations (>100μM) may result in uncoupling or activate surface K_{ATP} channels. When used at appropriate concentrations (10–30 μM) diazoxide displays mK_{ATP} specificity [115]. Recently we demonstrated that a low concentration of the complex II inhibitor atpenin A5 activates mK_{ATP} activity in isolated mitochondria and results in mK_{ATP}-sensitive cardioprotection [104]. Moreover, this concentration (1 nM) is without effect on bulk complex II enzymatic activity [169]. However, the antimycin-induced ROS generation was only seen at atpenin A5 concentrations (5–50 nM) that would influence complex II activity and subsequently the state of the co-enzyme Q_{10} pool.

Together, these findings suggest that the mK_{ATP} activity and cardioprotection mediated by complex II inhibition are independent of antimycin-induced ROS generation. The currently available evidence does not favor ROS generation as an intermediate signal between complex II, mK_{ATP}, and cardioprotection/IPC.

4.2 Energy sensing

The mK_{ATP} has been described in humans, rats, mice, plants, amoeba, *T. cruzi* and *C. elegans* [125,133,183–186]. Although the primary research interest in the mK_{ATP} field is cardioprotection, most of these species do not have a heart or suffer from any cardiac disease, suggesting that the channel may have an evolutionarily conserved physiologic role. If the mK_{ATP} is a super-complex of complex II, the phosphate carrier, mitochondrial ATP-binding cassette protein-1, ANT, and complex V, this may suggest a relationship between mitochondrial K^+ fluxes and bioenergetics. This view is compatible with the energy-sensing capabilities of the canonical surface K_{ATP} channel. While other metabolic sensing pathways such as mTOR [187], SIRT3 [188] and AMPK [189] may involve changes in complex II [190–192], they integrate more long term changes in metabolic state. In contrast, the complex II/mK_{ATP} system may respond more rapidly to energetic status. Since regulation of metabolism is known to be a strategy for protecting the heart against ischemia [97,193], any component which can regulate metabolism or the energetic status of the mitochondrion (e.g. mK_{ATP}) may also be a critical component of a cell's cardioprotective machinery.

The complex II/mK_{ATP} complex is hypothesized herein to exert control over metabolism in addition to "classical" respiratory control (i.e., state 3 / state 4), to meet the cell's energy demand. These mechanisms are shown in Figure 4. First, the introduction of electrons into the respiratory chain is competitive, such that the oxidation of succinate at complex II inhibits the oxidation of NADH at complex I [52]. Since complex II does not pump protons, the H^+/e^- stoichiometry is lower when electrons enter at complex II. Thus, the ratio of electron entry between complex II vs. I can lead to changes in the overall efficiency of Ox-Phos. This becomes an important mechanism of regulating metabolism, when coupled with the fact that complex II is activated by ATP. Under conditions of high ATP, complex II is active, thus inhibiting complex I, and lowering H^+/e^-, leading to inefficient Ox-Phos. Alternatively, when ATP is low, complex II activity is also low, so more electrons enter at complex I, and H^+/e^- rises to meet the demand for more ATP [52].

In a second novel mechanism of control, regulation of mitochondrial matrix volume by the mK_{ATP} channel may serve as a coupling link between ATP levels and the activity of the Ox-Phos machinery. Mitochondrial K^+ uptake is followed by osmotically obliged water,

resulting in matrix swelling [120]. Matrix volume controls efficient energy transfer (via the creatine kinase (CK) shuttle system), and controls the activity of matrix enzymes necessary for ATP production, and is critical to the regulation of energy metabolism [121,172,194–200]. As such, when ATP levels are high, the mK_{ATP} is inhibited, the matrix shrinks, and the resulting loss of inner/outer membrane contacts decreases the efficiency of the CK shuttle system. In contrast, low ATP levels facilitate K^+ influx via mK_{ATP}, leading to matrix swelling, which enhances inner/outer membrane contact and stimulates efficient shuttling of high energy phosphates.

As depicted in Figure 4, the combination of the influence of K^+ on mitochondrial volume and metabolism, with complex II activity and energy sensing capabilities, suggest that these phenomena may be components of a concerted mechanism to modulate Ox-Phos to meet energy demands. This proposed physiologic role of the mK_{ATP} also makes the channel an important component of how the cell responds to pathologic conditions (e.g., IR injury).

