Phospholipids and glycolipids mediate proton containment and circulation
along the surface of **energy-transducing** membranes

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Abstract

Proton bioenergetics provides the energy for growth and survival of most organisms in the biosphere ranging from unicellular marine phytoplankton to humans. Chloroplasts harvest light and generate a proton electrochemical gradient (proton motive force) that drives the production of ATP needed for carbon dioxide fixation and plant growth. Mitochondria, bacteria and archaea generate proton motive force to energize growth and other physiologies. Energy-transducing membranes are at the heart of proton bioenergetics and are responsible for catalyzing the conversion of energy held in high-energy electrons → electron transport chain → proton motive force → ATP. Whereas the electron transport chain is understood in great detail there are major gaps in understanding mechanisms of proton transfer or circulation during proton bioenergetics. This paper is built on the proposition that phospho- and glyco-glycerolipids form proton transport circuitry at the membrane's surface. By this proposition, an emergent membrane property, termed the hyducton, confines active/unbound protons or hydronium ions to a region of low volume close to the membrane surface. In turn, a von Grotthuß mechanism rapidly moves proton substrate in accordance with nano-electrochemical poles on the membrane surface created by powerful proton pumps such as ATP synthase.

Keywords: glycolipids; phospholipids; glycerolipids; energy-transducing membranes; proton bioenergetics; von Grotthuß mechanism; long distance proton transport.

1. History

The flow of protons through and on cellular membranes is central to bioenergetics, i.e. supply of energy for life. Peter Mitchell, the father of the field of proton bioenergetics, envisioned in 1961 [1] that the thermodynamic potential energy at equilibrium was coupled to a proton gradient across or within energy transducing membranes (i.e. proton motive force, *pmf*). While Mitchell's visionary "chemiosmotic" hypothesis is widely accepted today in its general terms, at that time few structural details of proton pumps or ATP synthase were available; as a consequence, Mitchell's revolutionary new hypothesis was severely debated (reviewed by [2]). Mitchell's concept of delocalized bulk-to-bulk proton gradients was confronted with the idea of a localized, i.e. limited distance of *pmf* coupled to ATP formation, membrane surface-to-surface gradient [3–5]. The latter mechanism was demonstrated in biological systems, where experiments systematically showed that protons do not immediately enter the bulk-water phase, but are in fact retained on membrane surfaces [6,7]. These findings stimulated the first publications suggesting membrane lipids as facilitators of lateral proton transfer [8,9].

Research activity on membrane-water interface proton transport intensified again in the 1990's. Two separate laboratories reported that protons ejected from cells of *Halobacterium salinarum* by archaeal rhodopsin, acting as a proton pump, did not enter immediately the bulk phase or medium [10,11]. Instead, protons effluxed by this light-energized proton pump hugged the membrane surface while traveling a relatively long distance on their journey to ATP synthases. These studies with purple membranes set the stage for several groups from different research areas to establish the importance of membrane surfaces in proton circulation [12–24]. Please note that a complete review of references in the history of this field is beyond the scope of

this paper. Furthermore, based on the thermodynamics of ATP synthesis, a membrane-water interface mechanism is apparently required for proton circulation in energy transducing thylakoid, bacterial and mitochondrial membranes [25,26]. Thus, it appears that the proton gradients established at membrane surfaces are in fact essential for proton pumping activity of integral membrane proteins of the respiratory chain complexes, e.g. ATP synthase [27–30].

While numerous lines of evidence confirmed a primary role of lipids in proton circulation along membrane surfaces, a unified model that accounts for the great variety and specificity of lipids in different types of membranes (Fig. 1; Table 1) is still missing. Given their negative charge, anionic lipids have been hypothesized as trap and conduction vehicles (proton wells) for proton transport [8], such that lipid-water interfaces would form a “network” for localized proton sources (pumps) and sinks (ATP synthases) [9,38,39]. A “proton trap” function for oxidative phosphorylation was also attributed to more specific lipids such as cardiolipin (CL) [40–42]. These models, however, do not fully integrate the distinct composition of lipids (e.g. the highly variable proportions of phospho- vs. glycolipids; anionic vs. zwitterionic vs. neutral lipids; as well as the absence of CL in thylakoid and most archaeal membranes) and their possible roles in the bioenergetics of energy transducing membranes. Moreover, the idea of a few lipid classes (e.g. CL) participating in the membrane’s interface proton transport is consistent with the localized proton gradients for ATP formation in respiratory supercomplexes [41–49]. The latter concept implies that proton sources and sinks are located nearby, i.e. within a limited distance for proton shuttling. However, this idea has been currently challenged by compelling data. These recent studies have provided evidence for segregated respiratory complexes into microdomains in *E. coli* [50,51], such that protons might have to travel long distances (thousands of Å) between efflux pumps and ATP synthase.

This paper is built on the proposition that lipids are **co-catalysts** in the bioenergetics of energy transducing membranes. Our challenge is to unify the roles of **phospho- and/or glyco-glycerolipids** typical of archaeal, bacterial, thylakoid and mitochondrial membranes (Table 1) into a general model for proton circulation hugging cell surfaces. We provide a critical assessment of lipidomics in membranes from all three domains of life, discussing the proposed model in face of current data on localized vs. delocalized respiratory complexes. This article features the roles of lipids in bioenergetics, which might **inform** broader ecological **understanding** such as life in extreme environments, primitive and extraterrestrial life, aging and age-dependent diseases.

2. Principles of proton circulation along membranes

2.1. Universality and essentiality of phospho- and glycolipids in energy-transducing membranes

Phospho- and/or glycolipids, **here defined and used throughout this paper as phosphate- and sugar-based glycerolipids, are characteristic building blocks of** all energy-transducing membranes (**Fig. 1; Table 1**). **In turn, energy-transducing membranes are** broadly defined here as any membrane involved with any aspect of proton bioenergetics or proton circulation. According to this definition a synaptic vesicle is an energy transducing membrane, as are plasma and excitatory membranes. Thus on total, **most** membrane surfaces in the biosphere are energy transducing, with chloroplast and mitochondrial membranes being the most **abundant**. The total surface area of these organelles (chloroplasts and mitochondria) is larger than the surface area of all other membranes combined. Note that phospho- and glycolipids are also components of

archaeal membranes even though ether-bound isoprenoidal chains as opposed to ester-bound fatty acids remained a hallmark of archaea during the course of their evolution.

Glycolipids are signature lipids of photosynthetic membranes [36,52]. The importance of glycolipids in ecology can be judged by the number of these building blocks produced annually, an amount estimated to be greater than two billion metric tons. This value is derived from tracer studies, which suggest more than ten percent of total fixed carbon ends up in chloroplasts with roughly one fifth of this fraction as glycolipids [53,54]. Obviously, these are rough figures but drive home the point that no other class of membrane lipids come close to this magnitude. Since glycolipids are present in thylakoid membranes of plants, algae and cyanobacteria (Table 1) this means that photosynthetic membranes enriched with glycolipids are the most abundant lipid class among all other membrane surfaces involved in proton bioenergetics. While glycolipids are considered as the most abundant, they tend to be found in specialized membranes such as photosynthetic membranes, but are otherwise missing in many membranes such as the inner membranes of *E. coli* and mitochondria. Conversely, phospholipids are universal and inseparable from energy-transducing membranes. In spite of their importance, the molecular roles of phospho- and glycolipids in proton circulation along membrane surfaces remain poorly understood.

