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# The mitochondrial complex II and ATP-sensitive potassium channel interaction: quantitation of the channel in heart mitochondria*

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**The mitochondrial ATP-sensitive potassium channel ($mK_{ATP}$) is important in cardioprotection, although the channel remains molecularly undefined. Several studies have demonstrated that mitochondrial complex II inhibitors activate the $mK_{ATP}$, suggesting a potential role for complex II in channel composition or regulation. However, these inhibitors activate $mK_{ATP}$ at concentrations which do not affect bulk complex II activity. Using the potent complex II inhibitor Atpenin A5, this relationship was investigated using tight-binding inhibitor theory, to demonstrate that only 0.4% of total complex II molecules are necessary to activate the $mK_{ATP}$. These results estimate the $mK_{ATP}$ content at 15 channels per mitochondrion.**

**Keywords:** complex II, $mK_{ATP}$, Atpenin A5, ischemic preconditioning

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## INTRODUCTION

The mitochondrial ATP-sensitive potassium channel ($mK_{ATP}$) is a critical component of the endogenous cardioprotective machinery of ischemic preconditioning (IPC). The activation of this channel protects against ischemia reperfusion injury *via* an unclear mechanism involving the prevention of mitochondrial calcium overload and reactive oxygen species overproduction, as well as mild swelling and uncoupling (reviewed in**:** Facundo *et al.*, 2006). Despite intense investigation, the identity of the $mK_{ATP}$ remains elusive. Pharmacological overlap between the channel and mitochondrial complex II (succinate dehydrogenase) led to the hypothesis that complex II may be a component of the $mK_{ATP}$ (Ardehali *et al.*, 2004). In this regard, pharmacological activators of the $mK_{ATP}$ were found to inhibit complex II (Ockaili *et al.*, 2001; Ardehali *et al.*, 2004; Wojtovich & Brookes, 2009; Wojtovich & Brookes, 2008). However, the effects of compounds such as diazoxide on the $mK_{ATP}$ were seen at concentrations sometimes orders of magnitude below those required for complex II inhibition. Therefore, the effect on complex II activity at high concentrations was divorced from the mechanism of channel activation. The relationship between complex II and the channel was further investigated using the complex II inhibitor Atpenin A5 (AA5) (Wojtovich & Brookes, 2009). AA5 is a potent and specific complex II inhibitor with an $IC_{50}$ of 10 nM (Miyadera *et al.*, 2003) yet like the $mK_{ATP}$ opener diazoxide, AA5 opens the channel at a concentration an order of magnitude below that (Wojtovich & Brookes, 2009).

To provide insight to the nature of the $mK_{ATP}$, tight-binding inhibitor theory was applied herein. This theory defines a tight binding inhibitor as one which exerts its effect on an enzyme catalyzed reaction at a concentration comparable to that of the enzyme. The theory has been used to determine the number of adenine nucleotide translocator molecules by titrating in its selective inhibitor carboxyatractylate (Streicher-Scott *et al.*, 1993; Brand *et al.*, 2005). In this regard, AA5 can be considered a highly selective inhibitor of complex II since an $IC_{50}$ of 10 nM is sufficiently low relative to the amount of protein present. Thus, by titrating AA5, the total number of complex II molecules as well as the number of complex II molecules resulting in the activation of the $mK_{ATP}$ channel, can be determined.

## MATERIALS AND METHODS

**Animals.** Sprague-Dawley rats, 200–225 g, were purchased from Harlan (Indianapolis, IN, USA) and housed on a 12 h light/dark cycle with food and water available *ad libitum*. All procedures were performed in accordance with the US National Institutes of Health "Guide for the care and use of laboratory animals", and were approved by the University of Rochester's Committee on Animal Resources.

**Cardiac mitochondria.** Mitochondria were rapidly isolated by differential centrifugation in sucrose-based buffer (300 mM sucrose, 20 mM Tris, 2 mM EGTA, pH 7.35, at 4°C) as previously described (Wojtovich & Brookes, 2008).

