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The protonmotive force – not merely membrane potential

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Reviewer 2: Antonio Galina

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Manuscript reviewed 2022-05-13: *Only major points included.*

Reviewer 2

The review made by Komlódi, T. and Tretter, L. entitled "The protonmotive force – not merely membrane potential", explains appropriately the historical definition of the *pmF* and its two components, the electrical membrane potential (Δpsi) and (ΔpH) thought the inner mitochondrial membrane. The manuscript discusses, and well explains, the use of cationic dyes, especially BCECF/AM to determine the ΔpH , TPP electrodes used to evaluate both components of *pmF* in isolated preparations of mitochondria. In addition, it makes clear, the not obvious use, of different H^+, K+ or K^+/H^+ ionophores used, as tools, to reveals and dissect the components of the *pmF*. The authors discuss yet the role of ΔpH, the smaller component of *pmF*, in reactive oxygen species (ROS) generation and on the determination of matrix pH in the reversal of the FoF1-ATPase. In ROS estimations in literature, they mainly focused of the RET generation that suffer a direct sensitivity on *pmF*. They also bring interesting bibliography and the controversial aspects and limitations of the influence of this important intermediate in mitochondrial energy conversion. In my opinion the contribution is very appropriate to the field and fits well to the BEC's scope. In fact, I feel that a more comprehensive and deep discussion would be very welcome.

The main points to be suggested to the authors is on second paragraph of the chapter 1.2 ΔpH, and relative to the Figure 1.

a- Could the ΔpH values in *pmF* be different depending if the mitochondria are oxidizing NAD-linked substrates or FAD-linked substrates?

b- Or a combination of multiple substrates?

This reviewer would be satisfied if the authors could explain and put this point explicit in the text.

Authors

ΔpH is dependent on the respiratory fuel substrates used, because differences in mt-membrane potential are observed using succinate (plus rotenone) and α-glycerophosphate (α-Gp) or α-ketoglutarate as we published previously (Komlódi, Tretter 2017; Mikulás et al 2021; Tretter et al 2007). The amount of protons pumped out from the mitochondrial matrix into the intermembrane space depends on the type of respiratory fuel substrates. In the case of glutamate and malate (NADH-linked

substrates) 10 protons whereas in succinate respiring mitochondria only 6 protons are pumped out from the matrix. Thus, in the presence of succinate, higher mt-membrane potential and lower ΔpH are supposed to be detected compared to the oxidation of a NADH-linked substrate. Additionally, the K_M and the V_{max} of enzymes participating in the metabolism of substrates can determine the mt-membrane potential and thus ΔpH. For example, K_M of the α-glycerophosphate dehydrogenase is relatively high, thus, 40 mM of α-Gp would be the saturating concentration, however, usually lower concentrations of α-Gp are used in respirometry studies which does not polarize the mt-membrane to the same extent as 40 mM α–Gp (Tretter et al 2007). Owing to the complexity of the system, further measurements are required to draw conclusion on ΔpH changes depending on the respiratory substrates.

Komlódi T, Tretter L (2017) Methylene blue stimulates substrate-level phosphorylation catalysed by succinyl-CoA ligase in the citric acid cycle. Neuropharmacology 123:287-98.

Tretter L, Takács K, Kövér K, Adam-Vizi V (2007) Stimulation of H_2O_2 generation by calcium in brain mitochondria respiring on α-glycerophosphate. J Neurosci Res. 85(15):3471-9.

Mikulás K, Hermann P, Gera I, Komlódi T, Horváth G, Ambrus A, Tretter L (2018) Triethylene glycol dimethacrylate impairs bioenergetic functions and induces oxidative stress in mitochondria via inhibiting respiratory Complex I. Dent Mater 34:e166-e181.