Data from comparative lipidomics and biochemical ecology tell us another interesting rule of thumb about the universal role of the lipid's headgroup. *E. coli* has been a genetic workhorse for mutant and recombinant analysis, and it is well known that a knockout or null mutant of its bulk molecular species of lipids, i.e. phosphatidylethanolamine (PE), is lethal [55]. However, researchers found that by simply supplementing $MgCl_2$ or engineering recombinants that express foreign genes for glucose or galactose transferases, growth of a PE-minus mutant

can be partially restored [56–59]. In the biosphere, especially in the ocean, a significant number of organisms are faced with a chronic shortage of phosphate in their environment. Cyanobacteria adapt to phosphate limitation by replacing their phospholipids with sulfoglycolipids [35], whereas eukaryotic phytoplankton substitute aminolipids for phospholipids [60]. Not only microorganisms substitute non-phospholipids for phospholipids under phosphate deprivation, but also higher plants are capable of this lipid substitution ([61] and references therein). The caveat of these data from the marine environment, plants and *E. coli* is that glycolipids and/or aminolipids can substitute for phospholipids under certain conditions (see section 2.2). The contrary has, however, not been observed.

The plant, *Arabidopsis*, is a model organism for genetic study and has proven to be especially valuable in demonstrating the essentiality of glycolipids, specifically galactolipids (reviewed by [61,62]). Mutants of *Arabidopsis* with deficiency in producing either mono or digalactosyl lipids display severe effects in growth and functional capabilities of their chloroplasts [63,64]. Moreover, it was demonstrated that glucolipids can substitute for galactolipids, but even this exchange in sugar headgroup, i.e. glucose for galactose, affects dramatically the maximal photosynthetic capacity of *Arabidopsis* [65]. Similarly, glucolipid substitution for galactolipids in the cyanobacteria *Synechocystis* lead to a significant reduction in photosynthetic activity and light energy utilization [66]. It is difficult to find a more thorough and convincing set of data defining the essential nature of a single class of membrane lipids in any organism. We suggest that phospholipids and glycolipids represent divergent solutions to the fundamental need in cell bioenergetics: the requirement for maintaining proton conduction along membrane surfaces.

2.2. Possible functions of anionic, neutral and zwitterionic lipids in membrane-water interface proton transport

This section is built on the concept that anionic lipids and bioenergetics are so tightly linked that they are fundamentally inseparable. Indeed, as stated by Haines [8], there exists no cellular membrane that lacks anionic lipids. The concept that negatively charged phospholipids (Fig. 1), including CL, act as proton antennas and conduction vehicles for membrane-hugging proton circulation is not a new idea [8,40]. It is well known that growth and activity of recombinants of both *Arabidopsis* and *E. coli* deficient in anionic lipids are severely compromised [67,68]. A closer look into the environmental data given in the section above reveals that cyanobacterial cells adapt to phosphate limitation by replacing their phospholipids with anionic sulfolipids [35]. Moreover, eukaryotic phytoplankton substitute zwitterionic betaine lipids for zwitterionic phospholipids, but not the anionic phosphatidylglycerol (PG) [60]. Note that in both cases the negatively charged headgroup is retained and phosphate is likely shunted to more critical metabolites such as for DNA synthesis.

Once again this data highlights the fundamental importance of the negatively charged headgroup rather than phosphate itself. Protons have a great affinity to “park” into this negatively charged headgroup [8,69,70]. Recent data defining the role of lipids in giant unilamellar vesicles as model to study the mitochondria inner membrane morphology [71–73] help us illustrating these properties. Subjecting these purely lipidic vesicles, containing a mixture of anionic cardiolipin and zwitterionic phospholipids typical of mitochondria (Table 1), to a local external acidification caused membrane deformations that resemble the cristae of mitochondria inner membranes. Further tests made with different headgroups of anionic phospholipids,

including PG, phosphatidyl-inositol (PI) and -serine (PS), resulted in distinct morphological changes [71–73]. More importantly, no changes in shape were noticed in vesicles containing exclusively zwitterionic phospholipids [71]. This data using a membrane model indicates that the electrostatic interaction between anionic phospholipids and protons results in deformation of lipid vesicles attributable to local changes in pH at the membrane surface.

Despite the multiple functions of anionic lipids in cell membranes, we suggest that anionic lipids are fundamental for proton circulation by simply placing protons on the membrane surface normal. This leads to the question: what are the functions of other membrane-associated zwitterionic phospholipids and sugar-based neutral lipids? It is well documented that phospholipids are organized in membranes via electrostatic interactions and hydrogen bonding of their headgroups, including the water molecules that are involved in the hydration shell of the polar headgroups [69,74–78]. This continuing “network” of water molecules in charged phospholipid headgroups (i.e. both anionic and zwitterionic lipids) would allow protons to move laterally along the membranes surfaces by a von Grothuß mechanism [79], instead of escaping immediately to the bulk [9,38,39]. This latter mechanism is described in more detail in the next sections. Recent data by Springer et al. [22] have shown that three different phospholipid membranes, whose proton affinities vary by 7 pKa units, enabled long-distance two-dimensional proton migration along planar membranes (70 µm). These authors concluded that protons are retained and diffuse in the membrane’s hydration layer as opposed to protons binding to fixed buffers such as anionic lipids, as previously suggested [8,40,41].

Cell membranes composed mostly of anionic lipids are rare in nature, and haloarchaea may represent the best-known exception. The cell membranes of these salt-loving archaea are composed mostly of anionic phospholipids [80], and their growth and viability are dependent on

relatively high concentrations of divalent cations (>75 mM Mg^{2+} ; [81]). Similarly, growth of a lethal PE-minus *E. coli*, composed solely of anionic CL and PG, can be restored by supplementing the media with divalent cations such as Ca^{2+} , Mg^{2+} and Sr^{2+} [56,58,59]. This requirement for divalent cations in membranes composed mostly of anionic lipids suggests that these ions may counteract the repulsive charges of negatively charged phospholipids in haloarchaea and PE-minus *E. coli*, thereby avoiding proton leakage. Thus based on these observations and the results from Springer et al. [22], it appears that a fundamental role of zwitterionic lipids may be related to the electrostatic organization of cell surfaces, balancing the net amount of negative charges, possibly for the purpose of proton transport along membrane surfaces.

A breakthrough in understanding how protons might be contained within a latent (not detectable by electron microscopy) layer on the membrane surface occurred in 2012. Wang and colleagues [82] used nanochemical methodology to synthesize an 18 Å-thick, H-bonded lattice, which they showed to act as a permeability barrier against protons (Fig. 2a). These authors synthesized this nanomembrane to demonstrate how archaeal and bacterial cells might use such H-bonded sugar lattices on their outer membrane surface as defenses against excessive acidity damaging to the cell. In retrospect, we suggest that their nanomembrane sugar lattice model can be directly applied to explain long-distance, membrane-water interface proton conductance on photosynthetic membranes of chloroplasts and cyanobacteria (Fig. 2b). In fact, a careful assessment of the lipidomics of *H. salinarum*, used as a biological model for long-distance proton transport [10, 11], reveals that the outer leaflet of archaeal purple membranes are covered by a triple sugar lattice of anionic sulfoglycolipids [85,86]. Similarly, neutral glycolipids are major lipids of *Sulfolobus* [87], another archaeal membrane used to experimentally probe long-

distance proton diffusion along membrane surfaces [12]. Collectively, these data illustrate that anionic, zwitterionic and neutral lipids (Fig. 1) can be integrated into a single category: H-bonded headgroup lattices for active proton or hydronium ion conduction or the “hyducon”.