4.3 Mitochondrial Fission/Fusion?

Recently however, an intriguing link has been proposed between a super-complex type mK_{ATP} channel (see section 3.3.2) and Charcot-Marie-Tooth disease type 2A [181]. Typically, this disease is associated with mutations in mitofusin 2 (MFN2), which is an important regulator of both mitochondrial fusion [201] and membrane potential [202]. Notably, the study found that a mouse model carrying an MFN2 mutation had a defect in both complex II (40% reduction) and complex V (30% reduction) [181]. Interestingly, the lost enzymatic activities were reversed by the mK_{ATP} inhibitor 5-HD. Furthermore, the phenotype could be mimicked by the mK_{ATP} channel opener diazoxide [181]. What remains unclear, is how a mutation in MFN2 (an outer membrane protein with the bulk of its structure facing the cytosol) communicates with complex II and V in the inner membrane. However, this study highlighted a role for complex II (and complex V) and the mK_{ATP} in disease. Further studies are needed to investigate the link between mitochondrial K^+ flux and fission/fusion.

5. Conclusions and Future Directions

Complex II inhibition plays a dual role in biology, being both a destructive force in many disease states and also a protective mediator in others. Complex II “the destroyer” is capable of generating ROS in a manner that may be different in quantity or quality from complexes I and III. The genes encoding complex II are protected in the nucleus and may represent a mechanism to protect the cell from devastating ROS production resulting from mutated complex II. Complex II “the protector” connects mitochondrial volume regulation and metabolism via the mK_{ATP} channel. Despite a role for mK_{ATP} in both physiological and pathological conditions, the underlying molecular structure of this channel and why it is evolutionarily conserved remains unknown. Since the phenomenon of the mK_{ATP} has been described in a range of organisms from plants to mammals, comparative genomics may yield promising candidates, as was recently applied to the identification of the mitochondrial calcium uniporter [203,204]. However, if the mK_{ATP} is composed of known mitochondrial components (e.g. complex II) this approach may not be successful. Pharmacologic evidence has suggested that the channel is molecularly related to the surface K_{ATP}, and while a significant pharmacologic overlap made this hypothesis attractive, model organism genomics has shown that a Kir subunit is not necessary for mK_{ATP} channel activity or protection against stress [154]. On the other hand, complex II (and other components in the super-complex) are implicated not only in energy sensing but also mitochondrial K^+ fluxes.

Despite recent reports [148], the quest for the precise molecular identity of the mK_{ATP} and the role of complex II in regulating it continue. In particular, although knockout mice for the

mK_{ATP} candidate gene ROMK are available, these mice exhibit renal pathology [205] which could the interpretation of experiments on cardioprotection. Clearly, a cardiac-specific $ROMK^{-/-}$ mouse would be a useful tool to elucidate the role of ROMK in cardioprotection. Furthermore, newly discovered inhibitors or activators of ROMK and other members of the inward rectifying K^+ channel family [206] will be useful tools, and (in the case of activators) potential therapeutics.

Although the complete loss of complex II in mammalian models is lethal [207], the generation of inducible tissue-specific knockout models may provide the tools necessary to address the role of complex II in cytoprotection. In such models, an acute loss of complex II activity in adulthood would be necessary, to avoid the contribution of developmental and metabolic affects associated with long term loss of function. Alternatively, microRNA regulation of complex II (e.g., by miR-210 [208]) may be a promising avenue to influence complex II levels, and is worthy of pursuit not only from a mechanistic angle, but also due to the increasing interest in miRNAs as therapeutic targets.

Finally, although complex II inhibitors are prevalent (Table 2), there is currently only one allosteric activator of complex II activity, namely ATP. Clearly such activators could have both therapeutic and investigational value, and the search for more such molecules should be encouraged.

Acknowledgments

This work was supported by an American Heart Association Founder's Affiliate Postdoctoral Fellowship award 11POST7290028 (to A.P.W.), and by US National Institutes of Health grants HL071158 (to P.S.B.), GM087483 (to P.S.B. & K.W.N.).

