The renaissance of mitochondrial pH

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Introduction

Mitochondria are multifunctional organelles involved in energy conversion, lipid metabolism, heat production, Ca²⁺ signaling, reactive oxygen species (ROS) production, and apoptosis. All of these functions rely on the ability of mitochondria to move protons across their inner membrane during oxidative phosphorylation (OXPHOS), the process that couples the oxidation of energetic substrates to the synthesis of ATP. According to the chemiosmotic theory first postulated by Mitchell (1975), the free energy (ΔG) released by the oxidation of highly reduced energetic substrates is used by the complexes I, III, and IV of the electron transport chain to generate a proton gradient across the IMM. The energy stored in the proton gradient is then used to drive the activity of the ATP synthase (complex V) that catalyzes the conversion of ADP to ATP within the mitochondrial matrix. The importance of mitochondrial proton transport is highlighted by the retention of genes coding for OXPHOS subunits within the mitochondrial genome. Mitochondria are endosymbiotic organelles, and virtually all of the ~1,500 genes required to build a functional mitochondria have been transferred to the chromosomes of the host cell, except for those coding for 13 polypeptides of the OXPHOS subunits, plus the ribosomal and transfer RNAs required for their synthesis. The 13 mitochondrial-encoded proteins include seven subunits of the respiratory chain complex I, one of complex III, three of complex IV, and two of the complex V, i.e., all the respiratory chain complexes that are involved in the transport of protons. The chemiosmotic theory is rooted in measurements of bioenergetics parameters, such as oxygen consumption, ATP production, pH, and membrane potential, in isolated mitochondria artificially maintained under different metabolic conditions. In intact cells, however, mitochondria are exposed to metabolic and environmental fluctuations, interact with other organelles, and receive inputs from cell signaling pathways. Therefore, data derived from experiments in isolated mitochondria cannot be readily transposed in vivo.

The generation of a proton gradient across the inner mitochondrial membrane (IMM) is an essential energy conservation event that couples the oxidation of carbohydrates and fat to the synthesis of ATP. Studies in isolated mitochondria have established that the chemical gradient for protons (ΔpHₐ) and the mitochondrial membrane potential (ΔΨₐ) contribute independently to the proton-motive force (Δp) that drives the synthesis of ATP. Because ΔΨₐ contributes most of the Δp and can be easily measured in intact cells with fluorescent dyes, most studies ignore the contribution of ΔpHₐ and only record changes in ΔΨₐ to track the metabolic state of mitochondria. ΔpHₐ, however, drives the fluxes of metabolic substrates required for mitochondrial respiration and the activity of electroneutral ion exchangers that maintain mitochondria osmolarity and volume, and recent studies indicate that the mitochondrial pH (pHₙₐₜₐ) plays an important and underappreciated role in physiological and pathological situations such as apoptosis, neurotransmission, and insulin secretion. In this Perspective, we discuss the putative roles of the pHₙₐₜₐ and review the different techniques used to measure pHₙₐₜₐ and ΔpHₐ in isolated mitochondria and in intact cells, focusing on our recent results obtained with genetically encoded pH-sensitive indicators. These measurements have revealed that the pHₙₐₜₐ is in dynamic equilibrium with the cytosolic pH and that spontaneous pHₙₐₜₐ elevations coinciding with ΔΨₐ drops occur in single mitochondria. Unlike the “superoxide flashes” reported with a pH-sensitive circularly permuted YFP (cpYFP), these “pH flashes” preserve the Δp during spontaneous fluctuations in ΔΨₐ; therefore, we propose that the flashes are energy conservation events that reflect the intrinsic properties of the mitochondrial proton circuit.

Abbreviations used in this paper: CHX, Ca²⁺–H⁺ exchanger; cpYFP, circularly permuted YFP; Δp, proton-motive force; ΔpHₐ, chemical gradient for protons; ΔΨₐ, mitochondrial membrane potential; IMM, inner mitochondrial membrane; IMS, intermembrane space; KHX, K⁺–H⁺ exchanger; mPTP, permeability transition pore; NHX, Na⁺–H⁺ exchanger; OXPHOS, oxidative phosphorylation; pHₚₛₐₜ, pH within the IMS; pHₙₐₜₐ, mitochondrial pH; PiC, Pₐₜ–H⁺ phosphate cotransporter; ROS, reactive oxygen species; UCP, uncoupling protein.

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In this Perspective, we will briefly describe the mechanism that maintains and regulates pH\textsubscript{mito} as established in isolated mitochondria and integrate this knowledge with more recent recordings of pH\textsubscript{mito} in intact living cells obtained with genetically encoded pH-sensitive probes, with a focus on our recent report that single mitochondria exhibit spontaneous pH\textsubscript{mito} elevations.