**Complex II enzymatic activity.** Complex II activity was determined spectrophotometrically as the thenoyltrifluoroacetone sensitive rate of succinate-driven, coenzyme $Q_2$-linked reduction of dichlorophenolindophenol (DCPIP), as previously described (Wojtovich & Brookes, 2008). Briefly, mitochondria (0.05 mg/ml) were added to a 50 mM $K^+PO_4^-$ buffer (pH 7.4, at 37°C) containing 100 μM EDTA, 40 μM DCPIP, 1 mM KCN, 10 μM rotenone, and 50 μM coenzyme $Q_2$. The reduction of

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**Abbreviations:** AA5, Atpenin A5; $mK_{ATP}$, mitochondrial ATP-sensitive potassium channel; SUR, sulfonylurea receptor; Kir, inwardly rectifying potassium channel

DCPIP ($\varepsilon$ = 21000 $M^{-1} \cdot cm^{-1}$) to $DCPIPH_2$ was initiated by addition of 20 mM succinate.

**$mK_{ATP}$ osmotic swelling assay.** Using a Beckman DU800 spectrophotometer, $mK_{ATP}$ activity was monitored at 520nm as light scatter (optical density) change due to $K^+$ uptake and swelling, as previously described (Wojtovich & Brookes, 2008). Briefly, mitochondria (0.25 mg/ml) were added rapidly to a stirring cuvette containing $mK_{ATP}$ swelling buffer (100 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 2 mM $KH_2PO_4$, 1 μg/ml oligomycin, pH 7.2, at 37 °C) and substrates/inhibitors as indicated in the figures. All measurements were taken within 1.5 h of mitochondrial isolation (Wojtovich & Brookes, 2008).

## RESULTS

### Complex II enzymatic activity and the measurement of complex II content by AA5 titer

AA5 is a potent and specific complex II inhibitor; therefore, the minimum amount of AA5 required to inhibit complex II activity equals the amount of complex II present. Complex II activity was inhibited successively by additions of AA5 and plotted as percent inhibition (Fig. 1). The amount of AA5 added was expressed as nmol AA5/mg protein. The minimum AA5 titer was determined as the intercept between the steepest slope and the maximal complex II inhibition (100%) (Fig. 1B). The titration of AA5 revealed a content of complex II of 0.209 nmol AA5/mg mitochondrial protein. The crystallization of AA5 with complex II determined that one molecule of AA5 binds per complex II molecule (Horsefield *et al.*, 2006) thereby yielding 0.209 nmol complex II/mg protein, or about 3% of total mitochondrial protein (assuming a molecular mass of 140 kDa for complex II).

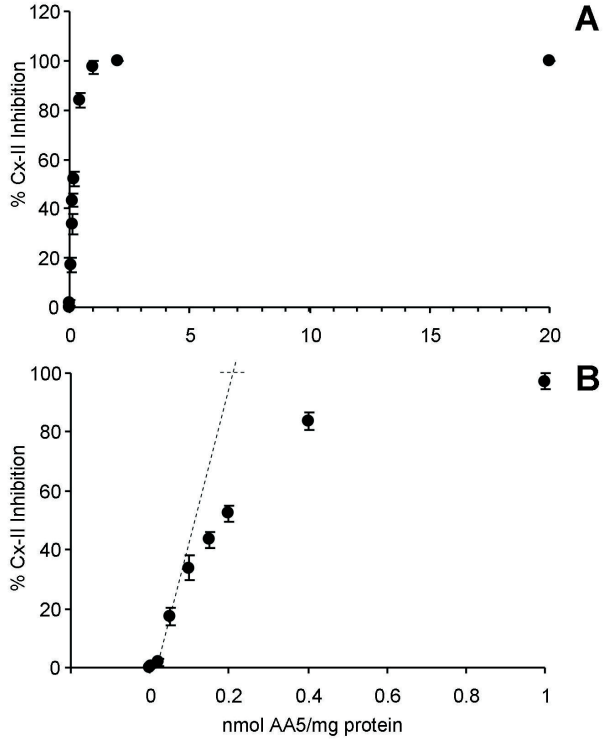


**Figure 1. Complex II content of rat heart mitochondria**
**(A)** The complex II content was determined *via* an AA5 titer of complex II activity. Complex II activity was measured and expressed as percent inhibition. **(B)** The expanded range of 0 to 1 nmol AA5/mg protein illustrates the determination of the steepest slope. Complex II content was measured as the intersection where the steepest slope in the AA5 titer crosses the maximal inhibition of complex II activity (100%) and is denoted with a dashed line. Data are mean ± S.E.M., n = 4.