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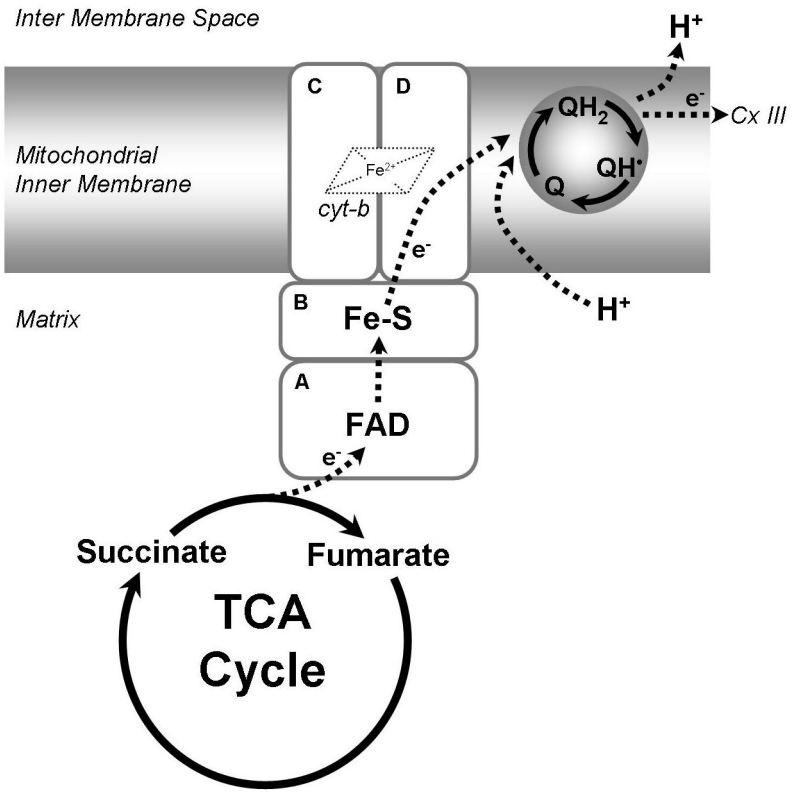

Figure 1.

Complex II: Complex II is the succinate dehydrogenase enzyme of the TCA cycle oxidizing succinate to fumarate. The succinate ubiquinone oxidoreductase enzymatic activity transfers electrons from succinate through the FAD moiety of SDHA to three iron sulfur centers of SDHB and finally to Coenzyme Q_{10} via SDHC/D. Although the enzyme containe a heme iron, its role in electron transfer is uncertain. Complex II acts to increase the pool of reduced Coenzyme Q_{10} (QH_2) in the mitochondrial inner membrane. Reduced Coenzyme Q_{10} then transfer electrons to complex III.

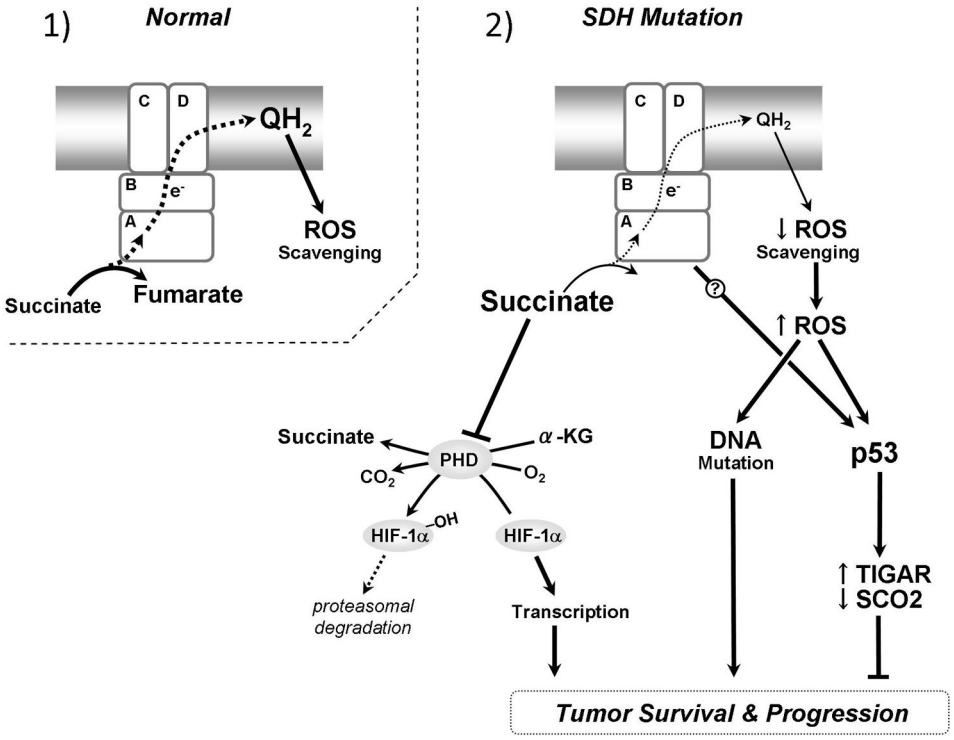

Figure 2.

