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The protonmotive force – not merely membrane potential

Timea Komlódi, László Tretter*

Department of Biochemistry, Semmelweis University, Budapest, Hungary

*Corresponding author:
tretter.laszlo@med.semmelweis-univ.hu

Abstract

The protonmotive force *pmF* establishes the link between electrical and chemical components of energy transformation and coupling in oxidative phosphorylation in the mitochondrial electron transfer system. The electrical part is corresponding to the mitochondrial membrane potential $\Delta\Psi_{mt}$ and the chemical part is related to the transmembrane pH gradient ΔpH. Although the contribution of ΔpH to *pmF* is smaller than that of $\Delta\Psi_{mt}$, ΔpH plays an important role in mitochondrial transport processes and regulation of reactive oxygen species production. Separate measurement of $\Delta\Psi_{mt}$ and ΔpH allows for calculation of *pmF*. Methods for monitoring $\Delta\Psi_{mt}$ such as fluorescence dyes are generally available, while determination of ΔpH is more challenging.

In this review, we focus on the application of the fluorescence ratiometric method using the acetoxymethyl ester form of 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF/AM) for real-time monitoring of the intramitochondrial pH in isolated mitochondria. Knowing the intra- and extramitochondrial pH allows for calculating the ΔpH. Application of specific ionophores such as nigericin or valinomycin, exerts the possibility to dissect the two components of the *pmF* in different directions. Furthermore, we tried to summarize those mitochondrial processes, such as production of reactive oxygen species, where the ΔpH has an important role.

1. Protonmotive force

The chemiosmotic theory on protonmotive force *pmF* (Δp, or $\Delta_m F_{H+}$) was postulated by Peter Mitchell in 1961 in four theorems describing that ATP synthesis is coupled to the electrochemical gradient across the mitochondrial inner membrane mtIM (Gnaiger 2020; Mitchell 1961, 1967; Mitchell, Moyle 1968). The oxidation of respiratory fuel substrates by electron transfer to O_2 is accompanied by H^+ translocation through respiratory Complexes I, III and IV from the matrix to the intermembrane space. This process results in a negatively charged matrix and positively charged intermembrane space. The proton and charge concentration difference create an electrochemical gradient called *pmF*. The electrochemical gradient is utilized to synthetize ATP in the electron transfer system ETS. The *pmF* has a chemical part $\Delta_d F_{H^+}$ related to ΔpH and electrical component $\Delta_{el} F_{p^+}$ corresponding to mitochondrial membrane potential $\Delta\Psi_{mt}$ (Gnaiger 2020),

$$\Delta_m F_{H^+} = \Delta_d F_{H^+} + \Delta_{el} F_{p^+}$$

$\Delta_d F_{H^+}$ is characterized by H^+ movement from the chemical partial force of diffusion caused by concentration gradient linked to H^+ potential difference. $\Delta_{el} F_{p^+}$ is the electrical partial force related to cation charge irrespective of the nature of the ion expressed per proton charge. The net distribution of ions (not only H^+) generates an internal electrical field on the two sides of mtIM. The cations move according to the electrical potential, from the positive side of the membrane to the negative side and the anions in the opposite direction.

The components of the *pmF* can be measured separately. Methods for monitoring $\Delta\Psi_{mt}$ are generally available, whereas determination of ΔpH is more challenging. In many studies contribution of ΔpH to *pmF* is ignored reporting only $\Delta\Psi_{mt}$ values, even in cases when a conversion from ΔpH to $\Delta\Psi_{mt}$ is not proven (shift between the two components can occur in the presence of specific ionophores; Komlódi et al 2018).

