**Reviewer #1**  
  
1. Introduction, first paragraph: Replace "oxidization" with "oxidation"

* Thank you for catching the typo. We have corrected it.

2. Page 6, first paragraph and Figure 2. "WB" is undefined and I could not find a reference to this abbreviation in the text. There is no elaboration on the relevance of "WB" in the Results that accompany Figure 2. The paper needs a summary of abbreviations.

* We apologize for the confusion. WB stands for Wurster’s blue, which is the common name for TMPD (tetramethyl-p-phenylenediamine). For consistency, we have replaced WB with TMPD in Figs. 2C & 2D. We also included the full names of TMPD and PMS in the figure legend.   
    
  3. Figure 3 & Page 6 Final paragraph. It is stated that oxaloacetate is the most potent SDH inhibitor, though Figure 3 appears to support that both malate and malonate are more potent.
* Malonate and malate are less potent than oxaloacetate because they achieve the same inhibitory effects at higher levels. For example, succinate oxidase activity is reduced by half in the presence of ~1 µM oxaloacetate, but ~200 µM (0.2 mM) malonate and ~400 µM (0.4 mM) malate are necessary to achieve the same effect.

4. Figure 5 and accompanying text on page 7. I read the paragraph that supposedly describes the results depicted in Figure 5 several times. For the most part, it doesn't appear that this text describes the data. What is the relevance of the data in Figure 5?

Accompanying text incompatible with the figure

* We are puzzled by this comment because the discussion in the second paragraph on page 7 specifically focuses on the succinate titration data in the presence of 250 nM atpenin under one condition and 50 µM stigmatellin under a different condition.
* Specifically, the paragraph
  + opens with the central theme to be discussed in the rest of the paragraph
    - “The difference in total and site-specific ROS production rates in the presence of stigmatellin or atpenin as the succinate concentration is increased is shown in Fig. 5.”
  + describes the mechanism of action of stigmatellin
    - “Stigmatellin competitively inhibits QH2 binding to complex III near the Rieske ISP site. Similar to when myxothiazol is present, the Q pool is fully reduced with stigmatellin, and quinol oxidation at the Qp site serves as an additional source of electrons for free radical production. So turnover at both electron input and output (reverse) of the enzyme complex results in the highest ROS production rates.”
  + compares and contrasts model analysis using these 2 data sets
    - “In the presence of either inhibitor, the FAD is the major source of ROS, and O2•- from the FAD site at low succinate concentrations is highest among ROS species. As the succinate concentration increases, H2O2 production from the FAD site exceeds the O2•- rate from this site. But as the succinate concentration further increases, the ROS production rate from the FAD site decreases due to succinate binding to the FAD, making it unavailable to interact with oxygen.”
  + recounts the mechanism of action exerted by atpenin
    - “However, when atpenin is present, electron transfer from the ISC to quinone or oxygen is blocked (47,48). “
  + The paragraph is then concluded with a more generalized notion that stigmatellin results in a more highly reduced state of the enzyme, resulting in an environment conducive for higher ROS production rates.
    - “Therefore, O2•- production from the FAD remains the dominate source of ROS, and the ISC produces no ROS when atpenin is present (Fig. 5C). In addition, the enzyme is more oxidized compared to when stigmatellin is present, so the amount of enzyme with a fully reduced flavin is lower. Thus, the rate of H2O2 production is lower.”

Relevance of data in Fig. 5

* An issue with studying ROS production in general and by SDH in particular is the use of inhibitors. As thoroughly visited in the Introduction (paragraphs 4 & 5), the use of inhibitors is further complicated by the other different environmental conditions that can influence ROS measurements. These environmental factors include but are not limited to the different substrate levels, the presence of other non-SDH substrate dicarboxylates and the presence of different kinds of inhibitors. These together result in discrepancies in experimental data reported by different groups, as well as the conclusions drawn from these data. Therefore, model construction and calibration require data sets obtained under different conditions. This process is briefly described in the Results (paragraph 1).
* However, we have included additional texts in the Results section to provide a rationale for why data sets using different inhibitors is necessary for model construction and calibration.
  + “Other inhibitors acting upstream or downstream of SDH can also influence the enzyme’s oxidation state. Some of the most common inhibitors used in experimental settings include myxothiazol and stigmatellin, which inhibits electron flow downstream of SDH and rotenone, which inhibits electron flow upstream of the enzyme complex.”
* The data presented in Fig. 5 were performed under different conditions from other data sets. The ROS data sets are presented in the order of increasing complexity. Specifically,
  + Fig. 4: Fixed environmental factors: myxothiazol and rotenone on one hand and atpenin on the other hand. Substrate level is varied.
  + Fig. 5: Fixed environmental factors: stigmatellin or atpenin at 2 different concentrations. Substrate level is varied.
  + Fig. 6: ROS production rates at a fixed substrate level. Environmental factors are varied: in the presence of Q reductase inhibitor (atpenin) or FAD site inhibitors (malate and oxaloacetate).
  + Fig. 7: The data set here combine environmental factors and substrate levels. In terms of environmental factors, fumarate is present as the non-SDH dicarboxylate substrate and at least another inhibitor is present (atpenin, myxothiazol or stigmatellin).

