Substrate Control in Mitochondrial Respiration and Regulation of Mitochondrial Membrane Potential

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Background

Substrate supply to mitochondria plays a key role in energy metabolism of the brain. Various neurological diseases are associated with specific enzymatic defects in the mitochondrial OXPHOS system including the tricarboxylic acid cycle.

Protocols for analysis of substrate-specific OXPHOS defects are traditionally performed in separate assays limited to a small number of titrations.

We developed protocols for multiple substrate-uncoupler-inhibitor titrations (SUIT), monitoring simultaneously mitochondrial membrane potential and respiration in the O2k-MultiSensor system.

Methods

We applied high-resolution respirometry (HRR) combined with an ion selective electrode system (**OROBOROS Oxygraph-2k MultiSensor system**, ISE) using tetraphenylphosphonium (TPP^+) as a reporter ion for simultaneous measurement of mitochondrial respiration, J_{O2}, and mitochondrial membrane potential, $\Delta\Psi$, at 37 °C in MiRO6, and 1 or 1.5 µM TPP^+.

Three mitochondrial preparations were compared from brain (C57Bl/6N mice, 2 months):

- **Isolated mitochondria (Imt)**
- **Supernatant after 3 min centrifugation of homogenate at 1300 *g* (Smt)**
- **Crude homogenate (Hmt)**

Coupling control and substrate control states [1, 2] were established sequentially in SUIT protocols. In calculations of $\Delta\Psi$, corrections were applied for side effects on the signal of the TPP^+ electrode induced by titrated chemicals, and for unspecific binding of TPP^+ [3, 4].

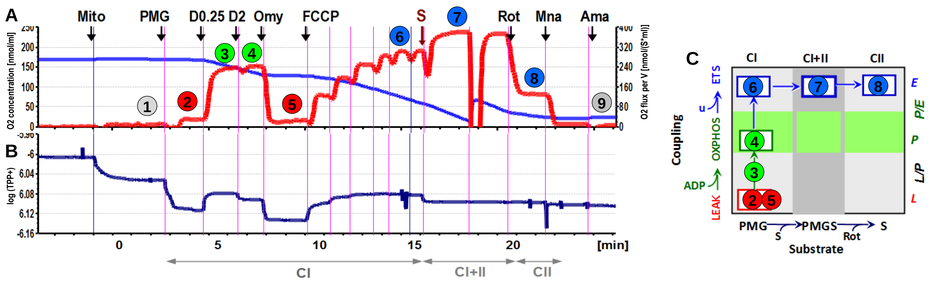

Figure 1. SUIT protocol with isolated brain mitochondria. **A:** Recording of oxygen concentration [µM] (blue line) and volume-specific oxygen flux J_{O2} [pmol·s^{-1}·ml^{-1}] (red line)**. B: log(TPP^+). Decrease of signal corresponds to increase of $\Delta\Psi$. C:** Coupling/substrate control diagram, with respiratory states:

1. ROX: Residual oxygen consumption without substrate and ADP; oxygen flux is corrected for ROX.
2. *L*: LEAK (CI) respiration in the presence of Complex I substrates pyruvate, malate and glutamate.
3. State 3 at high, not saturating ADP concentration (0.25 mM).
4. *P*: OXPHOS (CI) capacity after addition of saturating ADP (2mM).
5. *L*: LEAK (CI) respiration after ATP synthase inhibition by oligomycin (Omy).
6. *E*: Electron transport system capacity, ETS (CI), after FCCP titration (uncoupler, u).
7. *E*: ETS (CI+II) capacity after addition of succinate (10 mM).
8. *E*: ETS (CII) capacity after inhibition of CI by rotenone (Rot).
9. ROX: After inhibition of CII with malonic acid (Mna) and CIII with antimycin A (Ama).

Results

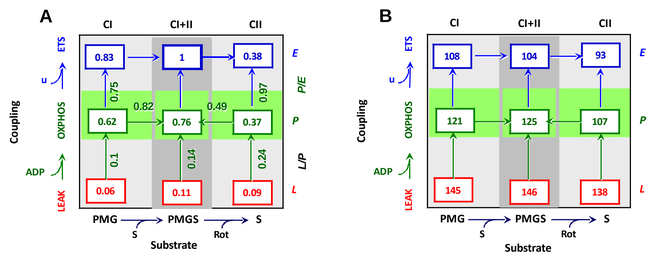

Figure 2. Coupling/substrate control diagrams summarizing five SUIT protocols with isolated brain mitochondria. **A:** Flux control ratios (*FCR*) normalized relative to ETS capacity with convergent CI+II electron input. **B:** Corresponding mitochondrial membrane potential, $\Delta\Psi$ [mV].

Figure 3. Coupling control and substrate control in brain mitochondria.
A, B: *FCR* and $\Delta\Psi$ for coupling states LEAK (*L*), OXPHOS (*P*), ETS (*E*) of CI, CI+II and CII.
C, D: *FCR* and $\Delta\Psi$ for substrates of CI, CI+II and CII within a coupling state (*L* or *P*).

Figure 5. Side effects of chemicals on the signal of the TPP^+ electrode. The trace shows titrations of the chemicals in a SUIT protocol at 1 µM TPP^+ without biological sample. Succinate, ADP, FCCP and ethanol-dissolved inhibitors affect the signal of TPP^+ beyond the theoretical dilution effect. Correction for titrations volumes >1 µl is necessary.

Preparations

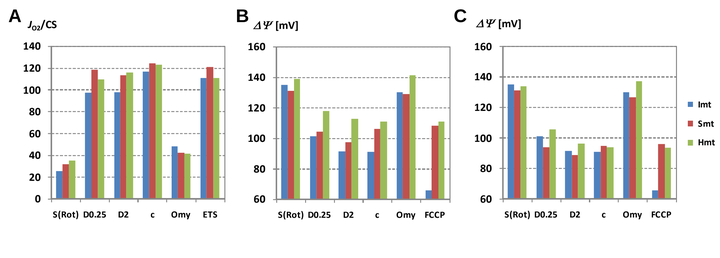

Figure 6. A: Oxygen flux in SUIT protocol with three mt-preparations normalized for citrate synthase (CS) activity. **B:** $\Delta\Psi$ calculated with binding constant for TPP^+ $K'_{in} = K'_{out} = 11$ [4]. **C:** $\Delta\Psi$ calculated with $K'_{out} = 100$ for Smt and $K'_{out} = 200$ for Hmt. **$\Delta\Psi$ and shifts of $\Delta\Psi$ between states are affected by K'_{out}.**

Fig. 4. SUIT protocols: Inhibition of CI and decrease of J_{O2} and $\Delta\Psi$ (dotted circles).

Figure 7. Relation between oxygen flux (J_{O2}/CS) and mt-membrane potential. Summary from five SUIT protocols with three brain preparations. $\Delta\Psi$ was calculated with $K'_{in} = K'_{out} = 11$ for all preparations.

Conclusions

- **Our results challenge the simplistic State 3/State 4 paradigm of mitochondrial respiratory coupling control and inverse regulation of $\Delta\Psi$.**
- **Complementary to coupling, substrate control exerts an influence on the complex relationship between oxygen flux and $\Delta\Psi$.**
- **J_{O2} normalized for CS was similar in isolated mitochondria and homogenates.**
- **External binding of TPP^+ in homogenates affects absolute values of $\Delta\Psi$ and shifts of $\Delta\Psi$ between states, partially resolved by changing K'_{out} for $\Delta\Psi$ calculation in homogenate preparations.**

References

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Acknowledgements

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