2.3. The “hyducon”: introduction to the general model

This section introduces a hypothesis based on the concept that H-bonded chains of water molecules in lipid headgroups are the proton conduits for energy-transducing membranes (Fig. 3). While the underlying concept is not new (see History section), our challenge is to integrate the hyducon model and the role and essentiality of phospho- and/or glycolipids membranes in the thermodynamic ecology of archaea, bacteria, plants and mitochondria. We first give a brief explanation of how hydronium ions potentially move along the membrane surface via a von Grotthuß mechanism. This concept has been used to explain proton transport across the membrane, such as through gramicidin [88] and ion channels [89–91], with several reviews available in the literature [92–96].

Negatively charged headgroups are known to interact with protons and other cations including Na^+ , K^+ , Mg^{2+} and Ca^{2+} [97,98]. Here, our attention is primarily on protons. According to the general model, anionic headgroups with attached protons are found within the H-bonded lattice of lipids along membranes. At this point, our model does not prove that lipids are proton wells, but rather demonstrates that the proton-carrying anionic headgroups are clearly in a position to participate in proton swapping via the von Grotthuß mechanism, with hydronium ions or H_3O^+ moving through this narrow channel (Fig. 3a). Protons leaving anionic headgroups and binding water will form more H_3O^+ that in turn influence the pool size of circulating protons in

the conduit. However, when a proton hops from an anionic headgroup to a water molecule it leaves behind a negative charge, which can be immediately filled by a H_3O^+ breaking away from another anionic lipid (Fig. 3b). Thus, proton swapping only slightly changes the net pool of H_3O^+ in the pipeline. However, this situation would change immediately should even a miniscule drop in H_3O^+ concentration occur for any reason. At this point, a net flow of protons from the headgroups to water would occur and vice versa. According to this scenario anionic lipids might be essential because they instantly give up their protons when needed and accept protons when too many are present. Thus, the H-bonded lattice of lipid headgroups might behave as a classic buffer against changes in pH and is fundamentally distinct from the proton well hypothesis [8,40–42]. The caveat is that this mechanism would be effective only if the protons in circulation are carried in a small volume within a hydrotion, preventing protons from leaving the membrane's surface.

A considerable increase in proton mobility at 2 Å radius compared with larger water-channel pores has been shown by molecular dynamics simulation, providing evidence of effective von Grotthuß water wire behavior when waters are in single file [99]. Other mechanisms of proton transfer include transport by soluble buffers such as phosphates [100] and the related phenomenon of water hydrolysis [101]. Although soluble buffers represent a labile source of protons at generally much higher concentrations (mM; for water ~55 M) than free protons (<µM), their diffusion rates are usually more than one order of magnitude lower than that of protons in water [94]. Indeed, a wide range of diffusion coefficients has been reported for proton transfer in lipid membranes, covering two orders of magnitude with the highest values comparable to those in water [10,11,18,22]. We quote DeCoursey [92] to conclude on this quandary: "... the speed of proton transfer may be less critical biologically than the

establishment of conditions that allow it to take place in a controlled and predictable manner.” In other words, we suggest that proton pathways along the membrane surface are inherently dependent on the blend of lipid structures, and that this emergent property has evolved under selective pressure to fulfill the energetic requirements of the cell.

Another matter of debate in proton transport along membrane surfaces is the reasons why protons stick to the surface and do not desorb to the bulk during long-distance transport. Recent papers by Medvedev and Stuchebrukhov [23,102] provide a theoretical rationale as to the implications of protons desorbing and re-adsorbing onto the membrane surface before equilibrating into the bulk. This possibility introduced a challenge to the field of proton transport along membrane surfaces. Wolf et al. [24] for example recognized: “We suspect the existing conundrum on the existence of the bulk-mediated diffusion pathway originates from the fact that the competing on-surface pathway is very sensitive to the membrane conditions”. In the same lines as the latter authors, we suggest that the surface-to-bulk proton exchange together with the possibility of proton leakage through the lipid bilayer [103,104] might be in fact the major reasons explaining the predominance of certain classes of membrane lipids associated with distinct environmental conditions (e.g. high/low pH and temperature; see section 5).

Long-distance proton transport by a hydrotion in membrane surfaces offers a multitude of benefits for bioenergetics. The ATP synthase is delicately regulated and requires precise management of *pmf* and metabolic homeostasis. A detailed description of this important topic is beyond the scope of this paper, but it is briefly considered here for completeness. The main point is that ATP synthase responds to numerous signals including changes in *pmf*, adenylyl phosphate levels and acidity [105]. The low concentration of protons, literally a million times smaller than that of other ions [96], circulating in the small volume of the hydrotion suggests that slight

changes in numbers of protons might be sensed by ATP synthase, in turn amplifying changes in energy status and homeostasis. These changes are part of an elaborate regulatory feedback loop modulating the activity of this powerful enzyme that is at the heart of proton circulation [106,107].

2.4. Proton arithmetic

The concept that proton transport occurs in a specialized structure with a miniscule volume raises many issues that we lump together as proton arithmetic. The underlying issue is how many hydronium ions take part in proton bioenergetics, especially if they are localized on the membrane surface. We do not yet offer a precise number but suggest some ways to gauge this number. Calculating the number of free protons taking part in proton circulation in a synaptic vesicle is a good place to start. How many protons does it take to charge a single synaptic vesicle with H_3O^+ needed to energize uptake of a standard load of glutamate, a neurotransmitter? Since only one ATP synthase (V-type consuming ATP) molecule, on average, is present per synaptic vesicle [108] the stage is set for the first lesson on proton arithmetic.

The number of protons in the cavity of a synaptic vesicle is estimated to be 0.1 per vesicle [109] and our calculation based on pH [110] and volume [108] is 0.07 protons per vesicle. For the cytoplasm of *E. coli* at pH 7 this number comes out to be 28 (Fig. 4), which is supported by back of the envelope calculations for protons in a typical bacterial cell estimated at 60 [112]. Proton arithmetic for chloroplasts is more complicated because of uncertainty about the volumes assigned to the stroma and thylakoid lumen. We suggest that at this point an order of magnitude is sufficient for these purposes. Interestingly, the numbers of protons are estimated at

0.6–3.6 in thylakoid membranes with a lumen pH of 6 [113]. Note that these fractions of protons are just a theoretical approximation based on pH and volume. However, they fundamentally demonstrate that only a few free/unbound protons participate in the cell's bioenergetics, consistent with the idea that proton availability is a key limiting factor controlling the rate of proton transfer in biology (see review by [94]).

