Supplementary Materials: Substrate-Dependent Differential Regulation of Mitochondrial Bioenergetics in the Heart and Kidney Cortex and Outer Medulla

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1. Mitochondrial Membrane Potential Calibration

Rhodamine 123 (R123) is widely used for dynamic measurements of membrane potential (ΔΨ) in isolated mitochondria. R123 is a fluorescent monovalent cationic dye whose transport across the inner mitochondrial membrane (IMM) is driven by ΔΨ. However, quantitative interpretation of changes in the measured R123 fluorescent intensity is not straight forward because of quenching and logarithmic relationship between R123 concentration and mitochondrial ΔΨ. This requires an appropriate calibration method for which we used a computational model of mitochondrial bioenergetics coupled with R123 dye transport across IMM and the partitioning of the dye into the mitochondrial matrix. In this paper, we used a calibration method developed by Huang et al. [1].

When mitochondria are added to the respiration buffer containing R123, mitochondrial ΔΨ drives R123 into the mitochondrial matrix. Higher ΔΨ drives more dye into the mitochondrial matrix, and hence, less dye remains in the extra-mitochondrial buffer space. The R123 fluorescent intensity thus changes with mitochondrial ΔΨ. Substrate- and tissue-specific comparisons of the time courses of R123 fluorescence in isolated mitochondria from heart, cortex, and outer medulla (OM) transitioning from state 1 to state 5 respiration are depicted in Figure S3.

For the present study, dynamic changes in the fluorescent intensity of R123 in isolated mitochondrial experiments were measured using a spectrofluorometer (Photon Technology International, Horiba Scientific Inc.). As described by Huang et al. [1], the relation between R123 fluorescent intensity and dye concentration can be described by the following empirical equation:

$$I_o\left(\left[R123\right]\right) = \frac{k_0\left[R123\right]}{1 + k_1\left[R123\right] + k_2\left[R123\right]^2 + k_3\left[R123\right]^3} \tag{0.1}$$

where $I_0([R123])$ is the observed fluorescent intensity; [R123] is the dye concentration; and k_0, k_1, k_2, and k_3 are kinetic parameters for the empirical equation (1.1). The values for the various kinetic parameter in equation (1.1) are from the study done by Huang et al. [1]; $k_0 = 8.15 \times 10^4$ M^{-1}, $k_1 = 5.16 \times 10^4$ M^{-1}, $k_2 = 1.01 \times 10^6$ M^{-2}, $k_3 = 3.91 \times 10^{12}$ M^{-3}.

The measured R123 fluorescent intensity is the sum of the fluorescent intensity of the dye in the mitochondrial matrix, the dye outside of mitochondria (i.e. in the buffer), and the dye bound to the mitochondria membrane:

$$I_{total}\left(\left[R123\right]\right) = V_e I_o\left(\left[R123\right]_e\right) + W_x I_o\left(\left[R123\right]_x\right) + \beta k_0 \alpha\left(\left[R123\right]_e + \left[R123\right]_x\right)/2 \tag{0.2}$$

where the first term corresponds to the volume-weighted intensity of the dye from the extra-mitochondrial buffer region, the second term corresponds to the volume-weighted intensity of the dye from the mitochondria matrix region, and the third term corresponds to the dye bound to the membrane. Here, V_e is the ratio of buffer volume to mitochondrial volume which is known from the experiment, W_x is the mitochondrial matrix fractional water space which is known to be 0.65 ($L_{water}/L_{mitochondria}$) [1], α is the membrane partition coefficient for R123, and β is the ratio of membrane bound R123 to free R123 in the aqueous phase. The parameters related to R123 transport are obtained from the study done by Huang et al. [1]; α = 4.49 and β = 0.33.