**Moving protons in and out of mitochondria**

Given the central role of mitochondrial proton transport in energy conversion, much effort has been devoted to unraveling the sophisticated molecular machinery that moves protons across the IMM. Protons are extruded from the matrix to the intermembrane space (IMS) by the respiratory complexes I, III, and IV during consecutive redox reactions that couple the free energy released during the transport of electrons from high to low redox potentials to the extrusion of protons (Dempsey et al., 2010). Crystal structures of the respiratory complexes have been obtained (Abrahams et al., 1994; Iwata et al., 1998), and the stoichiometry of H⁺ ejection was established as 10 H⁺ pumped for each pair of electrons entering at the level of complex I (Saraste, 1999). Because of the low permeability of the IMM to ions, including H⁺, the extrusion of protons by the respiratory complexes creates an electrochemical gradient for H⁺ across the membrane (ΔΨ\textsubscript{m}, more commonly expressed as Δp). Δp is the sum of an electrical gradient that constitutes the ΔΨ\textsubscript{m} and of a chemical gradient ΔpH\textsubscript{m} that reflects the pH difference between the pH\textsubscript{mito} and the pH within the IMS (pH\textsubscript{IMS}). From a chemiosmotic point of view, ΔΨ\textsubscript{m} and ΔpH\textsubscript{m} are independent components that equally contribute to the Δp driving the synthesis of ATP as H⁺ ions return to the matrix at the level of complex V (Mitchell, 1961). In addition to powering ATP synthesis, the potential energy stored in the H⁺ gradient also drives the transport of ions and metabolites across the IMM (Fig. 1). Some transporters rely only on ΔΨ\textsubscript{m}, for instance: Ca\textsuperscript{2+} uptake via the mitochondrial Ca\textsuperscript{2+} uniporter (Baughman et al., 2011; De Stefani et al., 2011), ATP–ADP exchange via the adenine nucleotide translocator (Krämer and Klingenberg, 1980; Klingenberg, 2008), or the import of mitochondrial resident protein via the translocase of outer membrane and the translocase of inner membrane complexes (Martin et al., 1991; Bauer et al., 1996). Conversely, several transporters rely exclusively on ΔpH\textsubscript{m}, such as the Ca\textsuperscript{2+}–H⁺ exchanger (CHX), K⁺–H⁺ exchanger (KHX), and Na⁺–H⁺ exchanger (NHX), whose molecular identities remain controversial (Nowikovsky et al., 2004; Jiang et al., 2009; Zotova et al., 2010). The P₁–H⁺ phosphatase cotransporter (PiC), which imports the phosphate required for ATP synthesis into the matrix, also relies on ΔpH\textsubscript{m} (Palmieri, 2004). Some transporters dissipate both ΔΨ\textsubscript{m} and ΔpH\textsubscript{m}, such as uncoupling proteins (UCPs), H⁺ channels that uncouple OXPHOS from ATP synthesis, and the permeability transition pore (mPTP), a nonselective ion channel whose opening initiates cell death by allowing the fluxes of ions and metabolites of up to 1,500 KD across the IMM.

![Diagram](https://example.com/diagram.png)

**Figure 1.** Determinants of the pH\textsubscript{mito}. Protons are pumped from the matrix to the IMS by the respiratory chain complexes I, III, and IV (green boxes) as electrons flow from reduced substrates in the matrix to O₂. The pumping of electrically charged protons generates a ΔΨ\textsubscript{m} of ~180 mV and a pH gradient (ΔpH\textsubscript{m} = pH\textsubscript{mito} – pH\textsubscript{IMS}) of ~0.9 pH units as the matrix becomes more alkaline than the IMS. The proton circuit is in thermodynamic equilibrium and changes in ΔΨ\textsubscript{m} result in opposing changes in ΔpH\textsubscript{m}, thus causing a decrease ΔpH\textsubscript{m} by altering the energy required for the pumping of protons by respiratory chain complexes. ΔΨ\textsubscript{m} and ΔpH\textsubscript{m} add up to generate a Δp used by the ATP synthase (blue-orange barrel) to generate ATP from ADP and Pi in the matrix. ΔΨ\textsubscript{m} drives Ca\textsuperscript{2+} uptake across the mitochondrial Ca\textsuperscript{2+} uniporter (MCU; blue cylinder) and ADP–ATP exchange across the adenine nucleotide translocator (ANT; brown ovals). Electroneutral H⁺–ion exchangers rely exclusively on ΔpH\textsubscript{m} to extrude Ca\textsuperscript{2+}, Na⁺, and K⁺ ions in exchange for protons (CHX, NHX, and KHX, respectively; brown ovals), whereas the PiC relies on ΔpH\textsubscript{m} to import the inorganic phosphate used for the synthesis of ATP (PiC; brown ovals). The coupling of H⁺ and ion fluxes implies that changes in the Na⁺, K⁺, Ca\textsuperscript{2+}, and Pi gradients can alter ΔpH\textsubscript{m}. UCPs and the mPTP (UCPs and mPTP; blue cylinders) dissipate both ΔpH\textsubscript{m} and ΔΨ\textsubscript{m}, to generate heat and to initiate cell death, respectively. Variations in pH\textsubscript{mito} reflect the equilibrium between proton pumping by the respiratory chain; Δp dissipation by the ATP synthase, UCPs, and mPTP; ΔpH\textsubscript{m} dissipation by KHX, NHX, CHX, and PiC; and adaptive responses to changes in cytosolic pH and in ΔΨ\textsubscript{m}. 

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(Kroemer et al., 2007). UCP1 is expressed in brown adipose fat where it acts as a proton channel to mediate adaptive thermogenesis (Cannon and Nedergaard, 2004), whereas the UCP2 and UCP3 isoforms, expressed in non-adipose tissues, do not appear to function as proton channels under basal conditions (Cadenas et al., 2002; Couplan et al., 2002) but only upon stimulation by fatty acids and purine nucleotides (Palmieri, 2004). The mitochondrial matrix pH, pH$_{\text{mito}}$, reflects the equilibrium between proton extrusion and proton entry into the matrix. Variations in pH$_{\text{mito}}$ therefore reflect the equilibrium between proton pumping by the respiratory chain and proton back-flux across the ATP synthase, across the KHX, NHX, CHX, and PiC, and across the UCP and mPTP. Variations in pH$_{\text{mito}}$ are also limited by mitochondrial H$^+$ buffers provided by the side chains of amino acids and by phosphates and bicarbonates, which dampen the variations in the free H$^+$ concentration during acid or alkaline loads. But because the pH$_{\text{mito}}$-buffering capacity ($\beta_{\text{mito}}$) is quite low at the physiological alkaline pH of the matrix (Poburko et al., 2011), pH$_{\text{mito}}$ changes mainly reflect the activity of H$^+$ fluxes across the IMM.