We have determined the size of a hyducon based on amount of polar lipids in a cell of *E. coli*, water molecules per lipid headgroup and volume of a water molecule. The purpose of this calculation, detailed in Fig. 4, is to understand the proton arithmetic inside the hyducon relative to that of *E. coli* cytoplasm. The rationale is that a variation in pH of 1 unit from pH 7 to 6, important to ATP synthesis, would need only a few protons (~3) added into the hyducon compared to more than 250 protons needed for the same ΔpH in the cytoplasm (Fig. 4). In support of our calculations, Cherepanov et al. [16] developed a model system and observed that a change in proton concentration of up to 10^{-6} M occurs at the membrane/bulk interface (or ~3 protons in the hyducon) when “pumps” ejected protons at the cell surface. We therefore suggest that the few protons released by pumps may cause a significant ΔpH shift in the hyducon, which could then be used to generate the electrochemical gradient that drives production of ATP.

We now compare the proton arithmetic with data on the time scale of proton bioenergetics. Ueno and colleagues have experimentally demonstrated in *E. coli* that the maximum velocity of an ATP synthase is around 230 revolutions per second [114]. This maximum velocity of an ATP synthase, with formation of 3 ATP/revolution, is equivalent to 690 ATP/s. Based on the ~1,000 ppm of subunits atpE or atpC of *E. coli* ATP synthase (Protein Abundance Database, <http://pax-db.org>) and estimated 3×10^6 proteins per μm^3 (or the volume of an *E. coli* [112]), the number of ATP synthases is in the range of 1,000 to 10,000 molecules/cell.

These calculations reveal a turnover of 0.7 to 7 million ATP/s/cell. Another way to gauge this number is experimental data showing that maximum yields of *E. coli* growing aerobically correspond to 12 billion ATP/cell [115; see also <http://bionumbers.org>]. Considering a generation time of 40 min. (or ~2,000 s) for *E. coli* cells during aerobic growth [116], the turnover can be estimate at 6 million ATP/s/cell. With a stoichiometry of 3-4 H⁺/ATP [117], the numbers of protons circulating per cell for ATP synthesis is in the order of 20 million H⁺/s.

Two major lessons can now be gleaned from proton arithmetic in *E. coli*: low availability of protons for bioenergetics and massive number of protons supplied to ATP synthases per second, a time scale in which protons at membrane surfaces theoretically equilibrate with the bulk [118]. Thus while the hyducton may not be an absolute requirement, it potentially represents an efficient pathway for proton transfer between sources and sinks along the membrane surfaces. Comparatively, bulk-mediated desorption and diffusion will cause proton loss into the bulk phase, even if membrane surface proton antennas (i.e. negatively charged lipids or proteins) are involved [17,24].

As concluded by Cherepanov and colleagues [16], the membrane-water interface may represent a potential pH barrier at cell surfaces allowing microbial life to exist in extremely alkaline environments. In addition to alkaliphiles, we suggest that the hyducton is likely essential to the bioenergetics of all life forms. From an ecological standpoint, however, it is important to recall that the benefits of the hyducton might come with several risks that include, among others, protons escaping from the membrane surface into the cytoplasm causing significant pH alteration and protons crossing the lipid bilayer [103,104; see section 5].

2.5. Lipid headgroups enable long-distance proton transport through the hyducton

If lipid headgroups buffer long-distance proton transport, what are their chemical features that could account for this property? In a recent *ab initio* molecular dynamics study, phosphates have been defined to possess the highest intrinsic proton conductivity of any known substance [119]. According to the authors, due to its peculiar H-bonded topology and weak solvent interaction, phosphoric acid is an exception among liquids with very strong H-bonds. The formation of ordered structures by strong H-bonds tend to result otherwise in very slow proton dynamics rather than high proton conductivity [119]. This important chemical specificity of phosphates may confer high conductivity upon phospholipid-based, energy-transducing membranes, enabling their function as a hyducton. It also reveals, however, that protons may readily leave the membrane normal, i.e. escaping to the bulk or across the lipid bilayer, should a slight change in the configuration of phospholipids occur at the cell's surface.

In order to evaluate the risks of proton leakage associated with phospholipids as hyductons, we revisit data showing that PE-minus mutants of *E. coli* are partially rescued by glycolipids and addition of divalent cations [56–59]. While glycolipids and divalent ions are not exact substitutes for PE, they can certainly balance the negatively charge density of the PE-minus membrane, thereby minimizing proton leakage from the hyducton. Compelling evidence for proton leakage control by divalent cations is found in archaeal membranes of methanogens composed mostly of anionic phospholipids. *Methanosarcina* species grown in low Mg^{2+} concentrations are observed exclusively as dense cell aggregates (cell packets). In contrast, cells grown in high Mg^{2+} concentrations transition from aggregates to single cells displaying comparatively higher growth yields [120,121]. These lessons from phospholipids are key for understanding the roles of glycolipids in energy-transducing membranes.

In section 2.1, we provided examples from photosynthetic membranes revealing that phospholipids cannot substitute for glycolipids. This picture is reinforced by data from Salzberg and Helmann [122] on *B. subtilis*. These authors reported a considerable slower doubling time of mutants lacking glycolipids relative to others lacking single phospholipids. Surprisingly, this study also revealed that compared to the wild type, viability and robust growth of single phospholipid mutants of *B. subtilis* stand in sharp contrast with nonviable strains of PE- and CL-minus mutants of *E. coli* [55]. While *B. subtilis* is devoid of an outer membrane, its presence in *E. coli* was suggested to account, in part, for the difference in viability and growth of recombinants [122]. However, no further explanation was provided as to the function of the outer membrane in bioenergetics. By interpreting the data in *B. subtilis* from a hyducon perspective we suggest: 1) glycolipids acting as proton shields enable robust growth rates of mutants lacking each major phospholipid; 2) glycolipids are likely tethered to specific membrane domains (e.g. near the entry/exit ports of major proton pumps) to minimize proton leakage. In other words, glycolipids in *B. subtilis* may provide the hyducon with proton containment, a concept idealized by Wang et al. [82] in their model for acidity resistance in archaea (Fig. 2). By analogy, we suggest that the outer membrane of *E. coli*, forming the periplasmic space (zone between the outer and inner membranes), represents the “roof above” for optimal function of the hyducon.

2.6. Phospho- and glycolipids acting as hyductons in energy-transducing membranes: lessons from ecology

In the gut, *E. coli* faces a number of toxic molecules such as soap-like bile salts and energy-uncoupling acids of their own making. In laboratory cultures, these acids accumulate as

byproducts of sugar fermentation, in levels high enough to kill most of these cells. Nikaido [123] has described the rationale and molecular architecture of the outer membrane of *E. coli*, with lipopolysaccharides located exclusively in the outer leaflet, as a defense against these and other toxic substances including antibiotics. Note that *E. coli* is often the cause of serious urinary tract infections, a disease treated with antibiotics [124]. We consider an excess of protons to be a potent toxin and suggest that the outer membrane shields the cell against this “problem” according to mechanisms described by Nikaido [123]. Interestingly, a complex network of H-bonds between neighboring sugar headgroups stabilizes the lipopolysaccharide leaflet, such that this layer acts as a permeability barrier from the outside in and simultaneously from the inside out. With the exception of porins openings to the cell’s exterior, we reasoned that the robust outer membrane architecture harnessed for blocking toxic compounds from entering the cell [123] might also act to prevent the loss of metabolites such as protons effluxed into the periplasmic space. In this scenario, protons in the periplasmic side of the inner membrane are localized in a relatively small volume compared to the total cytoplasmic volume of the cell. Thus, the outer membrane of *E. coli* has earmarks of a powerful permeability barrier for proton containment.