In isolated mitochondrial experiments, the extra-mitochondrial buffer volume (V_e) is usually thousands of times higher than the mitochondrial matrix volume (V_x), and hence the measured fluorescent intensity of R123 mostly reflects dye concentration in the extra-mitochondrial buffer space. Therefore, there is an inverse relationship between the measured fluorescent intensity and mitochondrial ΔΨ. The measured fluorescent intensity is usually maximum at the beginning of the experiments when no mitochondria is added into the buffer. Thus, the intensity can be normalized to its maximum value, which is obtained without mitochondria (see Supplemental Figure S3):

$$I_{normalized}\left(\left[R123\right]\right)=\frac{V_e I_o\left(\left[R123\right]_e\right)+W_x I_o\left(\left[R123\right]_x\right)+\beta k_0 \alpha\left(\left[R123\right]_e+\left[R123\right]_x\right)/2}{V_e I_o\left(\left[R123\right]_0\right)} \quad (0.3)$$

where $I_o([R123]_0)$ is the initial fluorescent intensity without the mitochondria and $[R123]_0$ is the original R123 concentration added to the buffer, which is known. In addition, the distribution of the dye under equilibrium condition follows the Nernst equation:

$$\frac{\left[R123\right]_x}{\left[R123\right]_e}=e^{zF\Delta\Psi/RT} \quad (0.4)$$

Assuming that the dye bound to the membrane can be approximated by $\alpha\left(\left[R123\right]_e+\left[R123\right]_x\right)/2$ [1], the mass balance of the dye can be written as

$$V_e\left[R123\right]_e+W_x\left[R123\right]_x+\alpha\left(\left[R123\right]_e+\left[R123\right]_x\right)/2=\text{constant} \quad (0.5)$$

which is equal to $V_e[R123]_0$ at time 0. Therefore, $[R123]_e$ can be obtained from

$$\left[R123\right]_e=V_e\left[R123\right]_0/\left(V_e+\alpha/2+\left(W_x+\alpha/2\right)e^{zF\Delta\Psi/RT}\right) \quad (1.6)$$

Using equations (1.1) and (1.3-1.6), the nonlinear relationship between R123 fluorescent intensity and mitochondrial $\Delta\Psi$ can be solved and the fluorescent intensity can be converted to actual mitochondrial $\Delta\Psi$. This model suggests: (i) a nonlinear calibration curve between experimentally measured R123 fluorescence intensity and mitochondrial $\Delta\Psi$, and (ii) both the R123 fluorescence intensity and the calibration curve are sensitive to the total concentrations of the dye and mitochondrial protein in the respiration buffer.

The model was parameterized and validated with kinetic measurements from isolated mitochondria to determine $\Delta\Psi$ from the R123 fluorescence intensity [1] as follows: (i) $[R123]_e$ and $\Delta\Psi$ are set to initial values, (ii) $[R123]_x$ is calculated from $[R123]_e$ and $\Delta\Psi$ based on equation (1.4), (iii) the corresponding intensities of $I_o([R123]_x)$, $I_o([R123]_e)$ and $I_o([R123]_0)$ are calculated from equation (1.1), and (iv) $\Delta\Psi$ is modified based on equation (1.2). Finally, $\Delta\Psi$ is estimated by minimizing the squared differences between the total observed fluorescent intensity from the data and the total calculated fluorescent intensity from equation (1.2), and estimated $[R123]_e$ and calculated $[R123]_e$ from equation (1.6). This way, mitochondrial $\Delta\Psi$ transients are predicted form the R123 fluorescence intensity under tissue and substrate-specific experimental conditions. Our MATLAB and Python source codes for mitochondrial $\Delta\Psi$ calibration from the R123 fluorescent intensity are available at https://github.com/xzhang0123/r123_membrane_potential_calibration. A simulation web application with sample data is available at https://r123plotly.herokuapp.com/.

References

1. Huang, M., et al., *Mitochondrial inner membrane electrophysiology assessed by rhodamine-123 transport and fluorescence.* Ann Biomed Eng, 2007. **35**(7): p. 1276-85.