Metabolic and cancer related consequences of mutations in the SDH complex: 1) wild-type SDH subunits form a functional complex II. Succinate is oxidized to fumarate keeping cellular succinate levels low. Electrons are transferred to Coenzyme Q_{10} which can act as an antioxidant maintaining low levels of ROS. 2) Mutations in any of the four SDH subunits inhibit or impair normal election flow. Left: Increased succinate enters the cytosol where it inhibits prolyl hydroxylase (PHD) mediated degradation of HIF-1α, resulting in translocation of HIF-1α to the nucleus and increased transcription of genes for tumor survival. Right: Disrupted electron flow through complex II leads to increased ROS (either directly or via less scavenging by reduced Co-enzyme Q_{10}). ROS can act directly as a mutagenic agent on DNA or as a signaling molecule activating p53. p53 responds to ROS by increasing genes responsible for inhibiting glycolysis and ROS scavenging and decreasing genes involved in stimulating Ox-Phos. Loss of p53 in conjunction with mutations in complex II can result in dysregulation of these genes.

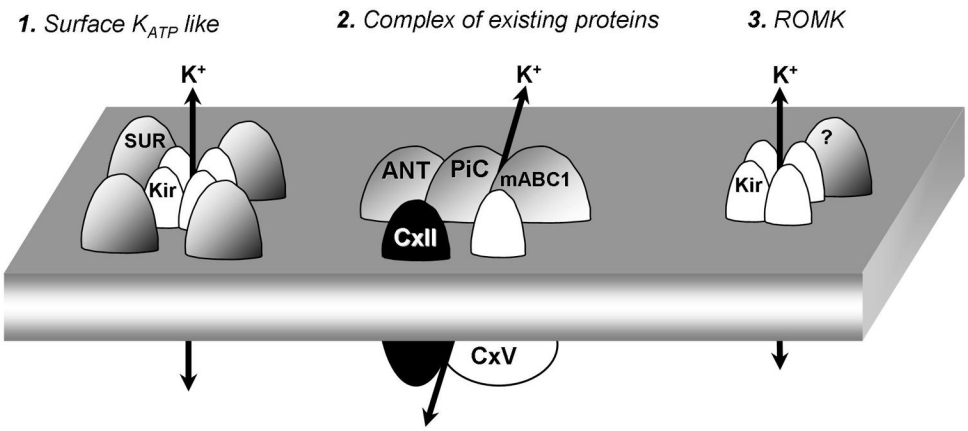

Figure 3.

Current models for the mK_{ATP}: 1) The mK_{ATP} mayo be composed of a Kir/SUR octamer. Evidence is based on the composition of known K_{ATP} channels from cell membranes and immunoreactivity of mitochondrial inner membrane fractions to anti-Kir and anti-SUR antibodies. 2) The mK_{ATP} may be composed of a super complex of known inner membrane proteins adenine nucleotide transporter, phosphate carrier, mitochondrial ATP binding cassette protein 1, complex II and complex V. This hypothesis is based on detection of mK_{ATP} activity in reconstituted proteo-liposomes containing these five proteins [137]. 3) The mK_{ATP} is proposed to be composed of a tetramer of the Kir1.1 protein (renoal outer medullary potassium channel, ROMK) [148].