The ecological function of the outer membrane as permeability barrier is also apparent in the extreme flight of hummingbirds. These birds require virtually the entire volume of the mitochondrial matrix to be filled with cristae, considerably enhancing the surface area for allocation of plentiful ATP synthase and respiratory proton pumps (as shown in Fig. 5). Presumably this adaptation reflects the need for mitochondria of breast muscle cells to energize wing beats of greater than 100 per second ([125] see also [84]). Mitochondrial bioenergeticists have known for decades about the restricted volumes of matrix and intermembrane space caused

by cristae morphology, but the main point here concerns the mechanism of proton circulation in this maze of cristae. According to the proton containment concept, the small volume of matrix and intermembrane space sandwiched between opposing cristae provides an upper boundary to each leaflet of the inner membrane. This tight arrangement in hummingbird mitochondria may be used to trap or localize protons that escape the phospholipid-based hyducon (Fig. 5). This proton containment strategy essentially enhances the efficiency of proton circulation to meet the extreme energy required by the flight of hummingbirds.

For the purpose of clarification, an outer membrane as proton containment of phospholipid-dominated membranes is not bioenergetically more effective than the glycolipid one. In fact, growth rates of the glycolipid-containing *B. subtilis* are comparable to those of *E. coli* [126]. Thus similar growth rates of mutants lacking phospholipids compared to the wild type in *B. subtilis* [122] may reveal glycolipids as a conservative strategy for harnessing energy. That is, conserving significant levels of protonic energy (i.e. by avoiding proton leakage) is equivalent to making more energy. Not surprisingly, sugar-based headgroups are widespread among archaea [127,128], which are especially well adapted for energy conservation in environments under chronic energy stress [129]. Glycolipids are also signature lipids of light-harvesting membranes, and this topic will be covered in detail in section 3.3. In summary, we suggest that the primary biochemical basis for glycolipid-containing membranes is both energy conservation and energy efficiency. Conversely, cell membranes lacking glycolipids such as *E. coli* and mitochondria need to balance the energy costs associated with the proton containment strategy (i.e. outer membrane) and the benefits of high proton conductance within the hyducon made of phospholipids. Lessons from ecology indicate control of environmental conditions on membrane

lipid composition, which in turn modulates the hyducon composition to meet the energy demands for life.

2.7. Grotthuß-type proton transfer in the hyducon and integral membrane proteins: effects of point mutations

The discussion below seeks to evaluate the proton transfer by a von Grotthuß mechanism at the cellular membrane level. The key to proton transfer by a von Grotthuß mechanism is the hopping of protons in a well-aligned H-bonded network ([92]; Fig. 3). Water wires organized via H-bonded amino acid side chains inside powerful proton pumps have been revealed by high-resolution X-ray crystallography (e.g. bacteriorhodopsin, cytochrome oxidases; see [94,130,131]). This H-bonded network is recognized as the main pathway of proton transfer across membranes, and thus its efficiency depends on close proximity of amino acids to the point of hydrogen bonding (e.g. reviewed by [94,96]). Indeed the essentiality of mobile water as gateways for proton transfer has been defined by point mutation of amino acid side chains within integral membrane proteins. Wraight [94] concluded that although key residues may result in irreplaceability, peripheral mutations do not alter the general functionality of the proton transfer pathway. These observations on efficiency of proton transfer by water wires in membrane proteins suggest a remarkable similarity to the organization of water molecules in lipid surfaces [77,78,98], and perhaps the functional capabilities of the hyducon as discussed below.

It is generally accepted that genetic manipulation of lipid metabolism in bacteria and yeast provides mutant strains lacking a range of membrane lipid classes with largely unaffected viability [55,66,122,132–134]. For instance, growth of CL-minus mutants of the yeast

Saccharomyces cerevisiae in glucose is largely unaffected relative to the wild type at 30°C, but displays severe defects in mitochondrial function and an apparent decrease in membrane potential [135,136]. Similarly to yeasts, *Arabidopsis* and *Synechocystis* mutants with substitution of a single sugar in essential digalactose headgroups could restore overall growth, but not the total photosynthetic capacity of these organisms [65,66]. The above-mentioned studies collectively suggest that a well-balanced lipid headgroup composition is likely required for optimal functioning of the hyducon (Fig. 3). This observation appears in contrast with the robustness to mutation of amino acids side chains for proton transfer in integral membrane proteins [94]. We attribute this difference to the fact that proton shuttling through the H-bonded network of lipid headgroups may occur at distances several orders of magnitude longer than that of amino acid water channels, e.g. 70 μm or 700,000 Å in planar membranes [22] vs. 18 Å in gramicidin [137]. We thus propose that the hyducon is an emergent feature, evolutionarily honed to provide an arrangement of H-bonded structures via lipid headgroups, which depends not just on the charged and non-charged nature of glyco- and phospholipids, but also on the characteristics of their lipid tails [83,84,138].

3. Case histories of hyducons chosen from all three domains of life

This section showcases the proposed bioenergetic relevance of the hyducon (Fig. 3) in four exemplary membranes that span the spectrum of life's domains. We start with the purple membrane of haloarchaea, on which early studies of long-distance proton transport were performed. We then discuss the hyducon in two idealized models for *E. coli*, highlight the importance of lipid composition for the hyducon in photosynthetic organisms including

chloroplasts of eukaryotes and cyanobacteria, and end by considering the hyducton in mitochondrial membranes.

3.1. Lipidomics of the purple membrane in haloarchaea

H. salinarum has been used as a model system for membrane-water interface proton transport [6,10,11]. In this haloarchaeon, the purple membrane (PM) converts light into chemical energy [139], i.e. proton pump, and oxygen is not evolved during this process. The PM is an island within the membrane of *H. salinarum* with an extremely high protein-to-lipid ratio (~75:25), featuring a membrane-spanning bacteriorhodopsin as the only protein of this domain [140]. Note that the PM may cover up to fifty percent of the total membrane surface of the cell [141], and protons must travel from the PM, which is devoid of ATP synthase, to ATP synthases localized in respiratory lipid domains as far away as 5,000 Å.

The membrane surfaces of *H. salinarum* are composed of both phospho- and glycosidic isoprenoidal diethers [142]. Interestingly, the distribution of these lipids on the leaflets of PM is asymmetrical [85,86]. As shown in Fig. 6a, whereas phospholipids (in green) are mostly present in the inner leaflet, the sulfated triglycosyl lipids (in yellow) are located at the outer leaflet of the PM. This asymmetrical localization of glycolipids to the exterior of the PM has been attributed to the vectorial nature of the proton pumping activity [85,130]. Here, we reinforce this idea suggesting that the triple-sugar headgroup provides essential containment for protons being transported in *H. salinarum*. That is, the H-bonded triglycosyl headgroups together with their anionic character, secured by negatively charged sulfated moieties at the top of these structures, are proposed to efficiently hold protons close to the membrane's surface. This conservative

proton containment strategy not only meets the permeability barrier properties of the hydrotion, but also focuses protons for long-distance transfer. Gradients created by powerful and opposing proton pumps, i.e. ATP synthase and bacteriorhodopsin, determine the directionality of proton transport (Fig. 6a).