Measurements in isolated mitochondria

The validation of the chemiosmotic theory implied precise measurements of the electrical and chemical component of the $\Delta p$ under well-controlled conditions, and until 1980, the preparation of choice was isolated mitochondria purified from liver by differential centrifugation. After attempts to impale giant mitochondria with microelectrodes (Maloff et al., 1977), physiologists relied on external K$^+$ and H$^+$-selective electrodes or on isotopes to measure $\Delta \Psi_m$ and $\Delta pH_m$ in suspended mitochondria (Mitchell and Moyle, 1969). The electrical component $\Delta \Psi_m$ was estimated by measuring the distribution of radioactively labeled lipophilic cations or by recording the changes in external [K$^+$] or the accumulation of matrix $^{36}$Rb$^+$ in the presence of the potassium ionophore valinomycin. This approach relies on the assumption that cations distribute according to the Donnan equilibrium and provided precise estimates of the distribution of K$^+$ or Rb$^+$ across the IMM. The chemical component $\Delta pH_m$ was estimated by measuring the distribution of radioactively labeled weak acids or bases, $^3$H-acetate or $^{14}$C-methylamine, assuming that the IMM is permeable to the uncharged but impermeable to the charged species (Nicholls, 1974), or by monitoring the changes in external pH after the lysis of mitochondria with detergents to estimate pH$_{\text{mito}}$, a calculation that requires the knowledge of the mitochondrial volume and of the buffering capacity of the mitochondrial matrix (Rottenberg, 1975). These measurements established that $\Delta p$ ranges from 180 to 220 mV depending on the metabolic state of the mitochondria, with $\Delta \Psi_m$ ranging from 50 to 180 mV and $\Delta pH_m$ from 0.5 to 1.2 pH units ($\text{pH}_{\text{mito}} = 8.2$–7.5 and $\text{pH}_{\text{out}} = 7$). Using the simplified Nernst equation ($E_m = -60 \log [H^+]/[H^+]_o$ at 30°C), the pH gradient can be converted into a diffusion potential and its contribution to the $\Delta p$ was estimated to be 30–70 mV, i.e., 17–30% of $\Delta p$, indicating that $\Delta \Psi_m$ is the main component of the $\Delta p$. These measurements provided the first quantitative estimates of the two components to the $\Delta p$ generated by mitochondria, grounding the chemiosmotic theory in solid scientific evidence and confirming several of its predictions. One of these predictions was the postulate that $\Delta \Psi_m$ and $\Delta pH_m$ add up to build $\Delta p$, which implied that, in respiring mitochondria, selective manipulations of $\Delta \Psi_m$ would induce compensatory alterations in $\Delta pH_m$ to preserve $\Delta p$. This was nicely demonstrated by Nicholls (1974) in isolated mitochondria equilibrated with valinomycin/K$^+$ and exposed to increasing amounts of K$^+$ to clamp $\Delta \Psi_m$ to varying voltages. In these conditions, $\Delta p$ remains constant as mitochondria are depolarized because the decreases in $\Delta \Psi_m$ are exactly balanced by opposite increases in $\Delta pH_m$ (see Nicholls, 2005, for a recent discussion of these findings). The compensation occurs over the whole range of voltages tested to the point that, when $\Delta \Psi_m$ is fully dissipated, the $\Delta p$ is contributed exclusively by $\Delta pH_m$. Conversely, when $\Delta pH_m$ is collapsed by the K$^+$/H$^+$ ionophore nigericin, $\Delta p$ is contributed exclusively by $\Delta \Psi_m$ (Lambert and Brand, 2004). These experiments demonstrated that the two components of the $\Delta p$ can vary widely without dissipating the stored energy, as $\Delta pH_m$ can fully compensate for imposed changes in $\Delta \Psi_m$ and vice versa.