1.1. Mitochondrial membrane potential

Lipophilic cationic fluorescence probes and ion-selective electrodes are most frequently used methods to measure changes of $\Delta\Psi_{mt}$ real-time. Lipophilic cations such as safranin are able to enter the negatively charged mitochondrial matrix, bind to anionic sides and create dimers or oligomers leading to decrease of the fluorescence signal due to fluorescence quenching (Akerman, Wikström 1976; Figueira et al 2012; Kauppinen, Hassinen 1984; Krumschnabel et al 2014). In the LEAK state, when $\Delta\Psi_{mt}$ is the highest (~170 mV), safranin accumulates in the mitochondrial matrix which is reflected in the decrease of the fluorescence signal (Figure 1A). If $\Delta\Psi_{mt}$ is low, e.g. in the presence of ADP in the OXPHOS state (ADP decreases $\Delta\Psi_{mt}$ by ~25 mV; Chinopoulos et al 2010), safranin partially remains in the extramitochondrial compartment which is shown by the increase of the fluorescence signal. Upon addition of an uncoupler (protonophore) $\Delta\Psi_{mt}$ would further decrease. A linear relationship between fluorescence intensity and $\Delta\Psi_{mt}$ can be observed for certain concentration ranges and ratios of safranin and mitochondria (Figueira et al 2012).

$\Delta\Psi_{mt}$ can be also estimated based on the distribution of a lipophilic cation such as tetraphenylphosphonium ion TPP^+ detected by an ion-selective electrode (Kamo et al 1979; Komlódi et al 2018; Rottenberg, 1984). The accumulation of TPP^+ in the mitochondria is described by the mitochondrial uptake and binding to the outer and inner

surface of the mtIM based on the Nernst equation. The advantage of this method is that the absolute $\Delta\Psi_{mt}$ expressed in mV can be easily determined. Upon hyperpolarization of $\Delta\Psi_{mt}$, TPP^+ accumulates in the matrix leading to decrease of extramitochondrial TPP^+ concentration accessible to the ion-selective electrode. Noteworthy, TPP^+ is more sensitive in the range of high $\Delta\Psi_{mt}$ values than safranin (Starkov, Fiskum 2003).