Given the specific association of sulfated triglycolipids with bacteriorhodopsins, these lipids are also predicted to be essential for the formation of purple membranes in haloarchaea [86,130,143]. Sulfated glycolipids can represent a significant proportion of total membrane lipids, up to 30% [142], especially in genetically modified strains of *H. salinarum* with high-bacteriorhodopsin content (e.g. L33 used by [144]). In contrast to previous studies, our recent lipidomics survey revealed the absence of these glycolipids in a *H. salinarum* wild type [32]. Collectively, these observations indicate that amounts of sulfated triglycolipids may be likely proportional to content and/or activity of purple membrane in *H. salinarum*. Indeed, based on ion binding motifs of their ATP synthases, haloarchaea in general are known to primarily use Na⁺ as coupling ion for bioenergetics [145], such that *H. salinarum* may abandon use of the purple membrane for ATP formation under certain conditions.

3.2. Electrochemical poles generated by microdomains of respiratory complexes in bacteria

Fluorescent imaging has been used to elucidate subcellular localization, mobility and stoichiometry of respiratory complexes in the inner membrane of bacteria such as *E. coli* [47] and *B. subtilis* [146] and thylakoid membranes of cyanobacteria [49]. These studies have collectively suggested co-localization of clusters of these complexes on the nanometer scale, e.g. ATP synthase and succinate dehydrogenase in *B. subtilis* [146]. These patches of bioenergetics

domains are mobile and resemble those of respiratory supercomplexes in mitochondria and plants [43–45,147]. Moreover, data by Liu et al. [49] provided additional evidence for dispersal of the respiratory complexes of cyanobacteria after induction by moderate light intensity. Liu et al. [49] further suggested that this redistribution of respiratory complexes matched the distribution of photosynthetic complexes, leading to electron exchange between both respiratory and photosynthetic electron transport chains. Note that in both cases, the generated proton gradient would benefit from transport by the hyducon linked to ATP synthase. The co-localization of ATP synthase within respiratory complexes also suggests relatively short-distance proton transfer in bacteria (Fig. 6b₁) as compared to the purple membrane hyducon examined above. However, most recent data from this field suggest that the concept of proton circulation in bacteria is entering a new era involving microdomains of respiratory complexes.

Perhaps the most exciting data involves mobile specialized bioenergetics domains, 100–200 nm in diameter, consisting of tens to hundreds of molecules of respiratory complexes, as exemplified in *E. coli* [50,51] (Fig. 6b₂). In these studies the labeled microdomains were NADH dehydrogenase (Complex I), cytochrome bd-I oxidase, cytochrome bo₃ oxidase, F_oF₁ ATPase, and succinate:fumarate oxidoreductase (Complex II). The surprising aspects of these data include the formation of bioenergetic microdomains composed of only one component of the electron transport chain, the relatively large numbers of enzyme complexes present (10 to 180 molecules) and the lateral movement of individual domains across the membrane surface. It was also suggested that ubiquinone shuttles electrons long-distances between redox complexes localized in membrane patches. Data from Llorente-Garcia and colleagues [51] suggests that there is both long-range electron transfer and delocalized pmf involved in the bioenergetics of *E. coli*. We

suggest that *E. coli* might have evolved a hyducton system for long-distance proton transport, linking delocalized sources to sinks for efficient energy production (Fig. 6b₂).

We further speculate that the network of respiratory complexes segregated into patches of several molecules, rather than a few co-localized supercomplexes (Fig. 6b₁), might create powerful electrochemical poles for proton bioenergetics. For example, 48 ATP synthase molecules working in close proximity [51] mean that the electrochemical poles in the vicinity of this bioenergetic microdomain would be expected to be stronger compared to a single molecule functioning alone (Fig. 6b). Thus to be energetically advantageous, it appears that the spatially segregated electrochemical poles may benefit from hyductons enabling long-distance proton transport in *E. coli*. The existence of these poles has potential revolutionary consequences in understanding proton transfer in general, including the distribution of ATP synthases in both thylakoid and mitochondrial membranes [148].

3.3. Chloroplasts: Hyductons as possible drivers of primary energy transfer in the biosphere

The model of Wang and colleagues (Fig. 2) discussed in some detail above is readily modified by simply coating both leaflets of photosynthetic membranes with a digalactose, H-bonded sugar lattice covering the lumenal region versus a monogalactose coating for the stromal membrane leaflet of the photosynthetic bilayer (Fig. 6c). Pioneering data on sidedness of thylakoid membranes of higher plants were published several decades ago [151,152]. These data show that transmembrane distribution patterns of monogalactosyldiacylglycerol (MDGD) and digalactosyldiacylglycerol (DGDG) lipids are universal among a variety of plants. However, the biochemical rationale to explain the remarkable asymmetry of thylakoid membranes in which

MDGD is highly enriched in the outer leaflet in contrast to DGDG enriched in the inner leaflet [52,152] remains a mystery. The importance of this question is highlighted by recognition that photosynthetic membranes displaying galactolipid asymmetry are the most abundant and essential membranes in the biosphere [53,54].

Although the asymmetry quandary remains unanswered, the hyducton model for chloroplasts as described below might help to explain biochemical functions related to the polymorphic membrane structure contributed by galactolipids (e.g. MGDG forms hexagonal II phase). Once again we borrow from the model of Wang and colleagues [82] who explained the membrane asymmetry of an extreme acidophilic archaea on the basis of a permeability barrier against toxic levels of acidity in the medium. Note that the asymmetry question of archaea centers on pH as low as pH 1-2 outside of the cell. Clearly, membrane components are denatured at such low pH, especially on the membrane surface. Thus, according to these authors, only the most vulnerable membrane surface (outer leaflet) is protected through pH homeostatic mechanisms guarding the cytoplasm against drastic shifts in pH. This concept led us to ponder that a digalactose shield against acidity might be more robust than a monogalactose, H-bonded lattice (Fig. 6c). This is consistent with the well-known quandary of lumenal pH of chloroplasts, which can become surprisingly acidic, well below pH 6, especially under conditions of high illumination [153]. Thus, placing the digalactose lattice on the lumenal leaflet [52,152] makes sense from the standpoint of a permeability shield against toxic levels of acidity. It has been proposed that the photosynthetic machinery begins to be denatured as pH drops as low as pH 4.5 in chloroplasts [113,150]. Why Mother Nature evolved a photosynthetic apparatus running so close to a toxic threshold of pH remains an open question.

In summarizing this section, we point to two essential functions for the galactose sugar lattices of photosynthetic membranes. The first involves acidity protection of photosynthetic machinery on the lumenal membrane surface. The second involves containment or localization of protons near the membrane surface as a prerequisite for efficient, long-range proton circulation. Surprisingly, the mechanisms for acidity tolerance and efficiency of proton circulation are proposed to be the same – an interface that facilitates lateral proton transport, but that simultaneously acts as a permeability barrier selective against both entry and exit of protons.