### Measurement of complex II content required for AA5-mediated $mK_{ATP}$ activation

The pharmacological overlap between complex II and the $mK_{ATP}$ suggests a regulatory role for complex II in channel activity. Interestingly, the potent complex II inhibitor AA5 is also the most potent $mK_{ATP}$ opener discovered to date (Wojtovich & Brookes, 2009). AA5 was titrated and $mK_{ATP}$ activity was monitored *via* swelling. The $mK_{ATP}$-mediated swelling relies on membrane-potential driven $K^+$ uptake and the AA5 titer was determined using different substrates. Concentrations of AA5 > 2.5 nM inhibited $mK_{ATP}$ activity when succinate was used as the substrate (Figs. 2C and D). However, this effect was not seen when glutamate/malate was used as the substrate (Figs. 2A and B) and thus we attribute the loss of $mK_{ATP}$ activity seen with succinate due to the inhibition of membrane-potential driven $K^+$ uptake (Wojtovich & Brookes, 2009). Channel activity was monitored at different concentrations of AA5 and plotted as percent of control (open channel). Again, the amount of AA5 added was expressed as nmol AA5/mg protein. The minimum AA5 titer was determined as the intercept between the steepest slope and the maximal $mK_{ATP}$ activity (control, 100%) (Figs. 2B and D). Using both complex I and complex II-linked substrates the complex II content required for AA5-mediated $mK_{ATP}$ activation was calculated to be either 0.864 or 0.816 pmol complex II/mg of protein, respectively. This is approx. 250-fold lower than the level of complex II enzymatic activity (*vide supra*).

## DISCUSSION

The results from Figs. 1 and 2, demonstrate the disconnect between $mK_{ATP}$ opening and complex II inhibition using an inhibitor of complex II, such that AA5 optimally opened the $mK_{ATP}$ channel at a concentration that had no effect on complex II activity. Furthermore, previous work has demonstrated that the opening effect of AA5 was still present under conditions that did not require complex II activity (e.g., glutamate/malate, and ascorbate/TMPD), suggesting a more direct role for complex II in the regulation of the channel and not secondary effects due to simply inhibiting complex II (e.g., changes in ROS generation by the complex) (Wojtovich & Brookes, 2009). The profile of activating the channel at concentrations which have no effect on total complex II activity is now reported for six compounds (nitro-linoleic acid, nitroxyl, malonate; 3-nitropropionate, Atpenin A5, and diazoxide) (Schafer *et al.*, 1969; Garlid *et al.*, 1997; Ockaili *et al.*, 2001; Ardehali *et al.*, 2004; Wojtovich & Brookes, 2008; Wojtovich & Brookes, 2009; Queliconi *et al.*, 2010). These compounds all inhibit complex II *via* different mechanisms and bind to different subunits of complex II, and emphasize the critical role complex II plays in the regulation or formation of the channel.

Given that AA5 is a tight binding specific inhibitor of complex II, the minimum amount of AA5 required to inhibit complex II equals the amount of complex II