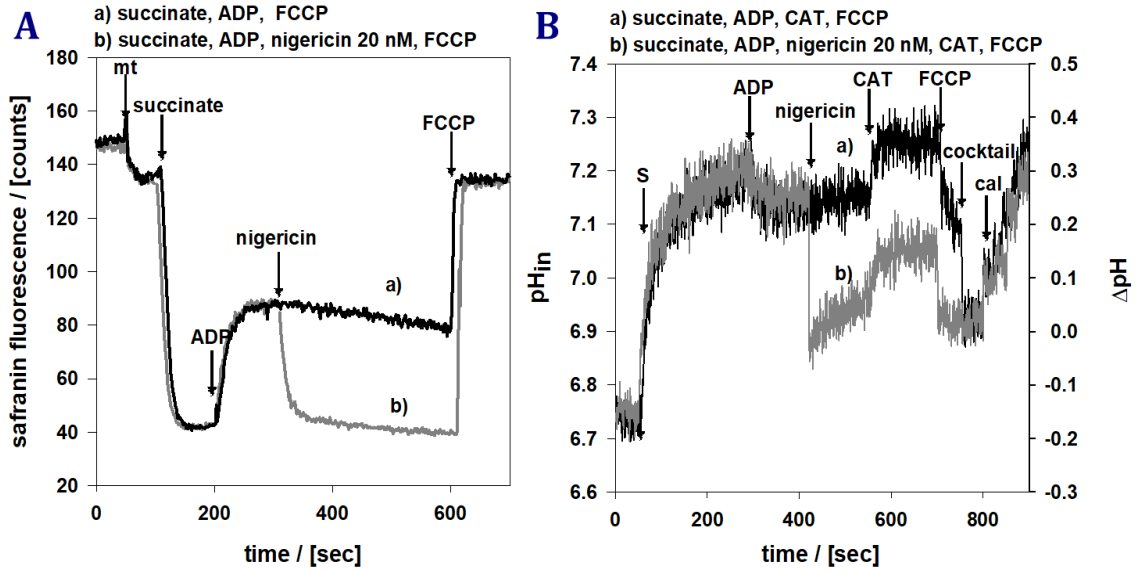

Figure 1. Mitochondrial membrane potential (A) and intramitochondrial pH pH_{in} and transmembrane pH ΔpH (B) in mitochondria isolated from guinea pig brain. Mt-membrane potential $\Delta\Psi_{mt}$ was measured by safranin fluorescence, pH_{in} was monitored by BCECF and ΔpH was calculated as described in detail by Komlódi et al 2018. Addition of succinate (5 mM) led to hyperpolarization of $\Delta\Psi_{mt}$ and increase of pH_{in}; ADP (2 mM) depolarized $\Delta\Psi_{mt}$ and decreased pH_{in} and ΔpH; nigericin (20 nM) increased $\Delta\Psi_{mt}$ and decreased ΔpH; carboxyatractilozide CAT (2 μM; inhibitor of the adenine nucleotide translocase) increased pH_{in} owing to H^+ accumulation in the intermembrane space; carbonyl cyanide-p-trifluoromethoxyphenylhydrazone FCCP (250 nM; uncoupler) decreased the proton gradient, therefore depolarized $\Delta\Psi_{mt}$ and decreased pH_{in}. A mixture of ionophores (8 μM nigericin; 2.5 μM gramicidin; 8 μM monensin) was added to equalize pH_{in} and extramitochondrial pH pH_{ex}. The BCECF fluorescence was calibrated by KOH. The measurements were carried out in the standard medium as follows: 125 mM KCl, 20 mM HEPES, 2 mM KH_2PO_4, 0.1 mM EGTA, 1 mM $MgCl_2$, 0.025 % fatty-acid free bovine serum albumin; pH 7.0.

1.2. ΔpH

Although the contribution of ΔpH to *pmF* is smaller than that of $\Delta\Psi_{mt}$, ΔpH plays an important role in mitochondrial transport processes such as transport of inorganic phosphate (Hoek et al 1970) or calcium influx (Bernardi, Azzone 1979).

Fluorescent indicators such as the acetoxymethyl ester form of 2,7-biscarboxyethyl-5(6)-carboxyfluorescein BCECF/AM (Jung et al 1989; Komlódi et al 2018) are widely used approaches to measure pH_{in}. Mitochondria are first loaded with the membrane-permeable esterified form of the indicator, which is then hydrolyzed by intramitochondrial esterases to non-permeable, free fluorophores, whose fluorescence depends on their protonation/deprotonation (Zółkiewska et al 1993). BCECF has the

advantage of having pH-dependent and pH-independent regions in its excitation spectrum, therefore, its fluorescence can be monitored at two excitation wavelengths allowing for ratiometric fluorescence (Han, Burgess 2010; Komlódi et al 2018). These data are correlated to pH values after equalizing intramitochondrial pH (pH_{in}) and extramitochondrial pH (pH_{ex}) using a mixture of ionophores as previously described (Komlódi et al 2018; Tretter et al 2007).

In isolated mammalian mitochondria, *in vitro* ΔpH depends on the composition of the respiration medium, substrates (pathway control state) and respiratory state (coupling control states). Addition of respiratory substrates e.g. succinate leads to alkalization of pH_{in} in the LEAK state (without ADP) owing to H^+ efflux from the mitochondrial matrix via respiratory Complexes (Figure 1). Addition of ADP decreases pH_{in} due to H^+ influx into the mitochondrial matrix via the proton channel of the ATP synthase. Uncouplers further decrease pH_{in}, ΔpH and $\Delta\Psi_{mt}$ due to proton translocation to the matrix. In a medium containing saccharose and low [K^+] (4 mM), the ΔpH was ~ 0.6-0.8, whereas at high K^+ concentration (~120 mM) ΔpH was 02.-0.3 in the presence of 2 mM phosphate (Komlódi et al 2018; Mitchell, Moyle 1968). Vajda et al 2009 also measured a ΔpH lower than 0.15 in a buffer with 120 mM KCl with 10 mM phosphate. Inorganic phosphate has an important role in regulation of matrix pH. P_i enters the mitochondrial matrix via the P_i/OH^- exchanger or via co-transport with protons resulting in acidification of the matrix and decrease of ΔpH. Decrease of pH_{in} is attributed to decrease of ROS generation, whereas alkalization is ascribed to elevated ROS release (Komlódi et al 2018; Selivanov et al 2008). In succinate-energized guinea pig brain mitochondria in the LEAK state the ΔpH was higher in the absence of P_i than in the presence of it (data not shown). Importantly, BCECF fluorescence can be easily calibrated after dissipation of ΔpH using ionophores followed by adding KOH solutions and measuring BCECF fluorescence and pH of the solution with glass electrode. The pH_{in} and pH_{out} are equal as a consequence of ionophore action (Komlódi et al 2018; Tretter et al 2007).

