Chemiosmotic coupling in Oxidative Phosphorylation: the history of a hard experimental effort hampered by the Heisenberg indeterminacy principle.

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ABSTRACT

Understanding how biological systems convert and store energy is a primary goal of biological research. However, despite the formulation of Mitchell's chemiosmotic theory, which allowed taking fundamental steps forward, we are still far from the complete decryption of basic processes as oxidative phosphorylation (OXPHOS) and photosynthesis. After more than half a century, the chemiosmotic theory appears to need updating, as some of its assumptions have proven incorrect in the light of the latest structural data on respiratory chain complexes, bacteriorhodopsin and proton pumps. Moreover, the existence of an OXPHOS on the plasma membrane of cells casts doubt on the possibility to build up a transversal proton gradient across it, while paving the way for important applications in the field of neurochemistry and oncology. Up-to date biotechnologies, such as fluorescence indicators can follow proton displacement and sinks, and a number of reports have elegantly demonstrated that proton translocation is lateral rather than transversal with respect to the coupling membrane. Furthermore, the definition of the physical species involved in the transfer (proton, hydroxonium ion or proton currents) is still unresolved even though the latest acquisitions support the idea that protonic currents, difficult to measure, are involved. It seems that the concept of diffusion of the proton expressed more than two centuries ago by Theodor von Grotthuss, is decisive for overcoming these issues. All these uncertainties remember us that also in biology it is necessary to take into account the Heisenberg indeterminacy principle, that sets limits to analytical questions.

INTRODUCTION

The "chemiosmotic theory" formulated by Peter Mitchell (Mitchell, 1961), a Researcher with an Anglo-Saxon training in chemistry, dates back to more than 50 years. The theory has universally been accepted since, although it immediately raised several controversies, which they lasted until today. An upgrading of the chemiosmotic theory appears necessary, having the enormous progress

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of bio analytic techniques defined the fine structure of the macromolecular complexes involved in oxidative phosphorylation (OXPHOS). This allows getting further insight into the real proton pathway, a key issue of the theory. In all evidence, it appears that a free proton osmosis would be impossible, as the proton has a huge destructive force and therefore would destroy any biological membrane it passes through. Moreover, in the last years, studies carried out by several laboratories allowed to overcome a basic postulate of the chemiosmotic theory according to which the aerobic synthesis of ATP is characterized by the need of closed compartments. In fact, it was demonstrated that many biological membranes, devoid of closed compartments and of mitochondria, conduct OXPHOS with high efficiency (Calzia et al., 2010)(Roehlecke et al., 2013)(Mangiullo et al., 2008)(Panfoli et al., 2012)(Ravera et al., 2011b)(Ravera et al., 2013)(Ravera et al., 2018) , challenging the idea that it is exclusive of mitochondria, thylakoid and bacteria. Of particular interest are the reports of the ability of plasma membranes to synthesize ATP outside the cell, as they harbour the five complexes of respiration (Mangiullo et al., 2008)(Arakaki et al., 2003)(Arakaki et al., 2007)(Kita & Arakaki, 2015) (Adriano et al., 2011)(Chang et al., 2012)(Lee et al., 2016).

The existence of an extra-mitochondrial aerobic ATP synthesis –driven by a machinery very similar to that expressed in mitochondria– challenges the concept of a transversal proton gradient built up across the cell wall with protons gathered on the outer side. New paradigms are needed to explain the basic mechanisms of aerobic metabolism. Here we debate the possibility to update the chemiosmotic theory, understanding the ultimate proton role, which could help in developing new strategies for innovative research centered on cellular bioenergetics.

The Chemiosmotic Theory and F_1F_o-ATPsynthase

The 1961 basic formulation of Mitchell's theory is schematically depicted in Figure 1, where ATP synthase is also indicated, differently from the original formulation where it, necessarily, was not depicted (Kagawa & Racker, 1966). In the original formulation, ATP synthesis was attributed to the membrane as a whole, as a generic subtraction of H^+ and OH^- to ADP and orthophosphate to form ATP.

The Theory is based on three basic postulates:

1) An electron transport chain that transfers H^+ from one side to other side of the membrane;
2) ATP synthase synthesizes ATP by translocation H^+ and vice versa for ATP hydrolysis
3) impermeability of the inner mitochondrial membrane to ionic species thereby including protons.

The experimental data in support of the theory came successively and are reported in literature as a huge amount of contributions. Reviews have been published and we refer to them for a complete documentation (Ernster & Schatz, 1981)(Hatefi, 1985), only the most significant issues being mentioned here. Particularly influential were the data produced in 1966 by Jagendorf & Uribe in the famous "acid bath experiment" (Jagendorf & Uribe, 1966). They obtained an ATP synthesis inducing a transmembrane leap of pH in chloroplasts *in vitro*. In the same year the Racker et al. (Kagawa & Racker, 1966) ascertained that the synthesis of ATP occurred on the so-called "spheres" referred to as F_1 subunits of the ATP-synthase. Since then the basic contribution of F_1F_o-ATPsynthase to the OXPHOS became clear. Developments in the molecular knowledge regarding F_1F_o-ATP synthase have been comprehensively addressed in many reviews (Walker et al., 1990)(Stock et al., 2000)(Junge & Nelson, 2015).