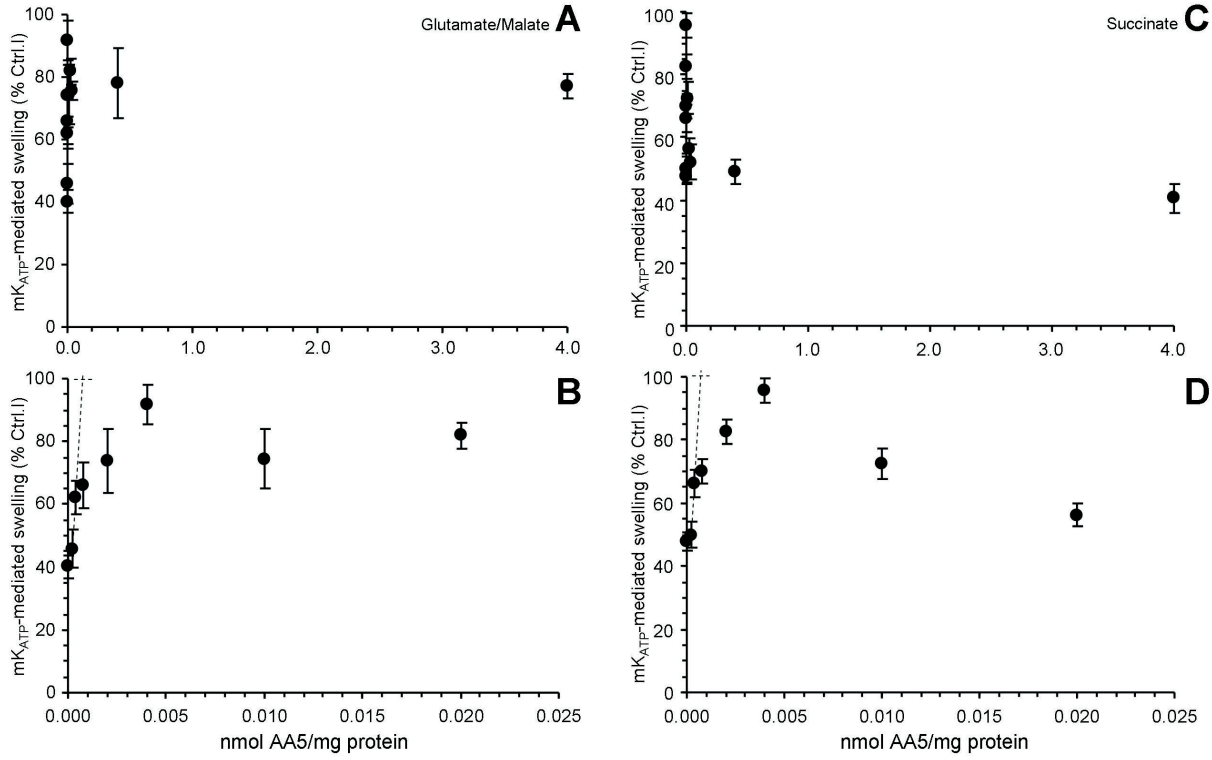


**Figure 2. Complex II content required for mK$_{ATP}$ activity**
The AA5 titer was investigated using both succinate **(A)** and glutamate/malate **(B)** to drive respiration. mK$_{ATP}$ activity was determined *via* mK$_{ATP}$-mediated swelling and expressed as a percent of maximal mK$_{ATP}$ activity (e.g. control, 100%). The range of 0 to 0.02 nmol AA5/mg protein was expanded illustrates the determination of the steepest slope using both succinate **(C)** and glutamate/malate **(D)**. Complex II content was measured as the intersection where the steepest slope in the AA5 titer crosses the maximal mK$_{ATP}$ activity (100%) and is denoted with a dashed line. Data are mean ± S.E.M., N≥8.

present, since there is a 1:1 ratio of complex II to AA5 (Horsefield *et al.*, 2006). The total number of complex II molecules per mg of mitochondria was determined from Fig. 1 by titrating AA5 and monitoring complex II activity. Assuming $8.7 \times 10^9$ mitochondria per mg of protein (Schwerzmann *et al.*, 1986), the number of complex II molecules per mitochondrion was estimated at 14,494 (0.209 nmol/mg protein). While the amount of complex II per mitochondrion is largely species and tissue specific, other reports of complex II content range 0.074 nmol/mg protein in rat liver (Schwerzmann *et al.*, 1986) to 0.330 mol/mg protein in cat skeletal muscle (Schwerzmann *et al.*, 1989). However, when comparing the same species and accounting for the difference in surface density (520 and 2,000 cm$^2$ of inner membrane/mg protein in liver and heart, respectively) the amount of complex II per mitochondrion is in close agreement (Schwerzmann *et al.*, 1986).