Measurements in living cells

The recognition that $\Delta \Psi_m$ is the major contributor of $\Delta p$ fostered the development of optical recording techniques to measure this parameter in intact cells. Since 1980, the preferred method is to use fluorescence lipophilic cations that distribute across the IMM according to the membrane potential. Fluorescent dyes such as TMRM, rhodamine, or JC1 provide a simple optical readout of the mitochondrial potential and enable the study of its dynamic regulation in intact living cells under physiological conditions. These dyes have become so popular that, in virtually all studies, the fluorescent $\Delta \Psi_m$ signal is thought to reflect the energization state of mitochondria, an assumption that equates $\Delta \Psi_m$ with $\Delta p$ and thus ignores the contribution of $\Delta pH_m$. As discussed in the preceding section, however, $\Delta pH_m$ contributes 20–30% of $\Delta p$ and can fully compensate for a loss in $\Delta \Psi_m$ when the mitochondrial potential is varied with an artificial K$^+$ conductance. To confidently establish the energization state of mitochondria, both $\Delta \Psi_m$ and $\Delta pH_m$ should be measured simultaneously to obtain a complete readout of $\Delta p$. Unfortunately, $\Delta pH_m$ is not only ignored but also more difficult to measure than $\Delta \Psi_m$, and very few studies so far have attempted to record
dynamic changes in ΔpH_m. Using radioactively labeled weak acid and bases, ΔpH_m was estimated around 1.0–1.2 pH units, contributing 60 mV to Δp in intact cells (Hock et al., 1980; Brand and Felber, 1984), but isotopic measurements are restricted to cell populations, do not allow real-time recordings, and do not provide any spatial information. Optical recordings of pH_m with pH-sensitive fluorescent dyes such as BCECF or SNARF brought the resolution down to the single-cell level. Using this approach, ΔpH_m was found to be ∼0.9 pH units in cardiac myocytes and to collapse with a different kinetic than ΔΨ_m during chemical hypoxia (Lemasters et al., 1995), whereas in MDCK cells, ΔpH_m was around 0.3 pH units and was dynamically regulated during metabolic inhibition (Balut et al., 2008). Because chemical dyes are not specifically targeted to mitochondria, cells must be simultaneously loaded with a fluorescent mitochondrial marker to distinguish between the mitochondrial and cytosolic pH signal; therefore, this approach is better suited for isolated mitochondria or permeabilized cells. To enable time-resolved in situ recordings of pH_m, an ideal fluorescent sensor should exhibit the following properties: (a) specific targeting to the mitochondrial matrix; (b) reduced toxicity compared with BCECF-AM or SNARF-AM, which generate harmful mitochondrial matrix; (c) rapid and reversible response to variations in pH_m; (d) high pH specificity to discriminate between pH changes and changes in ionic strength or in redox conditions; (e) high pH specificity to discriminate between pH changes and changes in ionic strength or in redox conditions; (f) ratiometric and nonratiometric GFP mutants have since been developed that exhibit an alkaline-shifted pK_a such as the YFP mutants H148G (pK_a = 8) and S65T/H114D (pK_a = 7.8) (Ebsiger et al., 1999), or the deGFP1 S65T/H114G/T203C (pK_a = 8) (Hanson et al., 2002). Using another strategy, Pozzan’s group (Abad et al., 2004) took advantage of the high pH sensitivity of the YFP-based Ca^{2+} sensors Camgaroos to generate a probe with an apparent pK_a of 8.5, mt-AlpHi, by replacing the Ca^{2+}-sensitive domain of the Camgaroo by a Ca^{2+}-insensitive module. In HeLa cells and primary cultured neurons, mt-AlpHi reported that basal pH_m levels were around 8.0 and increased heterogeneously upon stimulation with Ca^{2+}-mobilizing agonists, with some mitochondria alkalinizing and others not (Abad et al., 2004). In rat pancreatic β cells, sustained increases in pH_m, and in ΔpH_m, were observed with mt-AlpHi during glucose stimulation that correlated with an increase in mitochondrial ATP synthesis, indicating that pH_m is an important signal during nutrient-induced insulin secretion (Wiederkehr et al., 2009). Treatment with nigericin to prevent pH_m alkalinization blunted nutrient-induced ATP increase and insulin secretion (Akhmedov et al., 2010), indicating that pH_m and ΔpH_m control mitochondrial metabolism during cell stimulation (Wiederkehr, 2009). The new red-shifted RFPs are also promising tools to measure pH in living cells (Johnson et al., 2009), although their pKa values in the acidic range preclude accurate pH measurements in alkaline organelles (Jach et al., 2006; Shaner et al., 2008). pHRed (pK_a = 7.8) has been used to carry out simultaneous measurements of pH_m and ATP_m combined with Perceval (Tantama et al., 2011), and the availability of such alkaline-sensitive red-shifted fluorescent proteins will allow multicolor imaging of pH_m together with key parameters like Ca^{2+}, ATP, or ROS.

ΔpH_m is usually calculated as pH_m − pH_sto because the outer mitochondrial membrane has a high permeability to ions. The bulk pH_sto however, might not reflect the actual pH values achieved in the IMS, where H^+ is continuously ejected by respiratory chain complexes. Accordingly, recordings with a pH-sensitive YFP targeted to the outer surface of the IMM reported a pH of 6.8, i.e.,
slightly more acidic than the cytosol, and a $\Delta pH_{m}$ of 0.8 pH units (Porcelli et al., 2005). The pH on the IMS side of mitochondria cristae might be even more acidic than the bulk IMS pH because respiratory complexes are concentrated on these invaginations, which are connected to the IMS by small tubular junctions that constrain the diffusion of solutes (Scorrano et al., 2002). Indeed, electron cryotomography studies reported long ribbons of ATP synthase dimers assembling on tightly curved cristae edges (Strauss et al., 2008), an arrangement predicted to increase the surface density of protons in the curved membrane regions by ~0.5 pH units, thereby turning cristae into proton traps (Davies et al., 2011).

We have recently developed a new genetically encoded pH-sensitive probe, mito-SypHer, which we used to follow $\Delta pH_{m}$ changes during physiological activation of cells by Ca$^{2+}$-mobilizing agonists (Poburko et al., 2011). The probe was derived from HyPer, a cpYFP-based indicator for hydrogen peroxide very sensitive to alkaline pH, by mutating a cysteine residue to remove the probe H$_2$O$_2$ sensitivity. SypHer is highly sensitive to pH but insensitive to oxidizing and reducing agents, and has two maximal absorbance peaks at 430 and 490 nm that enable ratiometric measurements of the changes in environmental pH. By combining mito-SypHer with a fluorescent pH dye, we could record $pH_{mito}$ and $pH_{cyto}$ simultaneously to track dynamic changes in $\Delta pH_{m}$ in live cells. In HeLa cells, $pH_{mito}$ and $\Delta pH_{m}$ averaged 7.6 and 0.45 and, surprisingly, decreased together with $pH_{cyto}$ during activation of cells with Ca$^{2+}$-mobilizing agonists (Fig. 2). The rapid acidification of the cytosol reflected the activity of plasma membrane Ca$^{2+}$ pumps, and the cytosolic acid was readily transmitted to the mitochondrial matrix, predominantly via the KHX and Pi/H$^+$ symporter, thereby causing a mitochondrial acidification instead of the alkalization that was previously reported with mt-AlpHi in HeLa cells exposed to histamine (Abad et al., 2004) and in pancreatic β cells treated with glucose (Wiederkehr et al., 2009). The $\Delta pH_{m}$ decrease reflected the larger decrease in $pH_{mito}$ compared with $pH_{cyto}$ (Fig. 2), which in turn reflects the lower buffering capacity of mitochondria at physiological pH levels ($\beta_{mito} = 5$ mM at pH 7.8) compared with the cytosol ($\beta_{cyto} = 20$ mM at pH 7.4). Similar matrix acidification and $\Delta pH_{m}$ dissipation were observed in astrocytes exposed to glutamate, with the decreased $\Delta pH_{m}$ being associated with decreased O$_2$ consumption and reduced mitochondrial ROS generation (Azarias et al., 2011), suggesting that the mitochondrial metabolism of astrocytes decreases during neurotransmission, a mechanism that might increase local oxygen availability for neurons. The matrix acidification and $\Delta pH_{m}$ dissipation observed in HeLa cells and astrocytes appears at odds with earlier studies showing that cytosolic Ca$^{2+}$ elevations boost mitochondrial metabolism (Hajnóczy et al., 1995), but the rapid acidification evoked by the cytosolic Ca$^{2+}$ elevations was followed by a slow matrix alkalization as the cytosolic Ca$^{2+}$ signal subsided (Fig. 2), consistent with Ca$^{2+}$-dependent activation of matrix enzymes. Furthermore, the addition of micromolar Ca$^{2+}$ concentrations to permeabilized cells induced a slight and progressive matrix alkalization (Poburko et al., 2011). These findings suggest that cytosolic Ca$^{2+}$ elevations exert opposite effects on $pH_{mito}$, as they stimulate mitochondrial respiration, thereby increasing $pH_{mito}$, and at the same time generate large quantities of cytosolic acid that is transmitted to the mitochondrial matrix, thereby decreasing $pH_{mito}$. In cells that are essentially glycolytic such as cultured HeLa cells and astrocytes, the latter mechanism dominates and $\Delta pH_{m}$ decreases during Ca$^{2+}$ elevations. More fundamentally, these data indicate that the permeability of the IMM to protons is quite high in situ and thus appear to contradict the fourth postulate of the chemiosmotic theory, that mitochondria must be impermeable to protons to allow the generation of a $\Delta p$. However, the rapid pH equilibration was not caused by electrophoretic entry of protons but by the activity of electroneutral ion–H$^+$ exchangers, and our findings therefore remain consistent with the chemiosmotic theory, whose third postulate predicts the existence of exchangers coupling anion entry and cation extrusion to proton entry.

**pH elevations in single mitochondria**

Advances in live cell imaging revealed that mitochondria are morphologically and functionally heterogeneous within cells (Collins et al., 2002) and that rapid
fluctuations in \( \Delta \Psi_m \) occur in single mitochondria (Duchen et al., 1998; Hüser et al., 1998). The depolarization transients have been proposed to be triggered by Ca\(^{2+}\) elevations (Duchen et al., 1998), by openings of the mPTP (Hüser and Blatter, 1999; De Giorgi et al., 2000; Zorov et al., 2000; Jacobson and Duchen, 2002), by changes in the matrix concentration of adenine nucleotides (Vergun et al., 2003; Vergun and Reynolds, 2004), by the activity of the ATP synthase (Thiffault and Bennett, 2005), or by the opening of a H\(^+\)-selective channel (Hattori et al., 2005), and their functional significance is currently unknown. The fluctuations in \( \Delta \Psi_m \) coincide with transient elevations in matrix [Na\(^+\)] in astrocytes (Azarias et al., 2008), with ROS oscillations and NADH fluctuations in cardiac myocytes (Aon et al., 2003), and with superoxide flashes in skeletal muscle (Azarias and Chatton, 2011). The nature of the superoxide flashes is debated because flash activity persisted under anaerobic conditions and was abolished by all respiratory chain inhibitors including antimycin, which is known to boost superoxide production (Muller, 2009). In response to these criticisms, the authors performed additional experiments to show that the flashes are nearly abrogated during chemical and physical anoxia, and attributed the unexpected effects of antimycin to the unique mechanism of superoxide flash production (Huang et al., 2011). In plants, the cpYFP probe used to detect the putative superoxide flashes was found to be highly responsive to changes in matrix pH but insensitive to changes in matrix superoxide, raising the possibility that the fluctuations were pH and not superoxide flashes (Schwarzländer et al., 2011). Using our ratiometric pH-sensitive probe mito-SypHer, we and others observed spontaneous \( \Delta \Psi_m \) elevations of 0.4 pH units coinciding with increases in \( \Delta \Psi_m \) in individual mitochondria of HeLa cells (Fig. 3) (Santo-Domingo, J., and N. Demaurex. 2010. 16th European Bioenergetics Conference. Abstr. 15L.3; Santo-Domingo, J., and N. Demaurex. 2011. 65th Annual Meeting of The Society of General Physiologists. Abstr. 34) and of astrocytes (Azarias and Chatton, 2011). The \( \Delta \psi_m \) elevations had an abrupt onset and a slower recovery and their frequency was reduced by all respiratory chain inhibitors, a spatiotemporal and pharmacological profile similar to the superoxide flashes. To clarify the nature of the signal, we tested the pH and superoxide sensitivity of bacterially expressed SypHer and found the probe to be highly sensitive to pH but insensitive to superoxide in vitro (Santo-Domingo, J., and N. Demaurex. 2012. Biophysical Society 56th Annual Meeting. Abstr. 2907). Increasing the pH-buffering power of mitochondria delayed and decreased the amplitude of the \( \Delta \psi_m \) elevations, strongly suggesting that the elevations were caused by protons. Although this manipulation could alter mitochondrial function, it is unlikely to distort the kinetics of superoxide flashes exactly as predicted from the increase in pH-buffering power (Poburko et al., 2011). The rapid and transient elevations in SypHer ratio fluorescence observed in single mitochondria therefore reflect increases in matrix pH. Interestingly, we observed that enforced mitochondrial fusion increased the spatial extent of the \( \Delta \psi_m \) elevations, whereas fragmentation had the opposite effect, indicating that

![Figure 3. Alkalinization transients in single mitochondria. HeLa cells expressing mito-SypHer were recorded on a spinning disc confocal microscope at a frequency of 1.2 Hz. Ratio F480/F430 images from two cells exhibiting spontaneous alkalinization transients are shown, with warm colors denoting high ratio values. The \( \Delta \psi_m \) elevations occurred either in different regions of the mitochondrial network or repeatedly at the same location, but they always remained restricted to a particular mitochondrial cluster.](image-url)
mitochondrial fusion facilitates the propagation of $\Delta pH_m$ by functionally coupling mitochondria. The $pH_{mito}$ elevations persisted in cells permeabilized with solutions devoid of ions and, importantly, could be mimicked by artificial depolarization of mitochondria. These observations indicate that the $pH_{mito}$ flashes, which occur coincidentally with spontaneous decreases in $\Delta \Psi_{wm}$, reflect increased pumping by the respiratory chain during drops in $\Delta \Psi_{wm}$. A transient mitochondrial depolarization thermodynamically favors H$^+$ extrusion by decreasing the driving force for proton pumping by the respiratory chain complexes, and several studies in isolated mitochondria have confirmed this prediction by showing that an imposed decrease in $\Delta \Psi_{wm}$ increases the rate of proton extrusion and $O_2$ consumption (Talbot et al., 2007). Therefore, $pH_{mito}$ flashes reflect the intrinsic properties of the mitochondrial proton circuit.

These findings have important functional consequences, because other studies have linked superoxide flashes to altered mitochondrial respiration during oxidative stress–induced apoptosis (Ma et al., 2011). We propose instead that the flashes are alkalinization events that do not alter the ability of mitochondria to convert energy, but that, on the contrary, preserve the $\Delta p$ during spontaneous fluctuations in $\Delta \Psi_{wm}$. Spontaneous $\Delta \Psi_{wm}$ fluctuations are a well-known phenomenon thought to reflect alterations in mitochondrial metabolism. The observation that the $\Delta p$ remains constant during concomitant $\Delta \Psi_{wm}$ drops and $pH_{mito}$ flashes indicates that the ability of mitochondria to convert energy is preserved during these bursts of electrical and chemical activity.

In conclusion, the $pH_{mito}$, which was long neglected, is the object of renewed interest as GFP-based pH-sensitive indicators now allow recordings of dynamic changes in $pH_{mito}$ in living cells. The interpretation of $pH_{mito}$ changes is difficult because the steady-state pH of the organella reflects the combined activities of the respiratory chain and of mitochondrial H$^+$ transporters and is affected by variations in cytosolic pH and by variations in $\Delta \Psi_{wm}$. The observation that elementary fluctuations in $\Delta pH_m$ occur in single mitochondria and spread across the cell as mitochondria fuse provides new insights on the properties of the mitochondrial proton circuit and on the ability of mitochondria to propagate energy inside cells.

**REFERENCES**

Abad, M.F., G. Di Benedetto, P.J. Magalhães, L. Filippini, and T. Pozzan. 2004. Mitochondrial pH monitored by a new engineered green fluorescent protein mutant. *J. Biol. Chem.* 279:11521–11529. http://dx.doi.org/10.1074/jbc.M306766200

Abrahams, J.P., A.G. Leslie, R. Lutter, and J.E. Walker. 1994. Structure at 2.8 A resolution of F1-ATPase from bovine heart mitochondria. *Nature* 370:621–628. http://dx.doi.org/10.1038/370621a0

Ahmedov, D., M. Braun, C. Mataka, K.S. Park, T. Pozzan, K. Schoonjans, P. Rorsman, C.B. Wollheim, and A. Wiederkehr. 2010. Mitochondrial matrix pH controls oxidative phosphorylation and metabolism-secretion coupling in INS-1E clonal beta cells. *FASEB J.* 24:4613–4626. http://dx.doi.org/10.1096/fj.10-162222

Aon, M.A., S. Cortassa, E. Marbán, and B. O’Rourke. 2003. Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. *J. Biol. Chem.* 278:44754–44744. http://dx.doi.org/10.1074/jbc.M302673200

Azarias, G., and J.Y. Chatton. 2011. Selective ion changes during spontaneous mitochondrial transients in intact astrocytes. *PLoS ONE* 6:e28505. http://dx.doi.org/10.1371/journal.pone.0028505

Azarias, G., D. Van de Ville, M. Unser, and J.Y. Chatton. 2008. Spontaneous NA+ transients in individual mitochondria of intact astrocytes. *Glia* 56:342–353. http://dx.doi.org/10.1002/glia.20619

Azarias, G., H. Perreten, S. Lengacher, D. Poburko, N. Demaurex, P.J. Magistretti, and J.Y. Chatton. 2011. Glutamate transport decreases mitochondrial pH and modulates oxidative metabolism in astrocytes. *J. Neurosci.* 31:3550–3559. http://dx.doi.org/10.1523/JNEUROSCI.4378-10.2011

Balut, C., M. vandeVen, S. Despa, I. Lambrichts, M. Ameloot, P. Steele, and I. Smets. 2008. Measurement of cytosolic and mitochondrial pH in living cells during reversible metabolic inhibition. *Kidney Int.* 73:226–232. http://dx.doi.org/10.1038/sj.ki.5002632

Bauer, M.F., C. Sirrenberg, W. Neupert, and M. Brunner. 1996. Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. *Cell* 87:35–41. http://dx.doi.org/10.1016/0092-8674(95)01320-3

Baughman, J.M., F. Perocchi, H.S. Girgis, M. Plovanich, C.A. Belcher-Timme, Y. Sancak, X.R. Bao, L. Strittmatter, O. Goldberger, R.L. Bogorad, et al. 2011. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature* 476:341–345. http://dx.doi.org/10.1038/nature10234

Brand, M.D., and S.M. Pelber. 1984. Membrane potential of mitochondria in intact lymphocytes during early mitogenic stimulation. *Biochem. J.* 217:453–459.

Cadenas, S., K.S. Echtay, J.A. Harper, M.B. Jekabsons, J.A. Buckingham, E. Grau, A. Abuin, H. Chapman, J.C. Clapham, and M.D. Brand. 2002. The basal proton conductance of skeletal muscle mitochondria from transgenic mice overexpressing or lacking uncoupling protein-3. *J. Biol. Chem.* 277:2773–2778. http://dx.doi.org/10.1074/jbc.M109736200

Cannon, B., and J. Nedergaard. 2004. Brown adipose tissue: function and physiological significance. *Physiol. Rev.* 84:277–359. http://dx.doi.org/10.1152/physrev.00015.2003

Collins, T.J., M.J. Berridge, P. Lipp, and M.D. Bootman. 2002. Mitochondria are morphologically and functionally heterogeneous within cells. *EMBO J.* 21:1616–1627. http://dx.doi.org/10.1093/emboj/c21.7.1616

Couplan, E., M. del Mar Gonzalez-Barroso, M.C. Alves-Guerra, D. Ricquier, M. Goubert, and F. Bouillaud. 2002. No evidence for a basal, retinoic, or superoxide-induced uncoupling activity of the uncoupling protein 2 present in spleen or lung mitochondria. *J. Biol. Chem.* 277:26208–26275. http://dx.doi.org/10.1074/jbc.M202535200

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Davies, K.M., M. Strauss, B. Daum, J.H. Kief, H.D. Osiewacz, A. Rycosska, V. Zieckermann, and W. Kühlbrandt. 2011. Macromolecular organization of ATP synthase and complex I in whole mitochondria. Proc. Natl. Acad. Sci. USA. 108:14121–14126. http://dx.doi.org/10.1073/pnas.110362108

De Giorgi, F., L. Larigue, and F. Ichas. 2000. Electrical coupling and plasticity of the mitochondrial network. Cell Calcium. 28:365–370. http://dx.doi.org/10.1054/cell.2000.0177

De Stefani, D., A. Raffaello, E. Teardo, I. Szabó, and R. Rizzuto. 2011. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature. 476:336–340. http://dx.doi.org/10.1038/nature10290

Dempsey, J.L., J.R. Winkler, and H.B. Gray. 2010. Proton-coupled electron flow in protein redox machines. Chem. Rev. 110:7024–7099. http://dx.doi.org/10.1021/cr100182b

Duchen, M.R., A. Leyssens, and M. Crompton. 1998. Transient mitochondrial pH fluctuations in single rat cardiomyocytes. J. Cell Biol. 142:975–988. http://dx.doi.org/10.1083/jcb.142.4.975

Elsiger, M.A., R.M. Wachter, G.T. Hanson, K. Kallio, and S.J. Remington. 1999. Structural and spectral response of green fluorescent protein variants to changes in pH. Biochemistry. 38:5296–5301. http://dx.doi.org/10.1021/bi9902182

Hajnoczy, G., L.D. Robb-Gaspers, M.B. Seitz, and A.P. Thomas. 1995. Decoding of cytosolic calcium oscillations in the mitochondria. Cell. 82:415–424. http://dx.doi.org/10.1016/0092-8674(95)90430-1

Hanson, G.T., T.B. McAnaney, E.S. Park, M.E. Rendell, D.K. Yarbrough, S. Chu, L. Xi, S.G. Boxer, M.H. Montrose, and S.J. Remington. 2002. Green fluorescent protein variants as ratiometric pH sensors. 1. Structural characterization and preliminary application. Biochemistry. 41:15477–15488. http://dx.doi.org/10.1021/bi010609p

Hattori, T., K. Watanabe, Y. Uechi, H. Yoshioka, and Y. Ohta. 2005. Repetitive transient depolarizations of the inner mitochondrial membrane induced by proton pumping. Biophys. J. 88:2340–2349. http://dx.doi.org/10.1529/biophysj.104.041483

Hoch, J.B., D.G. Nicholls, and J.R. Williamson. 1980. Determination of the mitochondrial protonotive force in isolated hepatocytes. J. Biol. Chem. 255:1458–1464.

Huang, Z., W. Zhang, H. Fang, M. Zheng, X. Wang, J. Xu, H. Cheng, G. Gong, W. Wang, R.T. Dirksen, and S.S. Sheu. 2011. Response of mitochondrial calcium uniporter to different forms of mitoK-ATP stimulation. Free Radic. Biol. Med. 51:1937–1940. http://dx.doi.org/10.1016/j.freeradbiomed.2011.08.024

Hüser, J., and L.A. Blatter. 1998. Fluorochromes in mitochondria: membrane potential caused by repetitive gating of the permeability transition pore. Biochem. J. 343:311–317. http://dx.doi.org/10.1042/0264-6021:343:311

Hüser, J., C.E. Rechenmacher, and L.A. Blatter. 1998. Imaging the permeability transition pore in single mitochondria. Biophys. J. 74:2129–2137. http://dx.doi.org/10.1016/S0006-3495(98)77920-2

Iwata, S., J.W. Lee, K. Okada, J.K. Lee, M. Iwata, B. Rasmussen, T.A. Matsuyama, S., J. Llopis, Q.L. Deveraux, R.Y. Tsien, and J.C. Reed. 2005. Macromolecular organization of ATP synthase and complex I in whole mitochondria. Proc. Natl. Acad. Sci. USA. 102:20511–20516. http://dx.doi.org/10.1074/jbc.M109.019042

Klingenberg, M. 2008. The ADP and ATP transport in mitochondria and its carrier. Biochem. Biophys. Acta. 1778:1978–2021. http://dx.doi.org/10.1016/j.bbamem.2008.04.011

Knee, M., J. Farinas, Y. Li, and A.S. Verkman. 1998. Green fluorescent protein as a noninvasive intracellular pH indicator. Biophys. J. 74:1591–1599. http://dx.doi.org/10.1016/S0006-3495(98)77870-1

Krämer, R., and M. Klingenberg. 1980. Modulation of the reconstructed adenine nucleotide exchange by membrane potential. Biochemistry. 19:556–560. http://dx.doi.org/10.1021/bi00454a025

Kroemer, G., L. Galluzzi, and C. Brenner. 2007. Mitochondrial membrane permeabilization in cell death. Physiol. Rev. 87:99–163. http://dx.doi.org/10.1152/physrev.00013.2006

Lambert, A.J., and M.D. Brand. 2004. Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. Biochem. J. 382:511–517. http://dx.doi.org/10.1042/BJ20040485

Lemasters, J.J., E. Chacon, H. Ohata, L.S. Harper, A.L. Nieminen, S.A. Tesfai, and B. Herman. 1995. Measurement of electrical potential, pH, and free calcium ion concentration in mitochondria of living cells by laser scanning confocal microscopy. Methods Enzymol. 260:428–444. http://dx.doi.org/10.1016/0076-6879(95)60156-2

Llopis, J., J.M. McCaffery, A. Miyawaki, M.G. Farquhar, and R.Y. Tsien. 1998. Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. Proc. Natl. Acad. Sci. USA. 95:6803–6808. http://dx.doi.org/10.1073/pnas.95.12.6803

Ma, Q., H. Fang, W. Shang, L. Liu, Z. Xu, T. Ye, X. Wang, M. Zheng, Q. Chen, and H. Cheng. 2011. Superoxide flashes: early mitochondrial signals for oxidative stress-induced apoptosis. J. Biol. Chem. 286:27573–27581. http://dx.doi.org/10.1074/jbc.M111.241794

Maloff, B.L., S.P. Scordilis, and H. Tedeschi. 1977. Membrane potential of mitochondrial measured with microelectrodes. Science. 195:898–900. http://dx.doi.org/10.1126/science.841317

Martin, J., K. Mahlke, and N. Pfanner. 1991. Role of an energized inner membrane in mitochondrial protein import. Delta psi drives the movement of presequences. J. Biol. Chem. 266:18051–18057.

Matsuyama, S., J. Llopis, Q.L. Deveraux, R.Y. Tsien, and J.C. Reed. 2000. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. Nat. Cell Biol. 2:318–325. http://dx.doi.org/10.1038/35014006

Miesenböck, G., D.A. De Angelis, and J.E. Rothman. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Science. 286:1978–1980. http://dx.doi.org/10.1126/science.5301.15975-28210

Mitchell, P. 1969. Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. Proc. Natl. Acad. Sci. USA. 58:2340–2349.

Mitchell, P. 1975. Proton translocation mechanisms and energy transduction by adenine triphosphates: an answer to criticisms. FEBS Lett. 50:95–97. http://dx.doi.org/10.1016/0014-5793(75)80465-0

Mitchell, P., and J. Moyle. 1969. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature. 213:1175–1188.