2. Mitochondrial H_2O_2 emission calibration with the amplex red and horseradish peroxidase assay.

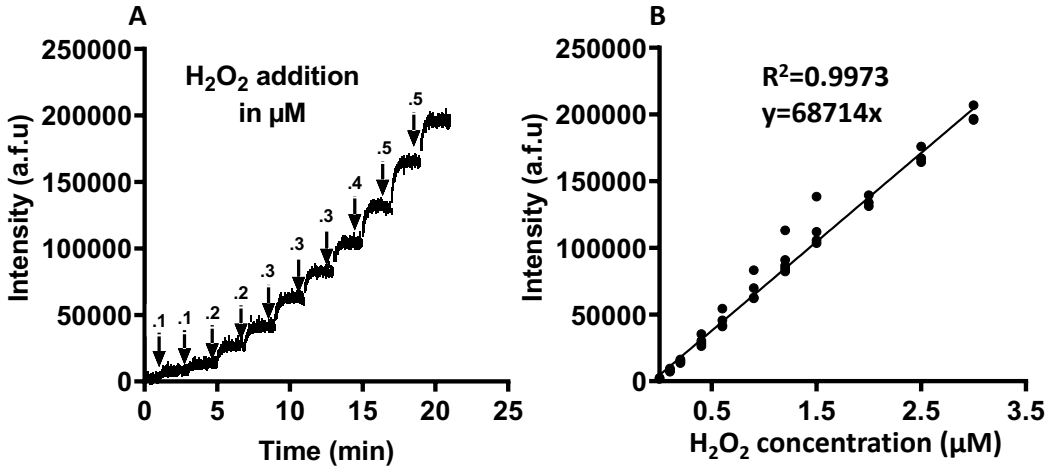

Figure S1: (A) Different concentrations of H_2O_2 starting from 0.1 µM with increments of 0.1 to 0.5 µM were sequentially added (arrows) to the reaction buffer containing amplex red and horseradish peroxidase and the resulting resorufin fluorescence intensity (a.f.u.) was recorded by the PTI spectrofluorometer. **(B)** The resorufin fluorescence intensity after each addition (arrows) is calculated and plotted as a function of the resulting H_2O_2 concentration (µM) for n=5-6 individual experiments. Based on the simple linear regression, H_2O_2 concentrations from 0.1 to 3.5 µM can be related to the resorufin fluorescence intensity with a high degree of confidence, as the correlation coefficient (R^2) was calculated to be 0.9973.