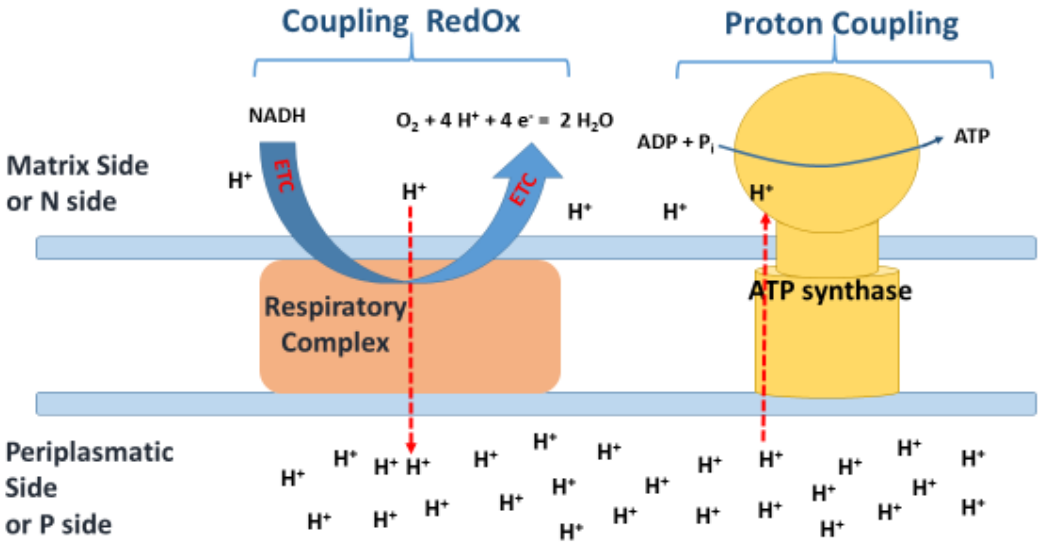

Figure 1: Schematic representation of the 1961 Mitchell chemiosmotic theory.
A delocalized coupling is depicted among proton extruded by the electron transport chain (ETC) and ATP synthesis. The overall process is arbitrarily divided in the two phases: the "Coupling RedOx", in which the proton movement is operated by the ETC, and the "Proton Coupling", in which proton movement is coupled with ATP synthesis, by F_1F_o-ATPsynthase.

The basic requirement for the OXPHOS is a coupling between redox processes, proton translocation and ATP synthesis. The global coupling can arbitrarily be divided in two distinct phases: a coupling between the oxidation-reductive process and the protonic translocation, referred as "RedOx

Coupling" (see left side of Figure 1) and the coupling between protons accumulated on the p-side of the membrane moving to the n-side through the ATP synthase, which determines the synthesis of ATP, here referred as "Proton Coupling" (see right side of Figure 1). About the first coupling, the recent review "*The mechanism of coupling between oxido-reduction and proton translocation in respiratory chain enzymes* " (Papa, Capitanio & Papa, 2018) evaluated the main characteristics, the recent experimentation and even the controversies around that. Considerable attention was devoted in the 80s and 90s of last century to clarifying the structural-functional details of the respiratory complexes (I, II, III and IV) and F_1F_o-ATPsynthase (Complex V). Important was the study of respiratory complexes organized in supercomplexes (Schägger & Pfeiffer, 2001)(Wittig et al., 2006)(Bianchi et al., 2004) with the demonstration that the loss of their aggregation leads to an increase in the production of reactive oxygen species (Bianchi et al., 2004)(Lenaz et al., 2016). The possible participation of Complex V has never been demonstrated (Wittig et al., 2006) . The study of supercomplexes has also benefited from the extraordinary surveys carried out on X-rays (Letts, Fiedorczuk & Sazanov, 2016).

By contrast, the "Proton Coupling" (see Figure 1-right) appears to be the most critical passage of the whole OXPHOS process. Literature reports a number of experiments performed with reconstructed systems (Yoshida et al., 1975)(Sone et al., 1977),i.e. F_1F_o-ATPsynthase incorporated into phospholipid vesicles, carried out about fifteen years after Mitchell's hypothesis. Vesicles obtained from the membranes from the purple *Halobacterium salinarium*, synthesized ATP as a result of illumination and it was thus demonstrated that illumination moved protons through the membrane supporting the synthesis of ATP. Decisive in 1977 (Sone et al., 1977) was the reconstitution experiment in artificial membranes of ATP synthase purified by *Thermophilic bacterium* that synthesized ATP thanks to a transient shift in membrane potential ($\Delta\Psi$) induced by valinomycin, allowing rapid passage of K^+ ions across a membrane on the sides of which different salt concentrations were set. These experiments demonstrated that proton translocation is the crucial step for the "Proton Coupling" between protonic movement and ATP synthesis. On this general topic, pivotal is the minireview of Wolfang Junge[15], which on one hand enhances the versatility of the F_1F_o-ATPsynthase nano-machine " *unique in converting electrochemical, mechanical and chemical forms of energy*" and on the other hand points out that there is still much to be understood about the chemical-physical basis of such process.

Controversies about the Chemiosmotic Theory

A long struggle was necessary for the chemiosmotic theory formulated by Peter Mitchell (Mitchell, 1961) to be widely accepted. The controversy, central to the history of bioenergetics for more than half a century, appears tackled by more than 200 articles (Weifu Lee, 2012) and to have lasted until the most recent years. Giovanni F. Azzone, many years ago (1972) published the manuscript "*Oxidative phosphorylation, a history of unsuccessful attempts: is it only an experimental problem*?"(Azzone, 1972) that already highlighted what did not convince in the theory and that wished for answers from the fine analysis of the macromolecular structures involved in chemiosmosis.

The harshest criticisms came from John Prebble (Prebble, 2001), which emphasized the lack of experimental data in support of the theory. Wolfang Junge effectively described in 2013 (Junge, 2013) the chronicle of the dispute, which even took harsh tones. His review "*Half a century of molecular bioenergetics*" examines many issues of bioenergetics, including chemiosmotic theory. Brown & Simcook in their article (Brown, Brown & Simcock, 2011) considered the motivations that have convinced the scientific community to accept the chemiosmotic theory, regardless of great skepticism welcoming it at the beginning. Authors note that: "*science shows tremendous resistance to change and it takes extraordinary perseverance to persuade the community*".