1.3. Ionophores

Ionophores are widely used compounds when studying $\Delta\Psi_{mt}$ or ΔpH in isolated mitochondria. Valinomycin is a K^+ ionophore and its effect is dependent on its concentration and on the K^+ content (Bernardi 1999; Komlódi et al 2018; Ligeti, Fonyó 1977). Valinomycin added in the nM range, in the presence of low K^+ concentration (~ 4 mM) increases pH_{in} which is explained by H^+ efflux and Pi/OH^- exchange leading to depolarization of $\Delta\Psi_{mt}$ and increase of mitochondrial respiration. Nigericin which is an electroneutral K^+/H^+ antiporter is widely used to shift ΔpH further to $\Delta\Psi_{mt}$ by decrease of pH_{in} and compensatory increase of $\Delta\Psi_{mt}$ (Bernardi 1999; Henderson et al 1969; Garlid, Paucek 2001; Komlódi et al 2018; Lambert, Brand 2004; Selivanov et al 2008). It is, however, important to note that nigericin added at the lowest possible concentration which caused the maximal hyperpolarization of $\Delta\Psi_{mt}$ in guinea pig brain mitochondria was not able to fully dissipate ΔpH leading to a decrease of P_i flux and of P_i concentration in the matrix, but it established a new equilibrium at a lower pH_{in} in the presence of high [K^+] (Komlódi et al 2018). Decrease of [P_i] in the matrix reduces F_1F_O-ATPase activity resulting in decrease of mitochondrial respiration in isolated mitochondria (Metelkin et al 2009).

2. Role of ΔpH in reactive oxygen species generation

It is well-known that production of mitochondrial reactive oxygen species (ROS) is sensitive to changes of the *pmF* components (Komlódi et al 2018; Lambert and Brand 2004; Selivanov et al 2008). In murine mitochondria, succinate-evoked reverse electron transfer (RET) in the LEAK state appears to promote the highest rate of ROS production which is highly sensitive to changes of the components of *pmF* (Votyakova, Reynolds 2008; Zoccarato et al 2011). It is generally accepted that decrease of the $\Delta\Psi_{mt}$ leads to a decrease in RET-evoked ROS formation supported by succinate (Korshunov et al 1997; Komlódi et al 2018; Lambert, Brand 2004; Selivanov et al 2008; Votyakova and Reynolds 2001), whereas hyperpolarization of the $\Delta\Psi_{mt}$ induces ROS production. Increase of the absolute pH rises succinate-supported ROS generation in the LEAK state due to the stabilization of semiquinone radicals (Komlódi et al 2018, Selivanov et al 2008). It is difficult to evaluate the direct effect of ΔpH on ROS production, because ΔpH usually changes in the same direction as $\Delta\Psi_{mt}$. For example, uncouplers cycling across the mtIM with protons decreasing both the $\Delta\Psi_{mt}$ and the ΔpH which leads to increase of respiration and decrease of ROS production. However, it is hard to evaluate whether the reduction in ROS production were caused by changes in $\Delta\Psi_{mt}$ or ΔpH. In order to determine which component of the *pmF* has a greater role in regulation of RET, ionophores such as nigericin (K^+/H^+ antiporter) and valinomycin (K^+ ionophore) can be used to dissect the components of the *pmF*. There is no general agreement on how these ionophores influence the RET-induced ROS generation. Nigericin hyperpolarized $\Delta\Psi_{mt}$, decreased ΔpH and moderately increased RET-driven ROS formation using succinate in brain and heart mitochondria isolated from guinea pigs (Komlódi et al 2018). Whereas valinomycin depolarized $\Delta\Psi_{mt}$, increased ΔpH and decreased the rate of ROS production using succinate as CHNO-fuel substrate. Based on these results it can be concluded that the $\Delta\Psi_{mt}$ is the dominant component of the *pmF* over ΔpH in modulation of succinate- or α-glycerophosphate-induced ROS formation in the LEAK state in guinea-pig brain mitochondria (Komlódi et al 2018). Lambert and Brand (2004), however, described that succinate-evoked RET depends more on ΔpH than on $\Delta\Psi_{mt}$ using nigericin, whereas Selivanov et al (2008) revealed that the acute pH rather than ΔpH is dominant over $\Delta\Psi_{mt}$ in regulation of RET. Although the literature is controversial on which component of *pmF* has a greater role in regulation of RET, it is obvious that the contribution of ΔpH to *pmF* is not negligible.