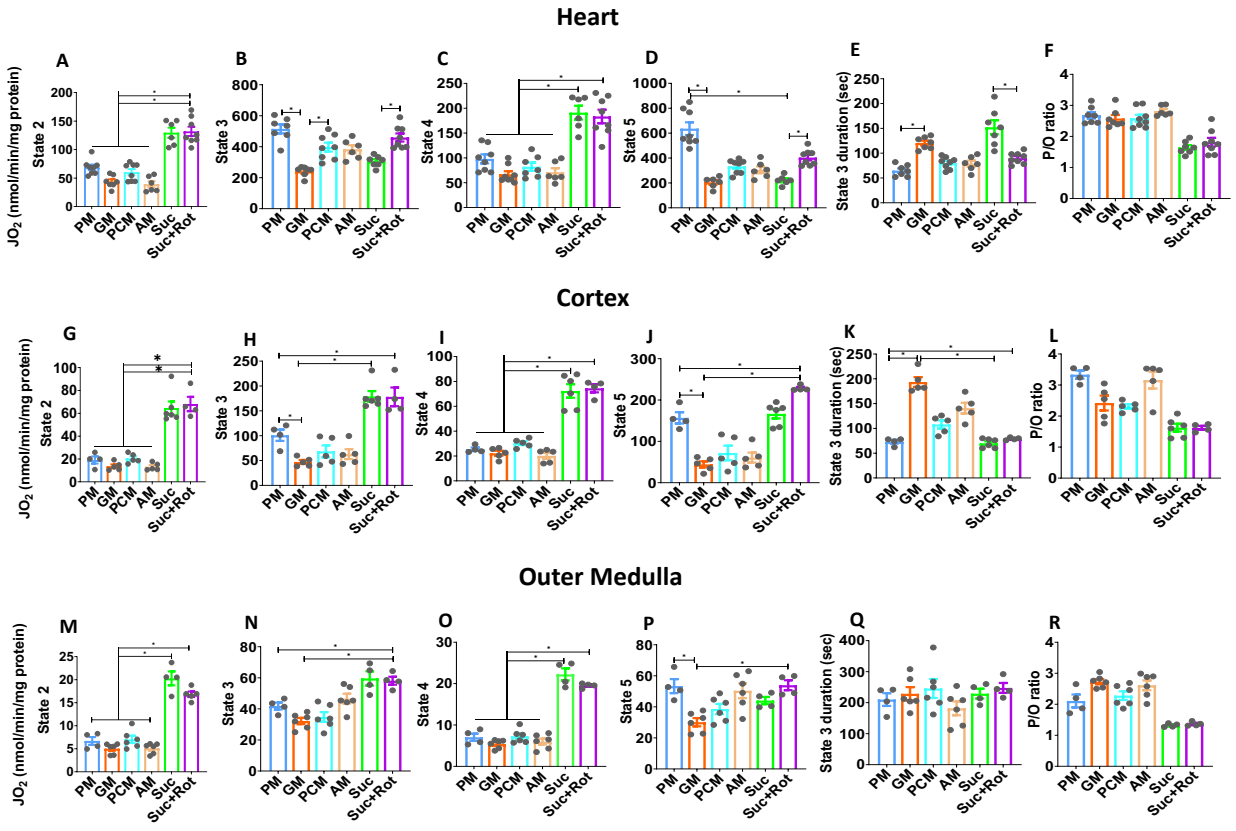

Figure S2: Substrate-specific isolated mitochondrial respiratory rates during different states and other derived parameters for heart, cortex, and outer medulla (OM), with mitochondria respiring under different substrate combinations, including pyruvate+malate (PM), glutamate+malate (GM), palmitoyl-L-carnitine+malate (PCM), alpha-ketoglutarate+malate (AM), and succinate±rotenone (Suc±Rot). Shown are respiratory rates for heart, cortex and OM: state 2 **(A-C)**, state 3 **(D-F)**, state 4 **(G-I)**, state 5 **(J-L)**, state 3 time duration (sec) **(M-O)**, and P/O ratio **(P-R)**. The respiratory rates are expressed as nmol/min/mg protein. Data are averaged over replicates n=4-6 for each substrates and tissue with ±S.E. Figures were generated using GraphPad Prism 9 software.