A controversial issue was the correlation between $\Delta\Psi$ and the proton motive force, often considered equivalent entities (Wiedenmann, Dimroth & von Ballmoos, 2008). It was postulated that a $\Delta\Psi$ with positive charge on the external p-side of the internal mitochondrial membrane and negative on the n-side in contact with mitochondrial matrix would let protons enter through the rotor F_o that synthesizes ATP in the matrix, thanks to its mechanical connection with the F_1 moiety. Protons would gather across the coupling membrane like chemical ions, creating a driving force for F_1F_o-ATP synthase to synthesize ATP, realizing the "Proton Coupling". However, the yield in ATP poorly correlates with bulk-to-bulk membrane potential (Krulwich et al., 1996)(Mulkidjanian, Heberle & Cherepanov, 2006) so that the basic chemiosmotic theory appears inadequate (Wolf, Grubmüller & Groenhof, 2014).

The awarding of the 1978 Nobel Prize for chemistry to Peter Mitchell cooled the dispute, but not definitively. In fact, in 1979, there was a heated confrontation published by TRENDS (Tedeschi & Rottenberg, 1979) between Henry Tedeschi, who disproved the idea that the metabolic activity of mitochondria could contribute to membrane potential and Hagai Rottenberg which instead defended Mitchell's theory. The original theory provides a protonated "delocalized coupling" (as depicted in Figure 1) while a "localized coupling" has seen Robert Williams as a great supporter (Williams,

1975). In the paper "*Proton-Electrostatics Hypothesis for Localized Proton Coupling*" J. W. Lee (Weifu Lee, 2012) reports a rigorous chemical/physical experiment in favour of localized coupling, demonstrating furthermore that the thylakoid membrane can be a "proton capacitor". The putative existence of a proton capacitor is a matter of great importance and later, H. A. Saeed and J. W. Lee (A. Saeed & W. Lee, 2015) showed that protons can actually accumulate on the membrane surface even though they never reside in the aqueous phase. Moreover, concerning the experimental verification of the "proton coupling", a recent elegant investigation in HeLa cells, bioengineered with green fluorescent protein as pH indicator inserted in respiratory complex III and in F_o moiety of ATP synthase, points to a localized coupling (Rieger, Junge & Busch, 2014).

A report entitled "*Proton migration along the membrane surface and retarded surface to bulk transfer*" by Heberle et al. (Heberle et al., 1994) interestingly reconciles the two visions, providing proof that proton transfer from a proton generator (bacteriorhodopsin) to an acceptor, (water-soluble pH indicators) is faster if occurring on the membrane rather than when protons are released in the aqueous bulk. Ferguson (Ferguson, 1995) emphasized Heberle et al.'s experiments, concluding that the delocalized coupling and lateral proton transfer (localized coupling), between the proton generator and user, occurs very rapidly on the membrane, as compared to the slower and transversal passage through the aqueous bulk. In this context, the recent paper form Von Ballmoos group observed that $\Delta\Psi$ and ΔpH are equivalent for the coupling with ATP-synthase (Wiedenmann, Dimroth & von Ballmoos, 2008). A primary role for membrane buffering on proton mobility in general can be hypothesised (Junge & McLaughlin, 1987). The experimental data (Capaldi et al., 1994)(Turina, Samoray & Graber, 2003) showing a close thermodynamic correlation between valinomycin-induced $\Delta\Psi$ and ATP synthesis in reconstituted systems are very important, but it seems plausible that they induce a transmembrane protonic flow that probably differs from the path in a native environment. Moreover, the eminent English chemist Robert Williams clearly rejected the hypothesis of the accumulation of protons from p-side: "*the p-phase corresponds to the infinitely extended external space. If protons are extruded into this "Pacific Ocean", they would be diluted and the entropic component of the pmf would be lost* " (Williams, 1978). Williams observed that "*I made it clear that protons in the membrane rather than an osmotic trans-membrane gradient of protons were required to drive ATP formation*" based on a series of considerations that excluded the presence of free protons from p-side(Williams, 1975). An elegant demonstration of Williams's localized coupling hypothesis came in 1976 (Yaguzhinsky et al., 1976) by an experiment in which purified ATP synthase was added to the octano-water interface. It was observed that protons accumulate in octane, a Brønsted acid, leading to ATP synthesis by ATP synthase. These data have

also been recently confirmed (Eroshenko et al., 2012). Eighteen years later, it was reported that :"*our results suggest that protons can efficiently diffuse along the membrane surface between a source and a sink (for example H+-ATP synthase) without dissipation losses into the acqueous bulk*" (Heberle et al., 1994). From all the cited data it can be concluded that protons (or protonic currents) are confined into the membrane, while proton exit from the membrane is to be considered only as a fallback way of escape, mostly *in vitro* reconstituted conditions.

Membrane Potential

A direct measurement of membrane potential of the mitochondrial inner membrane with microelectrodes was only be accomplished by Tedeschi, who showed the existence of a positive inside and negative outside mitochondrial membrane potential ($\Delta\Psi$) (Tupper & Tedeschi, 1969a)(Tupper & Tedeschi, 1969b). Such potential (contrary to the canonical) interestingly coincides with that calculated on the basis of the ionic species present on the membrane sides (Harris & Pressman, 1969). Clearly, knowledge of the entity and especially the sign of this potential is fundamental for understanding the basic functioning of chemiosmosis, as emerges from the already mentioned historical dispute between Henry Tedeschi and Hagai Rottenberg (Tedeschi & Rottenberg, 1979) .