3. Role of ΔpH and matrix pH in the reversal of F_1F_O-ATPase

The mitochondrial F_1F_O-ATPase is able to synthesize and hydrolyze ATP (Boyer 2002; Rouslin et al 1986; Walker 1994). F_1F_O-ATPase uses the *pmF* to generate ATP, thus, its reversal also depends on the component of the *pmF*. Since ΔpH is the smaller component of the *pmF*, the reversal of F_1F_O-ATPase mostly depends on $\Delta\Psi_{mt}$. The $\Delta\Psi_{mt}$ values at which the F_1F_O-ATPase starts hydrolyzing ATP called reversal potential is mainly controlled by the ADP/ATP ratio in the matrix, phosphate concentration, and H^+/ATP coupling ratio (Chinopoulos et al 2010 and 2011). Decrease of $\Delta\Psi_{mt}$ e.g. owing to inhibition of the ETS, results in the reversal of F_1F_O-ATPase allowing hydrolysis of mitochondrial ATP generated via substrate-level phosphorylation catalyzed by succinate-CoA ligase to maintain $\Delta\Psi_{mt}$ (Chinopoulos et al 2010 and 2011; Kiss et al 2014; Komlódi et al 2018; Lambeth et al 2004). Under this condition F_1F_O-ATPase operates in the reverse mode, whereas ANT in the forward mode. This has paramount importance under

pathological conditions, because in a specific $\Delta\Psi_{mt}$ range mitochondria can avoid using cytosolic ATP to maintain $\Delta\Psi_{mt}$, thus showing better survival rate for the cells. However, further decline of $\Delta\Psi_{mt}$ -despite of the ATP hydrolysis- leads to reversal of the adenine nucleotide translocase ANT (Metelkin et al 2009), thus, transporting cytosolic ATP into the mitochondria (Chinopoulos et al 2010). ANT has its own reversal potential which is controlled by the participating components such as the ADP/ATP ratio in the matrix and cytosol (Chinopoulos et al 2010).

Although ΔpH is the smaller component of the *pmF*, the question arises how it would affect the reversal potential of the F_1F_O-ATPase and ANT. Chinopoulos (2011) revealed that when ΔpH was kept constant using computer simulations, the reversal potentials of F_1F_O-ATPase and ANT were moved to the more depolarizing potentials. When pH_{in} and thus ΔpH was decreased, the reversal potentials were shifted to the polarizing potentials. Importantly, the reversal potential of F_1F_O-ATPase was more affected by decline of pH_{in} than that of ANT.

It has been recently published that individual cristae can have their own local $\Delta\Psi_{mt}$ thus depolarization might affect some cristae but not all (Wolf et al 2019). In line with this, Rieger et al (2014) observed that lateral pH gradient exists between respiratory complexes and the F_1F_O-ATPase leading to built-up of intracristal local *pmF*. Since the local *pmF* around the F_1F_O-ATPase was low during OXPHOS (Rieger et al 2021), the inhibitory factor 1, which is an endogenous regulator of F_1F_O-ATPase responsible for its dimerization (Campanella et al 2009), was required to block reversal of the F_1F_O-ATPase (Rieger et al 2021).

Abbreviations

$\Delta\Psi_{mt}$	mitochondrial membrane potential	IF1	inhibitory factor 1
ΔpH	transmembrane pH	mtIM	mitochondrial inner membrane
ANT	adenine nucleotide translocase	pH_{ex}	extramitochondrial pH
BCECF/AM	acetoxymethyl ester form of 2,7-biscarboxyethyl-5(6)-carboxyfluorescein	pH_{in}	intramitochondrial pH
		pmF	protonmotive force
		RET	reverse electron transfer
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone	ROS	reactive oxygen species
		TPP^+	tetraphenylphosphonium

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