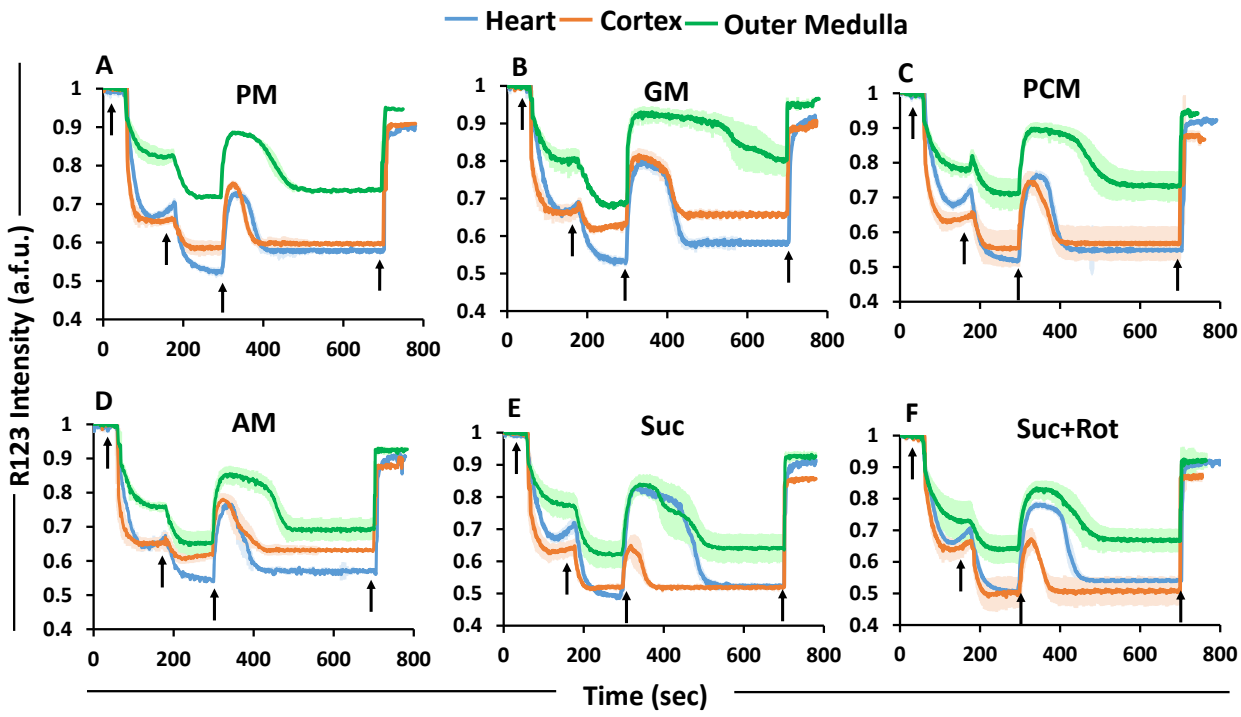

Figure S3: Substrate- and tissue-specific comparisons of time-courses of rhodamine-123 (R123) fluorescence in isolated mitochondria from heart, cortex, and outer medulla (OM) transitioning from state 1 to state 5 respiration under different substrate combinations. The timeline experimental protocol is as shown in Figure 1A. The R123 fluorescence intensity (a.f.u.) is normalized by its initial intensity. The substrate combinations used are pyruvate+malate (PM) (**A**), glutamate+malate (GM) (**B**), palmitoyl-L-carnitine+malate (PCM) (**C**), alpha-ketoglutarate+malate (AM) (**D**), Succinate (Suc) (**E**), and succinate+rotenone (Suc+Rot) (**F**). Each plot shows the comparisons of R123 fluorescence between the heart, cortex and OM mitochondria. Black arrows show the times (sec) of mitochondria (0.1 mg protein/ml for heart and 0.2 mg protein/ml for cortex and OM), substrates, ADP (200 µM for heart and 100 µM for cortex and OM), and FCCP additions to the PTI spectrofluorometer cuvette in that order. Data shown are the average of n=4-6 independent replicates ± S.E. for each substrate combinations and tissue.