The number of complex II molecules necessary to activate the mK$_{ATP}$ was determined from Fig. 2 using both complex II- and complex I-linked substrates. Both conditions yielded a similar result, such that 57 and 60 complex II molecules are necessary to activate the mK$_{ATP}$ for succinate and glutamate/malate, respectively. Therefore, although using a complex II inhibitor, only a small pool of inhibited-complex II molecules (e.g., 0.4%) are necessary to maximally open the channel thereby having a negligible effect on total complex II activity. The data presented herein also implies that the number of mK$_{ATP}$ channels present in a mitochondrion is relatively small, which would be hypothesized based upon the bioenergetic consequence of having large scale $K^+$ influx into the mitochondrion. Assuming one complex II per functional mK$_{ATP}$ channel, then the number of functional channels per mitochondrion is estimated at 60.

The mK$_{ATP}$ is hypothesized to resemble the molecularly defined surface K$_{ATP}$ (Paucek *et al.*, 1992; Mironova *et al.*, 2004) which is an octamer composed of four identical inwardly rectifying potassium channel (Kir) and four sulfonylurea receptor (SUR) subunits (Nichols, 2006). While recent evidence supports the existence of a bona fide $K^+$ channel, with pharmacological sensitivities resembling Kir6.2 (Wojtovich *et al.*, 2010), the role of a SUR in mK$_{ATP}$ composition remains elusive. While a short form SUR subunit was found in mitochondria, it is not a component of the mK$_{ATP}$ channel (Ye *et al.*, 2009). Without the molecular identity of the channel, the nature of the complex II and mK$_{ATP}$ interaction and the exact stoichemometry of complex II to mK$_{ATP}$ remains unknown.

It is possible that the mK$_{ATP}$ lacks a SUR subunit. Indeed, using arteries from SUR2$^{-/-}$ mice it was demonstrated that vasodilation responses to diazoxide and AA5 are independent of SUR ablation (Adebiyi *et al.*, 2008). Thus it can be hypothesized that complex II may replace SUR subunits, interacting with the mK$_{ATP}$ in a similar manner as the channel's own subunits (i.e., Kir/SUR). Thus, each functional mK$_{ATP}$ channel would contain 4 molecules of complex II, and based on the AA5 titrations herein this would yield an estimate of about 15 mK$_{ATP}$ molecules per mitochondrion. This number is based upon the assumption that every inhibited complex II molecule is able interact with a channel; however, it is more often the case that the modulator is present in ex-

cess and the exact number of channel may be even less. This low abundance could also account for the challenge of defining the $mK_{ATP}$ on a molecular level.

The results herein demonstrate the relationship between complex II and the $mK_{ATP}$, and provide an explanation for the conundrum of using a complex II inhibitor to open the $mK_{ATP}$ but at a concentration which does not inhibit complex II (Wojtovich & Brookes, 2009). Tight-binding inhibitor theory demonstrates that only a small pool of complex II molecules (0.4%) is necessary to activate the $mK_{ATP}$, thus leaving the bulk of complex II activity unaffected. This finding has implications for the specificity and mechanism of the $mK_{ATP}$ activator diazoxide. Similar to AA5, diazoxide activates the $mK_{ATP}$ at low concentrations (< 30 μM) (Facundo *et al.*, 2007; Wojtovich *et al.*, 2010), and inhibits complex II (>100 μM) (Schafer *et al.*, 1969; Dzeja *et al.*, 2003). Originally, the "side-effect" of complex II inhibition was divorced from the mechanism of action since the concentrations of diazoxide that activated the $mK_{ATP}$ are 3–10 times less than those needed for complex II inhibition. This data suggests that, like AA5, diazoxide may exert its effect on the $mK_{ATP}$ *via* complex II since only a small fraction of the complex II pool is necessary. This complex II-mechanism would also explain the specificity of diazoxide for the $mK_{ATP}$ over the surface channel (Garlid *et al.*, 1997), since complex II is not expected to be found at the cell surface.

In conclusion, complex II is an important regulator or component of the $mK_{ATP}$. The interaction between complex II and the channel is brought about through the inhibition of complex II activity. The mechanism of this interaction is not yet known. However, inhibition of the total complex II pool is not necessary and maximal activation the $mK_{ATP}$ *via* this mechanism requires 0.4% of the total complex molecules to be inhibited. At a time when the $mK_{ATP}$ identity remains elusive, the exploitation of the complex II-mediated channel opening mechanism provides a unique means to design potent and specific $mK_{ATP}$ activators.

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