3.4. Mitochondrial cristae as hyducton

Fig. 6d depicts a model for the hyducton in mitochondrial inner membranes. The main point of this model concerns the spatial segregation of proton sources (complexes I, III and IV of the electron transport chain) and sinks (ATP synthases), respectively at the flat and curved regions of the cristae membrane [154,155]. The localization of ATP synthase dimers, organized as ribbons at the curved regions of the inner membrane cristae is a common feature of mitochondrial membranes in mammals, fungi and plants, but not in chloroplasts, archaea and bacteria [148,155]. The hyducton may provide three major bioenergetic advantages for mitochondria. Firstly, protons generated by powerful pumps and moving along the hyducton on either side of the cristae will securely reach the ATP synthases organized as dimers at the cristae tips, minimizing loss of protons during delivery. Secondly, co-localization of rows featuring several ATP synthase dimers at the curved ridges of cristae [156] may create electrochemical poles for an optimum proton gradient, as in distributed microdomains of *E. coli* inner membrane (Fig. 6b₂). And finally, recent data by Klotzch et al. [30] provided evidence for a preferential

localization of the uncoupling protein 4 (UCP4) to the outside of cristae membranes, in the inner boundary membrane. These authors suggested that UCP4 does not compete for protons with major proton pumps, but may dissipate excessive proton gradients. The rationale is that when too many protons are available at the cristae hyducton, uncoupling proteins may release excess protons in a spatially segregated region of the mitochondrial inner membrane as a release valve.

5. Benefit/risk analysis of the hyducton

In developing the hyducton model described here, one of the first questions we asked was: how robust are hyductions, from an ecological standpoint? Recall that Wang and colleagues [82] were motivated to develop their model not as a conduit for proton transport but rather as a defensive shield protecting acidophilic archaea against toxic levels of acidity. The point is that the hyducton model was born in the world of extremophiles, more specifically a thermoacidophilic archaeon *Thermoplasma acidophilum* (Table A1). The acidity model above involves pH as low as pH 1-2 (Fig. 2a), which suggests that lowering seven units of pH does not breach the H-bonds in glycolipids. However, it is interesting to attempt to identify conditions or agents that rupture H-bonds of H-bonded glycolipid lattices resulting in uncoupling proton bioenergetics catalyzed by this structure.

Table A1 summarizes a selected list of extremophilic membranes and highlights the apparent limits of conditions for which glycolipids can function as hyducton lattices. The rationale for this data analysis lies in the assumption that H-bonded sugar lattices are involved in proton circulation and that breaching the H-bonded network would render the lattice useless. However, since H-bonds can instantly reform after breaking [194], agents or conditions that depolymerize the sugar lattice must be persistent. As shown in Table A1, glycolipids appear to

be not only widely distributed, but are also functionally robust inasmuch as they resist a range of moderate to strong stresses or environmental insults. For example, glycolipids of thermophilic cyanobacteria function at temperatures of 42 to 74°C in hot springs from Yellowstone National Park [195], whereas glycolipid-enriched membranes of hyperthermophilic methanogens function at least up to 100°C (e.g. *Methanopyrus kandleri*; [196]). The putative sugar lattice of the purple membrane of haloarchaea functions at near saturating concentrations of salinity (about 5M NaCl; [197]). Archaea often can be classified as energy extremophiles [129] and most of them synthesize glycolipid-based membranes [127,128]. The chloroplast of Antarctic eukaryotic phytoplankton is an example of an extremophilic organelle harvesting light near 0°C [84].

We next deal with poisons or inhibitors that might disrupt the permeability properties of a sugar lattice and thus, uncouple bioenergetics. Agents, chemicals and conditions that break H-bonds holding biopolymers together are well known. For example, both NaOH and LiCl₂ treatments disrupt H-bonds of chitin (glucosamine) polymers, which along with cellulose are the most abundant polysaccharides in the biosphere [198,199]. In fact, data in the chemical literature shows that hydroxyl ions (OH⁻) are very good receptors of H-bonds [199,200] and this property alone is a red flag suggesting OH⁻ might readily disrupt H-bonds of hydrotions. However, given that broken H-bonds can immediately reform and OH⁻-mediated H-bonds are fleeting, there is room for doubt that the strong donor function alone explains the H-bond-breaking behavior. Recently, the hydrogen (H) of OH⁻ has been shown to H-bond with water [201]. The point is that this dual nature of OH⁻ chemistry might allow this ion to not only readily attack the H-bonds of a sugar lattice, but also to pry open the bonds long enough to form molecular holes for protons to escape and OH⁻ to leak in. As shown in Table A1, it appears that glycolipids, which are otherwise widely distributed in high temperature and low pH conditions, are not prevalent in

obligate alkaliphilic haloarchaea [80,153] and *Bacillus* [34]. This is negative data, but it is consistent with OH⁻ acting specifically as a poison against H-bonded, sugar lattices.

Krulwich and colleagues [202] have reviewed the bioenergetics of alkaliphiles and it is clear that remarkable adaptations are required to live in an alkaline world. These include cell envelopes as permeability shields for membrane surfaces, such as the outer membrane in bacteria [123], peptidoglycan layers in archaea [203] and cell walls in cyanobacteria [204]. While alkaliphilic cyanobacteria (pH 9) are known [205] and seem to defy the OH⁻ rule, galactolipid-enriched membranes of these cyanobacteria may be located well away from direct contact with bulk OH⁻ in the environment, protected by their robust cell walls [204]. It seems clear from an ecological perspective that OH⁻ (alkalinity) in contrast to acidity disrupts glycolipid-based hyductions in direct contact with OH⁻. Apparently, building a H-bonded sugar lattice to support bioenergetics is not a common adaptation among alkaliphiles. Thus the hyduction model helps explain why obligate alkaliphilic haloarchaea and *Bacillus* lack glycolipids.

The final topic in this section involves the hyduction as being a unit of the light-harvesting membrane surface of chloroplasts and cyanobacteria, which support the most essential process in global ecology – photosynthesis (Fig. 6c). According to the sugar lattice hypothesis (Fig. 2), the H-bonds among polyhydroxy glycosidic headgroups create a lipid domain fixed in space. In essence, H-bonds are envisioned to polymerize the vast area of photosynthetic and other glycolipid membrane surfaces into a specialized, domain-like lipid structure where headgroups have limited lateral or rotational mobility. Excessive escape of protons to the bulk phase defeats the purpose of a hyduction in photosynthesis and protons leaking into the cytoplasm might be catastrophic, resulting in uncoupled bioenergetics.

Linus Pauling popularized H-bonds for organic chemists [206] while Watson and Crick did the same for biologists. Hydrogen bonds possess some ideal properties for proton bioenergetics and in previous sections we highlighted that photosynthesis, among other processes, depends on such bonding. Depending on their local environment, especially the presence or absence of water, H-bonds can be ca. 15 times stronger in membranes than van der Waals forces [207]. The fact that H-bonds display an entire spectrum of sub-covalent bond energies provides great versatility defining the properties of critical biological membranes. Recall that H-bonds are essential for specialized membrane lipids ranging from myelinated axons to photosynthetic membranes, the latter representing the major membrane lipid surface in the biosphere.