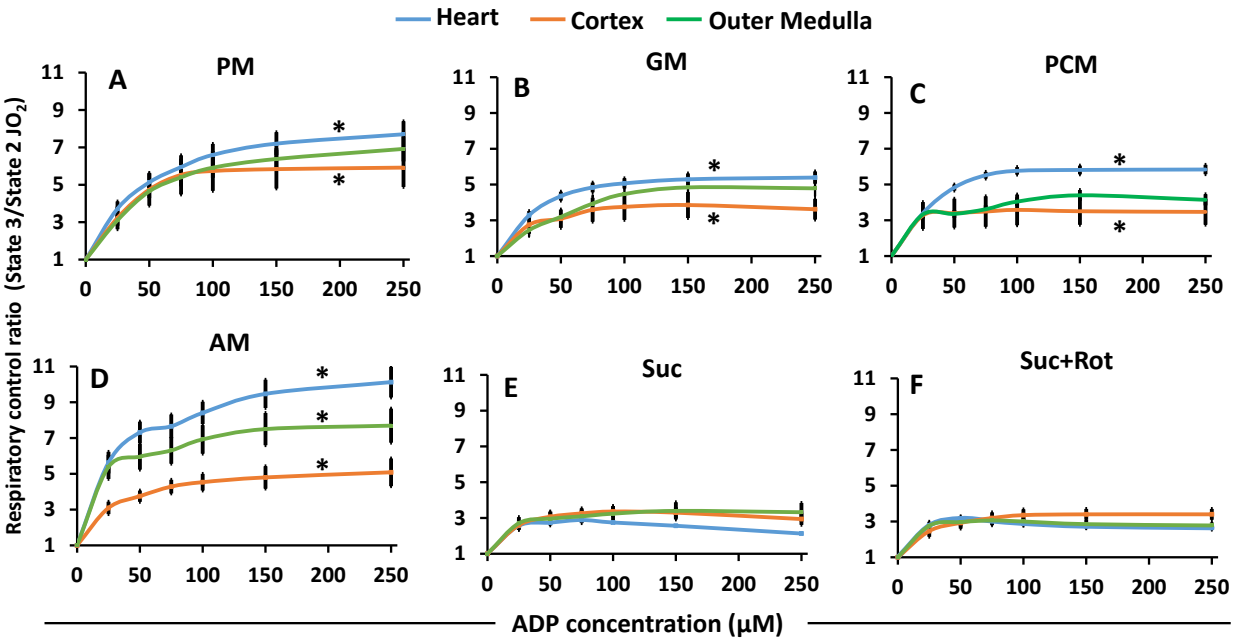

Figure S4: Substrate- and tissue-specific comparisons of respiratory control ratios (RCR) for isolated mitochondria from heart, cortex, and OM as functions of ADP concentrations. These data are derived from the O_2 consumption rates (OCR) data shown in Figure 9 as state 3/state 2 OCR with sequential additions of incremental ADP concentrations with different substrate combinations. The substrate combinations used are pyruvate+malate (PM) (**A**), glutamate+malate (GM) (**B**), palmitoyl-L-carnitine+malate (PCM) (**C**), alpha-ketoglutarate+ malate (AM) (**D**), Succinate (Suc) (**E**), and succinate+rotenone (Suc+Rot) (**F**). Each plot shows comparisons of RCR between heart, cortex and OM mitochondria. Data are shown as average of n=4-6 independent replicates ± SE for each substrate combination and tissue. The symbol '*' shows the statistical significance ($p < 0.05$) based on one-way ANOVA with repeated measures.

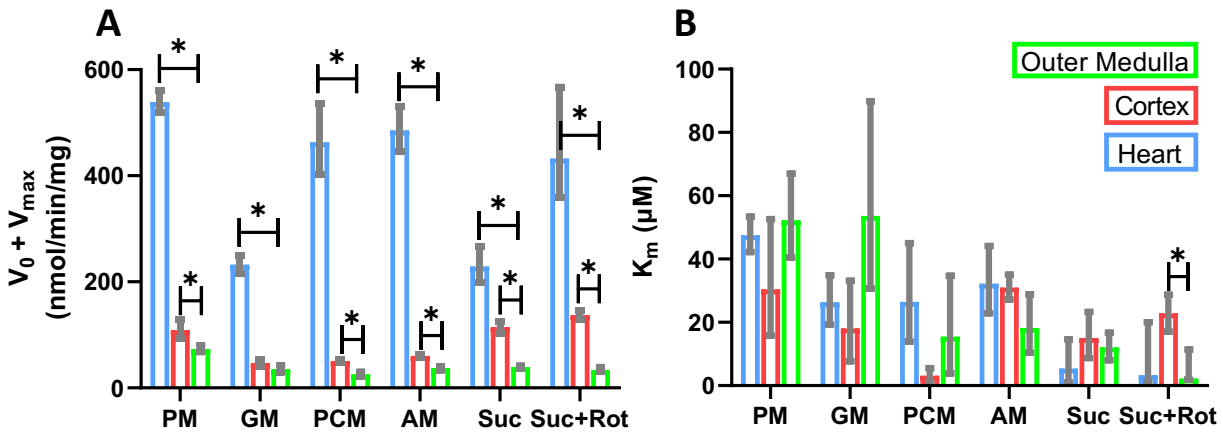

Figure S5: Substrate- and tissue-specific comparisons of (**A**) V_{max} (nmol/min/mg protein) and (**B**) K_m of ADP (μM) for isolated mitochondria from heart, cortex, and OM. Different substrate combinations included PM, GM, PCM, AM, and Suc±Rot. The tissue-specific mitochondrial state 3 respiratory rates (O_2 consumption rates; OCR) as functions of variable concentrations of ADP shown in Figure 9 were analyzed by fitting each of the substrate-specific OCR curves to Eq. 1 described in the Method section to estimate the V_{max} and K_m of ADP values. PM: pyruvate+malate, GM: glutamate+malate, PCM: palmitoyl-carnitine+malate, AM: alpha-ketoglutarate+malate, and Suc±Rot: succinate±rotenone.