5. Terminal sentence, first paragraph, page 8: Replace "require" with "required"

* Thank you for catching the mistake. It has been corrected.

**Reviewer #2**  
  
While the authors expand on their explanation, my concerns remain. This purely computational study is based on measurements of a complex protein, which can be influenced by protein quality and conditions of the in vitro experiments. There are 19 parameters, and as I stated in my original review, it is not clear to me that the insights are unique. Overall, I still feel that the manuscript is better suited for a specialized audience.

* We acknowledge that our manuscript appears heavy on the computational side. However, it is dangerous and conceptually wrong to think that a study is computational simply because the words computational and modeling are used. In fact, a lot of experimental data are included to calibrate and validate the model. As pointed out by the original Reviewer #2, the fact that the data come from different groups and were obtained under different conditions strengthens the model. In addition, we included data generated by our own group (Fig. 9 and Table 3). Altogether, the concern expressed here is ungrounded.

**Editor’s Suggestions to Address the Concerns by Reviewer #2**

1. Explains how such a model can be used study "metabolism" or other enzyme systems in an in vivo setting, (this could broaden the impact) This was not obvious to me.
   * This is a really good suggestion. We have included an additional paragraph in the Discussion (paragraph #) to explain how our SDH model can be integrated into larger scale model of mitochondria metabolism.
     + “”
2. Clearly present errors/variability in measures and parameter values from different preparations of mitochondrial from the same conditions, species, sex, etc. It looks to me that you only performed the measurements (in guinea pig) from one experiment with an N=3. What is the variance from day to day and animal to animal, etc.
   * We have now included individual data points from our in-house experiments using guinea pig (Fig. S2).

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| **Figure S2. Net hydrogen peroxide emission rates were measured in-house using mitochondria isolated from guinea pig cardiomyocytes.** The net hydrogen peroxide emission rate was monitored using the Amplex UltraRed assay (excitation 560 nm, emission 590 nm). A) Succinate titration. Mitochondria were added to the reaction mixture containing myxothiazol and rotenone. After 2 minutes of stabilization, succinate was added to the final concentrations of 50, 100, 200, 300, 500, 1000, 3000 or 5000 µM. B) Decylubiquinol (DQH2) titration was obtained at 200 µM and 5 mM succinate concentrations. The initial stage of the experiment is similar to the succinate titration. After 10 minutes following succinate addition, DQH2 was added to the final concentrations of 12.5, 25, 50, 75, 100, 150 or 200 µM. In all experiments, the final concentrations are 10 µM Amplex UltraRed, 1 U/mL HrP, 10 U/mL SOD, 4 µM rotenone, 2 µM myxothiazol and 0.1 mg/mL mitochondria. At least 3 replicates were performed at each succinate or DQH2 concentration. |

* To help the reader navigate through the different data sets used by different groups, we included experimental details from these studies in the figure legends as much as distilled from the original studies. Due to the large number of data points from all of the studies combined, the audience is referred to the studies for individual data points.

1. And lastly (point #3.), the paper in some places still reads like it is written for very specialized audience, so additional edits are needed.
   * We acknowledge that the technical aspect of the paper may not be familiar to everyone in the audience. However, the biochemistry on which the model is grounded is fundamental and of broad interest to the audience.
   * Faced with the challenge of balancing the technicality and content of the paper, we did our best to simplify the technicality of our modeling approach into a single paragraph (Results section, paragraph 1) in the previous round of revision.
   * Nevertheless, we have included an additional figure (new Fig. S1) that pictographically explains how our dynamic modeling process works. Text is included at the end of the second paragraph of the Results section to refer the audience to this new figure.
     + Text: “This dynamic modeling approach is summarized in Fig. S1.”
     + Figure

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| **Figure S1. Methodology overview.** The general modeling approach involves a dynamic three-staged process of model construction, calibration and validation. In the initial construction stage (blue), a rudimentary model emerges from structural, thermodynamic and kinetic data. In the corroboration stage (orange), outputs of the rudimentary model are compared to experimental data. The model details, as well as the model parameters, are adjusted until the model outputs are consistent with experimental data. At this point, the model is revised, and the associated parameter set is described as “optimized.” During the final stage of model validation (yellow), the model outputs are compared to experimental data that were not used in the calibration stage. The revised model is considered refined if its outputs are consistent with experimental data. Otherwise, the model is sent back to the calibration stage. |