To measure $\Delta\Psi$, laboratory tests currently utilize lipophilic fluorescent compounds whose response is considered to be related to $\Delta\Psi$. However, tests conducted with rhodamine have cast doubts on such correlation (Scaduto Jr. & Grotyohann, 1999) since these indicators inhibited the mitochondrial respiration, so they disturb the system. As such compounds dissolve into the membranes, they may reflect the membrane behaviour, in fact they inhibit a membrane intrinsic process, i.e. the OXPHOS, but do not interfere with $\Delta\Psi$. Surely, in vitro, proton passage across membranes can be forced with rapid movements of potassium ions by addition of valinomycin (Sone et al., 1977)(Schmidt & Gräber, 1987). A laboratory procedure utilizing valinomycin and also nigericin has been widely used to create a transient $\Delta\Psi$ operating a delocalized coupling linking $\Delta\Psi$ and ATP synthesis, but this does not exclude that in the native membranes a localized coupling would operate, independently of $\Delta\Psi$.

A crucial issue: the Membrane Permeability to Protons

In an old study (1986) Giovanni Felice Azzone and coll. highlighted the uncertainties of the proton cycle (Zoratti et al., 1986). In the same year Grzesiek and Dencher (Grzesiek & Dencher, 1986) showed that the phospholipid membranes are intrinsically permeable to protons. Data show that phospholipid membranes, normally impermeant to ionic solutes (transversal or permeability diffusion coefficients varying between 10^{-12} to 10^{-14} cm/sec), exhibit a significant proton

permeability, varying from 10^{-3} to 10^{-9} cm/sec. Such variability may be justified by a buffering capacity of the membranes for protons: proton diffusion value could depend on the higher or lower degree of pre-existing protonation. Recently, the proton leak through lipid bilayers was modelled as a concerted mechanism (Grzesiek & Dencher, 1986)(Tepper & Voth, 2006)(Shinoda, 2016). Tepper and Voth (Tepper & Voth, 2006) provided a theoretical interpretation of proton permeability, based on the formation of transient membrane spanning aqueous solvent structure. High proton permeability has also been confirmed in liposomes, independently form their phospholipid composition (Brookes, Hulbert & Brand, 1997). It is clear that a high degree of permeability to protons is *per se* in contrast to the third of the aforementioned basic postulates of the chemiosmotic theory. With regard to the relationship between membrane and aqueous phase, many observations confirm the existence of a layer of water molecules on the two sides of the membrane which to some extent isolate it from the aqueous phases present on its two sides (Mulkidjanian & Cherepanov, 2006)(von Hansen, Gekle & Netz, 2013)(Kundacina, Shi & Pollack, 2016).

Proton solvation.

The actual proton path across the membrane, their putative concentration on both sides of the membrane and the consequent membrane potential have been the object of countless studies. A central issue is the knowledge of the actual chemical species of the proton: free, or in the form of H_3O^+? This depends on the phase in which the proton is located. Protons possess peculiar chemical properties, being essentially an atomic nucleus. Free protons do not exist in the aqueous phase, being solvated to H_3O^+, from which the extraction of a proton would be virtually impossible. In fact, in the transition from H_3O^+ to free proton a strong energy barrier higher than 500 meV, must be overcome as specified in the paper: "*Proton in the well and through the desolvation barrier*" (Mulkidjanian, 2006), which corresponds to the enormous amount of 262.400 Cal/mol (Zhan & Dixon, 2001). An immense literature exists on the subject (Mulkidjanian, 2006)(Weifu Lee, 2012)(Cherepanov, Junge & Mulkidjanian, 2004)(Mulkidjanian et al., 2005). An interesting report (Bal, Kurowska & Maret, 2012), not sufficiently taken into account, calculated the number of free protons (actually in the form of H_3O^+) in the volume of a mitochondrion, which is of the femtoLiter order of magnitude. This study demonstrated, starting from basic physical chemical data (Avogadro number, ionic water product, mathematical pH expression and mitochondrial volume), that free protons in a mitochondrial periplasmic space are too few (less than ten) to support any process dependent on proton translocation in the aqueous bulk across the membrane and absolutely inadequate to support the thousands of ATP synthase molecules present in a mitochondrion. Moreover, the pH value inside the mitochondrion resulted to differ by 0.5 units from what

previously believed (Żurawik et al., 2016). Moreover, the huge energy associated with proton solvation would have a negative consequence: a free membrane proton would quickly be "sucked" by the near aqueous phase, releasing the huge energy associated with the solvation process, to the detriment of the membrane.

Grotthuss mechanism and proton translocation through the membranes.

A putative mechanism for proton diffusion was hypothesized more than two centuries ago by Theodor von Grotthuss (Grotthuss, 1805) and is synthetically explained by Kreuer: "*Proton diffusion according to the Grotthuss mechanism occurs much faster than molecular diffusion because it is uncoupled from the self-diffusion of its mass*" (Kreuer, 1996). Protons would not diffuse as a mass, rather as a charge, the latter moving between water molecules or protonable groups of suitable macromolecules of the membranes. The Grotthuss mechanism allows better understanding of the possible ways in which protons or species derived from them move in biological systems. DeCoursey published a review entitled "*Voltage-gated proton channels and other proton transfer pathways*", which exhaustively analyzes proton movement in water and biological membranes (Kreuer, 1996), including HV1 channels which specifically transfer protons in aqueous phase, therefore actual acidity from one side of the membrane to the other. The mechanism of the voltage gated proton channel H_V1 is not as yet resolved, as emblematically stated by DeCoursey in the paper entitled "*The voltage-gated proton channel: a riddle, wrapped in a mystery, inside an enigma*" (DeCoursey, 2015). Two mechanisms have been proposed for H_V1 as schematically depicted in Figure 2, where on the left side is represented the so-called "frozen water" mechanism, in which the channel traps one or more molecules of water which allows protons to pass through with Grotthuss-style proton hopping, as in the typical case of Gramicidin (Pomès & Roux, 2002). On the right side of Figure 2 is depicted a passage of proton with protonation/deprotonation of ammino acid side-chain, that would realize the so-called "proton wire", already proposed in the 1978 paper "*Molecular mechanisms for proton transport in membranes*" by Nagle & Morowitz (Nagle & Morowitz, 1978). The topic was consolidated successively by Nagle & Nagle (Nagle & Tristram-Nagle, 1983). DeCoursey in a debate recently published on the Journal of Physiology supports the mechanism shown in the right part of Figure 2 that provides the action of proton wires through the membrane (DeCoursey, 2017), while Bennettt & Ramsey (Bennett & Ramsey, 2017a)(Bennett & Ramsey, 2017b) support a mechanism of passage through molecule of water as schematized on the left of Figure 2. Interestingly, both mechanisms are based on the Grotthuss proton movement between i) water molecules in the case of

the water channel (on the left) and ii) between side chains of amino acids in the case of protons wires (on the right).

The proton movement in membranes (both biological and artificial) has been analyzed by many rigorous chemical/physical studies. The article -"*Proton Holes" in Long-Range Proton Transfer Reactions in Solution and Enzymes: A Theoretical Analysis*- shows that other compounds in addition to water are involved in the "proton hopping" (Riccardi et al., 2006a), and is interesting that a quantum-mechanical approach is applied (Riccardi et al., 2006b) . The article "*Grotthuss mechanisms: from proton transport in proton wires to bioprotonic devices*" presents devices such as proton diodes, transistors, memories and transducers, semiconductor electronic devices that use the Grotthus mechanism (Miyake & Rolandi, 2016).

Notably, H_V1 only allows the passage of protons balancing their concentration between two aqueous compartments separated by a membrane. This property does depend on the membrane potential $\Delta\Psi$, similarly to other ion membrane devices abundant in biological membranes (for example the Na^+ and K^+ voltage gated channels), acting exclusively on the conformation of the H_V1 protein. It appears therefore that to carry protons through the membrane there are ad hoc structures that deeply differ from the respiratory complexes, both for the function finality, and for

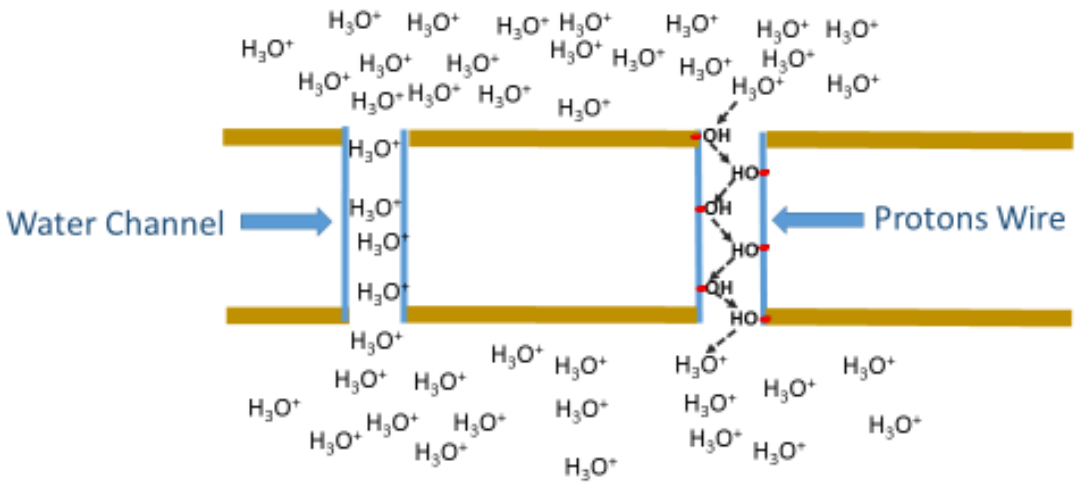

Figure 2: Mechanism for H^+ transfer through the membrane by H_V1.
Left, water channel model: the water molecules allow protons to pass through with Grotthuss-style H^+ hopping. Right, proton wire model: a charge migration occurs (through with Grotthuss-style H^+ hopping) on polar groups of side chains of amino acids of H_V1.

the molecular mechanism. Moreover, the process would be quantitatively linked to an unwanted acidification of a closed compartment. From these and other evidences it can be concluded that the native proton movements in the respiring membranes, when there is no need for acidification of the milieu on one side of a membrane, must take place entirely inside the membrane (Morelli et al., 2013). As far as the respiratory complexes are concerned, on the other hand, it is theorized that the proton and membrane potential movements are mutually dependent, in that the pumping of protons would *generate* the membrane potential, which is impossible for thermodynamic considerations that we will elaborate later on.

Moreover this comparison between proton movement in support of the OXPHOS and the actual protonic movement in nature sheds light on the fact that when protons are really transferred through a membrane i) they are never in the form of free protons and ii) are subject to the Grotthuss-style proton hopping. Hence the need for chemiosmotic theory to be revised in the light of all this emerges.

Plausible proton pathways inside the respiring membranes.

Having established the clear divergence between the pathways of the solvated proton and of the proton alone, in light of the detailed molecular structural data now available we can seek for the proton plausible pathways inside the respiring membrane, even if it is a just a plasma-membrane. The respiratory complexes able of handling protons are Complex I, III and IV, whose structural studies have benefited of the progress of X-ray analysis and of cryo-microscopy. Excellent investigations are available on the subject, here for simplicity we mention the studies on Complex I (NADH: ubiquinone oxidoreductase) from Leonid Sazanov and collaborators (Baradaran et al., 2013)(Sazanov, 2015) and Complex IV (Cytochrome c Oxidase).

Numerous structural X-ray studies were conducted on Complex I from *Escherichia coli* (Efremov & Sazanov, 2011), *Thermus thermophiles* (Baradaran et al., 2013), and from mammalian ovine (*Ovis aries*) mitochondria (Fiedorczuk et al., 2016) and with cryo-microscopy on Complex I from *Bos taurus* (Vinothkumar, Zhu & Hirst, 2014)(Zhu, Vinothkumar & Hirst, 2016). The complexity of the macromolecular aggregation of Complex I is impressive: in the mammalians it is formed by as many as 45 polypeptides and its assembly needs an unknown number of chaperons, so indispensable that the impairment of just one of them (B17.2L) causes of a progressive encephalopathy (Ogilvie, Kennaway & Shoubridge, 2005). Authors state that: "*results demonstrate that B17.2L is a bona fide molecular chaperone that is essential for the assembly of complex I and for the normal function of the nervous system.*"

As only 5 alpha-helices have been found in the Complex I structure, in order to allow for proton translocation, it was necessary to postulate the existence of two hemi channels: one from the matrix (n) side to the centre of the respiratory complex, referred here to as the "proton entrance hemi channel " and the other from the centre to the periplasmatic (p) side here indicated as "proton exit hemi channel". However, only the latter was well identifiable in Complex I (Efremov & Sazanov, 2011). Instead, the entry pathway has not been identified with certainty so much so that we only can talk about putative pathways labelled with "? "(Efremov & Sazanov, 2011). Furthermore, it clearly emerges from the X-ray studies that there is an obvious proton tunnelling at the centre of the Complex I. Comprehensive studies on the protonic movement inside Complex I have been carried out by the Helsinki Bioenergetic Group of Martin Wikström, which highlighted uncertainty margins on the stoichiometry of protonic extrusion that appears closer to 3 $H^+/2e^-$ (Wikström & Hummer, 2012) instead to the classic 4 $H^+/2e^-$. Also, in the review di Verkhovskaya & Bloch (Verkhovskaya & Bloch, 2013) (of Helsinki Bioenergetic Group) four mechanisms for proton translocation are proposed and the "proton entrance half channel" is not identified with certainty, while the "proton exit half channel" is clearly identifiable. Emblematically on the website of the Helsinki Bioenergetic Group it is written "*...the mechanism of proton transfer in Complex I remains completely enigmatic*".

As far as Complex IV (Cytochrome c-oxidase) is concerned, the proton translocation of has been studied in depth (Brzezinski, 2004). In their recent review, Martin Wikström & Vivek Sharma talk of an "anniversary": "*Proton pumping by cytochrome c oxidase - A 40 year anniversary*" (Wikström & Sharma, 2018). Among the many quotations in this review the Chemical Review of the Wikström group stands out (Wikström, Krab & Sharma, 2018). It goes into the details of the possible molecular processes carried out by Complex IV (Wikström, Krab & Sharma, 2018) , thereby including proton translocation. The topic is complex and more putative pathway of protons are well developed in the review , that we can not here detail here (Wikström, Krab & Sharma, 2018). For the "proton entrance half channel" for each molecule of oxygen reduced to water they need 4 H^+ and it is hypothesized that another 4 H^+ for a total of 8 enter through this channel. It seems unlikely that there exists equivalence between protons that exist as mass and link to water molecules, and protons that should move as a charge, according to the Grotthuss mechanism.

Proposal for a localized Complex I-ATP Synthase coupling

Taking into account what reported above, it is possible to trace a plausible proton pathway within the respiring membrane. Just in 2006 a direct proton transfer was proposed to couple the Complexes I, III, IV respiratory pathway with FoF1-ATP Synthase (Papa, Lorusso & Di Paola, 2006).

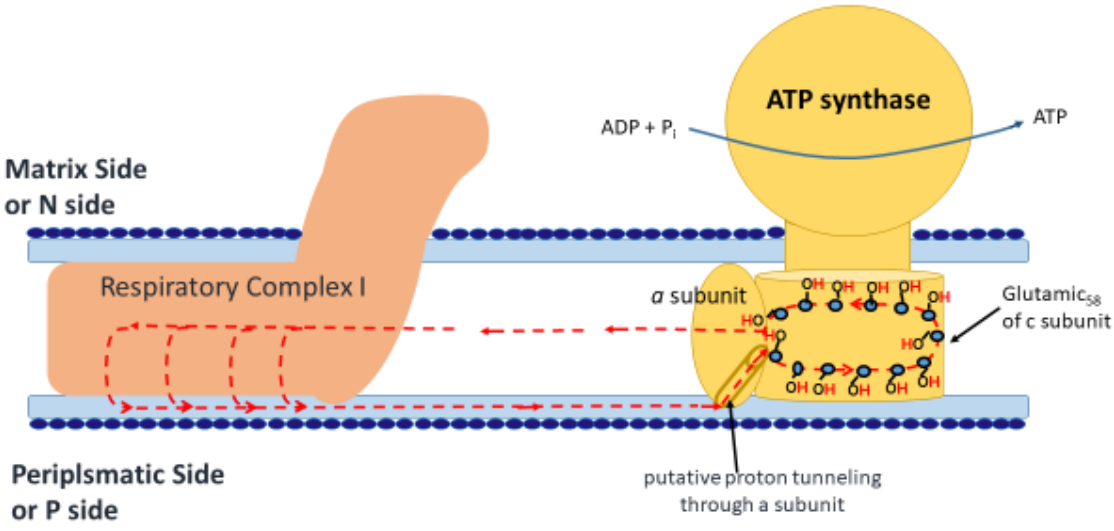

Figure 3: A possible H^+ circuit inside respiring membrane.
The water insulating shield on both side of the membrane are shown by blue ellipsoids. The image proposes that the H^+ (red dotted line) are transferred to the Glu 58 (E58) at the centre of subunit c through subunit a of ATP synthase by proton tunnelling. H^+ would flow from the periplasmic side, always bound to phospholipid heads. This can be arranged in each layer of the membrane.