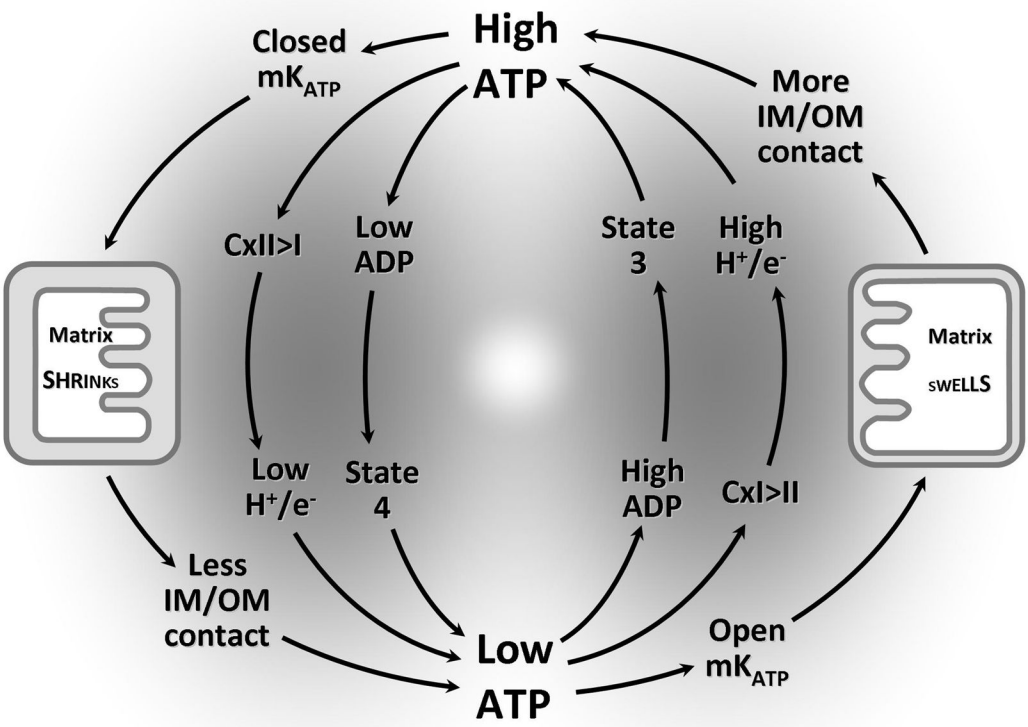

Figure 4.

Complex II/mK$_{ATP}$ interaction in energy sensing: "Classical" respiratory coupling is shown in the center, in which mitochondria transition between quiescent (state 4) and phosphorylating (state 3) conditions depending on availability of ADP. Additional levels of control are shown in outer circuits, including: (i) ATP is a complex II activator, so low ATP leads to preferred electron entry via complex I, which in turn increases the efficiency/stoichiometry of Ox-Phos (H^+/e^- ratio), increasing ATP synthesis (right side). The reverse is true when ATP is high (left side). (ii) Low ATP dis-inhibits (opens) the mK$_{ATP}$ channel, leading to K^+ and water entry and swelling of the matrix (right side). This leads to greater contact between the inner and outer membranes (IM/OM), enhancing the creatine kinase shuttle and ATP availability for the cell. When ATP is low, the cycle reverses, as ATP closes the mK^{ATP} channel, the matrix shrinks, and IM/OM contact decreases, lowering ATP transport to the cytosol (left side).

Table 1

Complex II mutant phenotypes associated with cancer and other diseases.

Subunit	Gene Locus	Phenotype	Malignancy	MIM #	Reference
SDHA	5p15.33 [55]	Cardiomyopathy	?	613642	[209]
		Leigh Syndrome		256000	[55,210–212]
		Mitochondrial Cx II deficiency		252011	[55,210–212]
		Paraganglioma type 5		614165	[213]
SDHB	1p36.13 [214]	Cowden-like syndrome	High	612359	[215]
		GIST		606764	[216]
		Paragangliomas and GIST		606864	[217,218]
		Paraganglioma type 4		115301	[10,219–223]
		Pheochromocytoma		171300	[10,22,224]
SDHC	1q23.3 [217]	GIST	Low	606764	[216]
		Paragangliomas and GIST		606864	[217]
		Paraganglioma type 3		605373	[8,225]
SDHD	11q23.1 [226]	Carciniod Tumors Intestinal	Low	114900	[227]
		Cowden-like syndrome		612359	[215]
		Merkel Cell Carcinoma (Somatic)		-	[227]
		Paragangliomas and GIST		606864	[217]
		Paraganglioma type 1		168000	[9,221,224,228–232]
		Pheochromocytoma		171300	[229,233]
SDHAF1	19q13.12 [234]	Mitochondrial Cx II deficiency		252011	[234]
SDHAF2	11q12.2 [12]	Paraganglioma type 2	?	601650	[12,235]

Legend. GIST, Gastrointestinal stromal turmors (GIST); Cx II, complex II.

Table 2

Complex II modulators.

Reagent	Conc.	Effects	mK_{ATP} effect
ATP	mM	Activate [102,162]	Inhibit [102,182,198]
Atpenin A5	nM	Inhibit [104,114,173,174]	Activate [104]
Diazoxide	μM	Inhibit [171]	Activate [102,115,182,198]
HNO	μM	Inhibit [108,109]	Activate [108]
LNO2	μM	Inhibit [108]	Activate [108]
Malonate	mM	Inhibit [137]	Activate [102,104,137,181]
3-nitropropionate	mM	Inhibit [236,237]	Activate [113,137]
Oxaloacetate	μM	Inhibit [52,102,238,239]	
Harzianopyridone	nM	Inhibit [114]	
carboxin	μM	Inhibit [114]	
HQNO	μM	Inhibit [114]	
TTFA	μM	Inhibit [114]	

Legend. HQNO, 2-heptyl-4-hydroxyquinoline N-oxide; TTFA, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione