BEC BIOENERGETICS COMMUNICATIONS
Bioenerg Commun 2025.7r
https://doi.org/10.26124/bec.2025-0007r
Open peer review
Open Access

Open peer review and author's responses Protonmotive force - from motive protons to membrane potential

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Bioenerg Commun 2025.7. https://doi.org/10.26124/bec.2025-0007

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Manuscript reviewed 2025-06-10: *Some minor points excluded.*

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Manuscript reviewed 2025-06-01: *Some minor points excluded.*

Reviewer 1

The manuscript entitled "Protonmotive force – from motive protons to membrane potential" has the potential to be a valuable contribution to the BEC educational series. The accuracy and detail of the manuscript are highly commendable. It is crucial for readers and scientists in the field to understand how the protonmotive force, less frequently measured than discussed, can be correctly understood and rigorously evaluated.

To ensure that the manuscript is accessible and comprehensible to a broader audience and meets the educational criteria, the current version should be revised. Several comments and suggestions have been provided in an attempt to enhance the educational value of the manuscript. We encourage the author to enhance the educational perspective of the article by extending certain sections and explanations to make the concepts more accessible and understandable for a broader audience.

Here are some suggestions to improve the readability and quality of the manuscript.

Figures should be identified (Figure 1, Figure 2, etc.) and should include an explanatory legend to help readers understand the figure and its purpose.

Potential Figure 2 (following the order of appearance) should be more specific. The H^+ "cathodic" side is identified as the mitochondrial matrix compartment, followed by the mitochondrial inner membrane (mtIM); however, the anodic part where the protons are located is not identified as the mitochondrial intermembrane space or outer space. This is relevant and should be addressed in the text.

The pH differences are defined in the text (Notes on page 3) as "$\Delta\mathrm{pH}$: pH difference across the mitochondrial inner membrane (mtIM), due to high pH on the cathodic matrix side and lower pH on the anodic outer side." Protons are pumped by Complexes I, II, and IV from the matrix to the intermembrane space; however, $\Delta\mathrm{pH}$ is mostly measured between the matrix and the outer space (outside mitochondria).

Potential differences between the pH at the mitochondrial intermembrane space and the outer space should be discussed and explained. This will help readers better understand this process and how $\Delta\mathrm{pH}$ contributes to the protonmotive force (pmF) and modulates the transmembrane potential difference ($\Delta\Psi$), as referred to in the manuscript, how pmF_d contributes to pmF and could affect pmF_el.

Author

Several explanations have been added to the text (e.g., p. 3): "Definition of the anodic compartment varies according to decisive experimental details determining where the anodic pH is measured: at the outer phase of the mtIM, in the intermembrane space between the mtIM and the mitochondrial outer membrane (mtOM), or – most frequently – in the extramitochondrial incubation medium as the outer space." In the Notes (p. 4), the definition has been adjusted, and an additional reference is added [5], critically addressing this issue: "$\Delta\mathrm{pH}$: pH difference between high pH on the cathodic matrix side and lower pH on the anodic outer side [5, 6], when the direction of transport is defined as $a \rightarrow b$."

Reviewer 1

Figure 3 or the first figure that is in section 2. 'Motive': It is not self-explanatory, and it doesn't illustrate the two points addressed in the first paragraph of this section, mainly the proton differences between the matrix and the intermembrane space or outer space (clarify).

Author

Further clarification is provided in the new legend to Figure 3: "Translocation in a compartmental system. The motive amount of H^+ (arrows from compartment b to a) is distinguished from the activity of H^+ within the compartments."

Reviewer 1

Figure 4 is clear but added a new formula ($1 = \Phi_a + \Phi_b$). But there is no mention of the symbols and why it is introduced until the end of the section. This is why it is important to have a figure legend to be self-explanatory and more educational (easier to follow).

Author

Clarified in the new legend to Figure 4.

Reviewer 1

Figure 8, if we follow text rationale Z^- at the matrix space does not cross the mitochondrial inner membrane but HZ does from the intermembrane space or outer space to the matrix. This counterions could be better defined or give an example to facilitate readers understanding. Could Z^- at the matrix space be a protein? Could Z^- at the outer space be a Pi^-? Same annotation "Z^-" different behaviour it makes the paragraph unclear.

In addition, these sentences should be extended for better understanding “HZ dissociates in the cathodic matrix to Z^- + H^+, maintaining the low number of protons in the matrix at a steady state **while building up the electric potential difference adding negative charges** to the matrix.” How are the negative charges incorporated into the matrix regulated when pmF changes? Do they diffuse to the outer space? Are they co-transported?

Author

Additions to the text (p. 9): “(e.g. OH^-)” and “(e.g. inorganic phosphate). Additionally, exchange diffusion of H^+ against Z^+ (e.g. K^+) counteracts the pH difference across the mtIM.” Module 3 is shown in Figure 8, extended by “exchange diffusion” (Figure legend). Figure 8 has been modified to provide a better explanation.

Reviewer 1

Page 6; Notes on F “The protonmotive force pmF is exergy per advancement, $\partial G/\partial_{\mathrm{m}}\xi_{\mathrm{H+}}$” along the text advancement is defined as $\mathrm{d_m}\xi_{\mathrm{H+}}$.

Author

The definition of advancement is unchanged when showing either the change of advancement as $\mathrm{d_m}\xi_{\mathrm{H+}}$, or the partial derivative as, $\partial G/\partial_{\mathrm{m}}\xi_{\mathrm{H+}}$. This is clarified by adding “change” in the text (page 5), “indicated as a partial derivative” (page 8), and “partial” (page 10).