However, in a recent review (Papa, Capitanio & Papa, 2018) the concept of transmembrane proton motor force to move the ATP Synthase is reinforced, although it is noted that many aspects of coupling are not yet clarified. Clear cut consideration was proposed some years ago (1991) by Akeson & Deamer(Akeson & Deamer, 1991) about speed of proton translocation through putative proton channel as limiting step for ATP synthesis by FoF1 ATP-synthase. For the sake of simplicity, we only examine the coupling between the Respiratory Complex I and the F1Fo-ATP synthase. The existence of a proton pathway at the centre of Complex I is quite clear, and we can hypothesize that the protons are sent to the well-identified four "exit half channel", as shown in Figure 3. The proton donor at the centre of Complex I has already been tentatively identified in the phospholipid cardiolipin (CL) (Morelli et al., 2013), which appears essential for the OXPHOS. However, for example, phosphatidylethanolamine (PE) is a valid phospholipid for the functioning of the OXPHOS (Tasseva et al., 2013). The experimentation recently carried out by Von Ballmoos group (von Ballmoos et al., 2016)(Nilsson et al., 2016) is clear, and also unconventional, on the role played by phospholipids. In fact, pure phosphatidylcholine (PC) is excellent for the coupling, while it increases when the membrane is formed by PC + PE. By contrast, it is dramatically inhibited if

the membrane is formed by PC + CL, incredibly diverging from the traditional role assigned to CL. This research is also important because experiments are performed with a reconstructed system, more adherent to the " H^+/ATP coupling ". Here, the driving force that feeds ATP synthase was not the rapid transfer of K^+ generated by valinomycin, a widely used method, but the *bo_3 oxidase* of Escherichia coli, analogue to the respiratory Complex I of vertebrates.

It emerges that all the membrane phospholipids can act as mobile proton transporters, hindering their relocation in the near aqueous medium, so that protons are never free. The exergonic process of proton solvation would release an impressive amount of energy (262.400 Cal/mol) (Zhan & Dixon, 2001) which, if not transferred on a generic acceptor, would generate heat, devastating not only for the membrane but also for the whole cell integrity. Complex I would transfer protons to the p side of the membrane, but these would not be dispersed in the aqueous bulk. In fact it is well documented the existence of a barrier of water molecules attached to the membrane, determining the lateral displacement of protons on the phosphate heads of phospholipids (Prats, Tocanne & Teissié, 1985)(Teissié et al., 1985)(Serowy et al., 2003) to meet a sink that is the ATP synthase subunit that through a proton wire (Panfoli et al., 2017)(Brändén et al., 2006) would leads the proton to the rotor (see Figure 3), accommodating on the central glutamic or aspartic residue (depending on the species) of the c subunit which in variable numbers from 8 to 15 make up the rotor Fo (Watt et al., 2010). As Fo rotates it is conceivable that the proton returns to the centre of Complex I, an highly hydrophobic environment, thus closing the protonic circuit. This last passage, from the rotor exit to the centre of the respiratory complex, appears plausible, for the operativity of the Brownian motion (diffusion) of particles in a highly anisotropic environment that can occurs efficiently (Yaguzhinsky et al., 1976)(Saffman & Delbrück, 1975) and a direct passage of the proton exiting the subunit at the entrance to the Complex is theoretically possible. Moreover the investigations of the Von Ballmoos group have produced exhaustive studies with reconstructed systems (von Ballmoos et al., 2016)(Nilsson et al., 2016)(von Ballmoos, Wiedenmann & Dimroth, 2009), in which a direct transfer of proton on the p-side of the membrane from Complex I to ATP-synthase is evident. Their recent paper also demonstrates that proximity to the membrane between the respiratory complex and ATP synthase is required for the "proton coupling" (Nilsson et al., 2016) . However, the fact remains that the last part of the protonic circuitry (i.e. from Fo moiety of ATP-synthase to respiratory complexes) is only hypothetical.

Extramitochondrial oxidative phosphorylation

The Chemiosmotic Theory(Mitchell, 1961) as it was formulated is a process that can only take place in organelle possessing double membrane systems forming closed compartments to entrap

protons, such as mitochondrial cristae, bacteria and thylakoids. Here, for the sake of simplicity, we have considered the mitochondrial inner membrane, even though in recent years, it has been shown that the oxidative phosphorylation (OXPHOS), coupled to aerobic ATP synthesis, also occurs in extra-mitochondrial districts, as rod outer segment (OS) disks (Calzia et al., 2014)(Calzia et al., 2013)(Bianchini et al., 2008)(Panfoli et al., 2009), myelin sheath (Ravera et al., 2009) (Ravera et al., 2011b)(Morelli et al., 2011)(Ravera et al., 2015), plasma membrane (Mangiullo et al., 2008) (Arakaki et al., 2003)(Arakaki et al., 2007)(KITA & ARAKAKI, 2015)(Adriano et al., 2011)(Chang et al., 2012)(Lee et al., 2016)(Taurino & Gnoni, 2018)(Taurino et al., 2016)(Gao et al., 2017) extracellular vesicles shedding form cells, such as exosomes and microvesicles, which seem to carry an unsuspected metabolic signature (Panfoli et al., 2016). In particular, recent data indicate an active extramitochondrial OXPHOS in the endoplasmic reticulum of platelets which both have elevated ATP need but possess very little mitochondria (Ravera et al., 2018).

It is worth noting that photoreceptor OS, specialized subcellular compartment that contains lesser molecular system as compared to bulk cytosol, have allowed important discoveries. In fact, Transducin, discovered in the rod OS in 1981, is considered the G protein prototype (Fung, Hurley & Stryer, 1981); in 1986 the OS were proven to contain a considerable amount of the second messenger cyclic GMP (Fesenko, Kolesnikov & Lyubarsky) and finally in 2009 data emerged accomplishing the discovery of an extramitochondrial OXPHOS (Panfoli et al., 2009), coupled to aerobic ATP synthesis in the OS(Panfoli et al., 2012) (Bruschi et al., 2018).

Interestingly, the synthesis of extracellular ATP by F_1F_o-ATPsynthase on plasma membrane has been recently demonstrated in human neutrophils (Gao et al., 2017) confirming data previously obtained (Mangiullo et al., 2008) (Taurino & Gnoni, 2018)(Taurino et al., 2016)(Karelin, Demidova & Globa, 1992)(Quillen et al., 2006)(Ravera et al., 2011a)(Kim et al., 2004)(Ma et al., 2010). Since the plasma membrane potential is positive on the outside and negative on the inside, it would favor the ATP hydrolysis rather than the synthesis. This datum is in line with the postulated independence of F_1F_o-ATPsynthase activity from the membrane potential and therefore it is plausible that this synthesis of ATP depends on the proton intramembranous coupling.

Conclusion

The impressive amount of experimental data cited appears globally in contrast with the above cited three assumptions underlying the chemiosmotic theory. First of all, it excludes that the protons can accumulate on the coupling membrane surface, whose high permeability would dissipate, and essentially since it would correspond to an extreme acidity incompatible with any vital process. Secondly, since diffusion does occur, it is clear that the membrane potential is irrelevant to

translocation. Thirdly, it can be excluded that the respiratory complexes operate a transmembrane proton transfer from the aqueous bulk, where the proton would exist as hydroxonium ion. This appears to rule out the actual possibility that ATP synthase can overcome the energy barrier, higher than 500 meV (Mulkidjanian, 2006) required to extract the proton from water, leaving space for the localized coupling hypothesis (Cherepanov, Junge & Mulkidjanian, 2004)(Mulkidjanian et al., 2005) as dehydration/hydration reaction from hydroxonium ion to free protons would require 262.400 Cal/mol (Zhan & Dixon, 2001). Which are the actual processes acting on the FoF1-ATP synthase? There is no certainty, as highlighted by the emblematic title of an article by John Walker "*The ATP synthase: the understood, the uncertain and the unknown*" (Walker, 2013). In this controversial scenario a decisive contribution is undoubtedly the sophisticated bioengineering experiment that labelled the IV respiratory complex and ATP-Synthase with proteins of the GFP family, to experimentally observe a local ΔpH triggered by the respiratory substrate galactose (Rieger, Junge & Busch, 2014). Observations were conducted in cultured HeLa cells. The authors conclude: "*the observed lateral variation in the proton-motive force necessitates a modification to Peter Mitchell's chemiosmotic proposal*". The experimentally proven lateral proton motive force is in line with the hypothesis of localized coupling. Indeed, today a constellation of clues leads us to hypothesize the existence of protonic currents internal to the membrane, with the formation of possible circuits travelled by the positive elementary charge, thus realizing a localized coupling that excludes an osmotic nature of the process. To trace this circuit, at least for the possible coupling between respiratory Complex I and ATP-synthase it appears realistic that Complex I may transfer protons from its central part to the periplasmatic side, allowing them to travel on the membrane surface thanks to the heads of phospholipids (Prats, Tocanne & Teissié, 1985)(Teissié et al., 1985) finally entering by tunnelling in the subunit a of the ATP-synthase (Panfoli et al., 2017)(Ivontsin, Mashkovtseva & Nartsissov, 2017) (see Figure 3). Moreover several studies show that the membrane is isolated from the aqueous bulk thanks to a layer of water molecules on both sides of the membrane, which consolidates the idea that the membrane is radically distinct and isolated from the liquid phase (Kundacina, Shi & Pollack, 2016). Considering the isolated two phases, the only way evolution could pursue to link proton movement to ATP synthesis was a nanomachine connecting the proton movement inside the membrane to the deformation mechanics of the F1 sphere immersed in the aqueous phase.

We can reasonably assume that the proton movement inside the membranes occurs as a charge, according to the proton hopping Grotthuss mechanism with the establishment of "protonic currents" inside the membrane (Tepper & Voth, 2005). In the non-biological field we find a remarkable

adherence to this theory for the development of protonic devices (as proton diodes, transistors, memories and transducers, semiconductor electronic) (Miyake & Rolandi, 2016) that could replace devices widely used in electronics. It is surprising that the two areas, the biological and the physical-chemical one, have ignored each other. Since in the history of biology the application of physic-chemical methodologies has led to dramatic advances in biology (we may remind the reader that the resolution of the DNA structure (WATSON & CRICK, 1953) was obtained with the fundamental application of X-ray crystallography, developed in 1913 by Williams Henry Bragg and his son Lawrence for the study of inorganic crystals) it is desirable that this fusion of knowledge can be realized in the years to come. Furthermore, the classical mechanical approach cannot be used to approach these currents. In fact, any time there is a movement of charge bound to a mass, the dualism that cannot be assessed by classical mechanics, instead quantum mechanics must be applied, and in this perspective lay the promising recent quantum-mechanical approach by Riccardi et al (Riccardi et al., 2006b) , Ivontsin et al. (Ivontsin, Mashkovtseva & Nartsissov, 2017). There is the need to take into account the Heisenberg indeterminacy principle as Philip Hunter already highlighted: "*A quantum leap in biology. One inscrutable field helps another, as quantum physics unravels consciousness*" (Hunter, 2006).

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501 **Abbreviations used**

502 OXPHOS, Oxidative Phosphorylation; ΔΨ, membrane potential; HV1, potential-dependent proton
503 pump; Complex I, NADH: ubiquinone oxidoreductase; ETC: Electron Transport Chain; CL:
504 cardiolipin; PE: phosphatidylethanolamine; BR: bacteriorhodopsin.

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