Reviewer 1

When the author referred to Peter Mitchell's defined modules, it would be beneficial to explain them throughout the text for educational purposes. This is especially important for modules 3 and 4, which are less familiar to scientists new to the fields of bioenergetics or mitochondrial physiology. For instance, Module 4, or coupling membrane, is only mentioned in the summary and not elaborated upon in the text.

Author

Peter Mitchell’s modules 1 to 4 are now shown again in Figures 1, 2 and 8.

Reviewer 1

Next paragraph stated that “Oversimplified textbook conventions are challenged by rigorously incorporating stoichiometric numbers $\nu_{\mathrm{H^+}}$ and the charge number $z_{\mathrm{H^+}}$ in the equations defining the advancement of proton translocation and the protonmotive force.” To improve the understanding of the sentence and be more educational how the textbook conventions are challenged should be exemplified and how the incorporation of “stoichiometric numbers $\nu_{\mathrm{H^+}}$ and the charge number $z_{\mathrm{H^+}}$” proves so.

Author

Adding an Appendix, this is fully addressed in the new Table A1: “General expressions of physical chemistry are compared with equations simplified in the context of bioenergetics." It is further addressed in the conclusions, with the final Eq.(12) explicitly incorporating the stoichiometric numbers $\nu_{\mathrm{H}_i^+}$ and the charge number z_{H^+}: "The electric and diffusive nature of the two components of the pmF are distinguished from the electrical and chemical formats of the pmF. For conversion of formats with different units, the Boltzmann constant k and elementary charge e, $kT = fT{\cdot}e$ [J/x], provide the link between the electromotive constant, $f = R/F$ [J/C], the gas constant, $R = f{\cdot}F$ [J/mol], and the Faraday constant, $F = R/f$ [C/mol],

$$\Delta\tilde{\mu}_{\mathrm{H}^+}: \quad pmF_{\underline{n}} = -RT \cdot \ln(10){\cdot}\Delta\mathrm{pH} + \Delta\Psi_{\mathrm{p}^+}{\cdot}(z_{\mathrm{H}^+}{\cdot}F) \qquad [\mathrm{kJ/mol}] \qquad (12a)$$

$$\Delta\mathrm{p}: \quad pmF_{\underline{e}} = fT/z_{\mathrm{H}^+} \cdot \left(\ln a_{\mathrm{H}_a^+} \cdot \nu_a + \ln a_{\mathrm{H}_b^+} \cdot \nu_b\right) + \Delta\Psi_{\mathrm{p}^+} \qquad [\mathrm{V}] \qquad (12b)$$

For $\nu_b = -1$, the endergonic direction of proton transport is $b \rightarrow a$. In most textbook representations, neither the charge number z_{H^+} nor the stoichiometric numbers $\nu_{\mathrm{H}_i^+}$ are shown explicitly in the equations defining the protonmotive force. Eq. 12b uses the sum of stoichiometric chemical potentials defining the diffusive partial protonmotive force pmF_{d} (Eq. 6), related to $-\Delta\mathrm{pH}$ as shown in Eq. 12a (Table A1).

Reviewer 1

How does the cytosolic pH correlate with pH at the intermembrane space where the H^+ are transferred or translocated? This is not explained in Section 2. 'Motive'. This point was stressed before and it could be important for the readers to understand pmF. How the $\Delta\mathrm{pH}$ is measured? What are the limitations?

Author

I hope that this important topic is now clarified by reference to the different experimental approaches of measurement of the anodic pH (p. 3).

Reviewer 2

This manuscript provides an outstanding overview of the proton motive force and its bioenergetic foundation. The explanations and narratives are clear and relatively accessible for the non-expert and should be particularly useful to the BEC readership familiar with HRR and mitochondrial physiology to solidify their understanding of this important topic. Along these lines, I have suggested the addition of two additional figures or charts to help the reader visualize the relevant connections between the pmF and common respiratory states (*L*, *P* and *E*) and related impacts on the rate of electron flow (OX "upstream") and ADP phosphorylation ("PHOS" downstream). There is good discussion of this already, but figures illustrating these concepts would benefit more visual learners and thinkers.

Section 3; paragraphs 3-5: This is a particularly accessible and important section for a typical HRR experimentalist reader and would perhaps benefit from a simple figure illustrating the relationships between pmF, mtIM permeability in the *L*, *P* and *E* states. Those new to bioenergetics and HRR nomenclature will often confuse *L* and *E* states in terms of “coupling” and “leak”, and this paper and discussion could help to resolve their confusion (especially with a good summary illustration).

Author

New Figure 7 is added.

Reviewer 2

In the conclusion section, it may be helpful to the reader to add a brief statement here (and perhaps earlier in the paper) that clarifies how an increasing pmF can both “power” the ATP synthase and favor a slower rate of electron transfer (i.e., substrate oxidation). Without these brief returns to the physiological concepts, the non-expert reader may miss the forest for the trees and not benefit as much as he/she could from this outstanding review.

Author

A paragraph is added in the conclusion section: “The positive endergonic protonmotive force is generated by the electron transfer system ETS during oxidation of reduced fuel substrates (OX) and counteracts the negative exergonic driving force of the ETS. As the pmF approaches its maximum under conditions blocking phosphorylation, leak respiration *L* is suppressed and primarily compensates for proton back-diffusion across the coupling membrane. In contrast, respiration *P* in the OXPHOS state is stimulated as the pmF is lowered by driving ATP synthesis (PHOS). Uncouplers collapse the pmF, abolishing the counterforce and fully stimulating electron transfer capacity *E*.”