6. Conclusions

The hyducton hypothesis provides a testable model for proton transport and utilization, but many uncertainties remain. We suggest that the hyducton is vital to proton bioenergetics and proton circulation on a global scale. Some of the most convincing data supporting the fundamental roles of the hyducton in ecology come from genetic analysis of galactolipid knockout mutants of plants. We interpret this data as evidence that the biosphere's primary productivity is dependent not only on galactolipids, but also on the emergent property of proton conductivity enabled by these membrane lipids. In the absence of membrane glycolipids, the numerous great cycles of nature including the C, O and N cycles would not function as they do today. Outside the photosynthetic world, many archaeal membranes important to these cycles, such as the ones from methanogens and ammonia oxidizing archaea, are enriched in glycolipids (Tables 1 and A1). However, in spite of the massive production of glycolipids in the biosphere,

phospholipids (particularly the ones carrying a negative charge) are more ubiquitous and essential in all life forms. In one sense the concept of negatively charged phospholipid headgroup acting directly as a biocatalyst for proton bioenergetics comes from the hypotheses that anionic phospholipids act as proton antennas for parking protons in circulation [8,40]. We suggest that anionic lipids behave not only as proton antennas, but also act as circuits for proton delivery (Figs. 3 and 6). This is an exciting area for future research.

According to the hyducon hypothesis, the directionality of proton flow is expected to allow protons to travel distances of tens of thousands Å (e.g. cristae of hummingbird mitochondria, Fig. 5). Recent data from *E. coli* suggest that a limited number of electrical poles occur on the surface of the cytoplasmic membrane [50,51]. These poles, both positive and negative, are formed on the membrane surfaces when key proton pumps such as ATP synthase aggregate together and thus work in unison as a specialized bioenergetic microdomain for pumping protons. This brings a Mitchell's delocalized electrochemical gradient and a von Grotthuß mechanism of proton movement into the picture in *E. coli*. Two centuries ago von Grotthuß showed that protons in water move from all directions to a negative pole, and this rule may apply to proton transfer along the inner membrane of *E. coli*. It is interesting to note that a von Grotthuß mechanism may also play an essential role in proton transfer within ATP synthase and other proton pumps important in proton bioenergetics [105,131]. Thus, it is not a complete surprise that once protons exit a proton pump to the membrane surface, a von Grotthuß mechanism mediated by anionic lipid headgroups might transfer this ion via hyducon to its entry portals on another species of proton pump.

The concept of the hyducon as presented in this work remains a hypothesis. If the hyducon hypothesis withstands testing, it carries grand implications for evolution of emergent

732 properties at the cellular scale (e.g. likely to act as a selective pressure on membrane lipid
733 composition) as well as for physiology associated with global warming, human diseases and
734 aging.

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Table 1. Distribution of phospho- and/or glyco-glycerolipids in energy-transducing membranes. a = anionic, z = zwitterionic, Phos = phospholipids, SulfG = sulfoglycerolipids, Glyco = glycolipids, Ref. = references.

Cell/Organelle	a-Phos	z-Phos	a-SulfG	Glyco	Ref.
<i>Nitrosopumilus maritimus</i>	4			96	31
<i>Halobacterium salinarum</i>	75			15	32
<i>Escherichia coli</i>	22	78			33
<i>Bacillus subtilis</i> *	74	12		6	34
<i>Synechococcus</i> WH8103	3		52	44	35
<i>Prochlorococcus</i> MED4	2		66	32	35
Chloroplast (spinach)	11	4.5	6.5	78	36
Mitochondria (bovine heart)	24	76			37

*other polar lipids included glycophospholipids and aminolipids.

FIGURE CAPTIONS

Figure 1. Structures of phospho- and glyco-glycerolipids in the three domains of life. (A) Major characteristics of archaeal lipids relative to bacterial and eukaryal lipids. Note that archaeal polar lipids are ether-linked to side chains composed of isoprenoids, whereas bacterial and eukaryal polar lipids are ester-linked to side chains most often composed of fatty acids. (B) Phospholipids are intrinsically charged. The attached functional group to the phosphate moiety (e.g. glycerol, ethanolamine) determines the final charge of the phospholipid. These lipids are generally anionic (in red) or zwitterionic (in green). Glycolipids are most often neutral (in gray), but may also display negative charge as the sulfolipids (in red). Sugar moieties can vary greatly in composition (e.g. galactose, glucose). Glycolipids may also carry charged functional groups such as the sulfated glycolipids, which are anionic. PA=phosphatidic acid; PG=phosphatidylglycerol; CL=cardiolipin; SQ=sulfoquinovosyl; PE= phosphatidylethanolamine; PC=phosphatidylcholine; G=glycosyl.

Figure 2. Membrane adaptation in archaea and bacteria to toxic levels of environmental acidity includes placement of glycolipids on the outer leaflet of the membrane surface. (A) This

membrane synthesized by Wang and colleagues [82] shows how H-bonding of polyhydroxyl groups of sugar residues forms a lattice that might protect cells against damage by extremely low pH (acidity). These scientists used nanochemical methods to build a model sugar lattice whose H-bonded molecular architecture blocked diffusion of H^+ (hydronium ions, H_3O^+). (B) We have modified their model to explain how long-distance proton circulation may occur within glycolipids tethered by H-bonds on membranes surfaces such as in photosynthetic membranes. The focus here is on sugar headgroups in mediating proton transfer. However, it is important to recall that the often highly unsaturated tails of glycolipids represent by far the largest pool of membrane lipids in the biosphere. The conformation dynamics of the tails enables shuttling of electron carriers through the membrane bilayer [83, 84].

Figure 3. The hypothesized hyducton model. (A) Lipids comprising membrane surfaces are attached to each other via H-bonds, forming a narrow lattice with a minuscule volume (the hyducton), similar to the tethered glycolipids in Fig. 2. Protons attached to anionic lipids are in position to participate in proton circulation. Given the small numbers of protons in circulation, any change in proton concentration on the membrane surface will enable a net flow of protons from anionic headgroups to the water contained in the hyducton (red dotted line). (B) Protons are transported by a von Grotthuß mechanism, i.e. protons hopping through H-bonded water molecules contained in the hyducton. By giving up protons for circulation, anionic lipids are again free to accept available protons.

Figure 4. Proton arithmetic using a cell of *E. coli* as a model*. (A) Differences in volume between cytoplasm and the hyducton. (B) Variation in the number of protons available in the cytoplasm and the hyducton according to pH values. * Parameters used for calculation: cell dimension (0.5 and 2 μm in width and length, respectively); 2.2×10^7 total polar lipids [111]; ~ 15 water molecules per lipid [77, 98]; volume of a water molecule (3×10^{-26} L; [112]); Avogadro's number (6.02×10^{23}).

Figure 5. Electron micrograph of mitochondria. (A) Liver mitochondrion displaying a dozen of cristae structures in its matrix (courtesy of Daniel S. Friend). (B) Hummingbird breast muscle mitochondrion showing juxtaposition of two to three dozens of cristae structures, which are known as an adaptation to the extreme nature of their flight with wing beats of hundreds per

second [84, 125]. By doubling the number of cristae, as compared to liver mitochondria in (A), hummingbirds' mitochondria decrease dramatically the volume of their matrix, providing a small volume sandwiched between the outer and the inner membrane (proton containment or roof above). We suggest that this morphological arrangement is crucial for localization and thus proton circulation along membrane surfaces, an adaptation to maximize ATP production.

Figure 6. Hyducton model unifies lipidomics and bioenergetics. (A) The purple membrane of haloarchaea. These integral membrane proteins can cover up to 50% of total membrane surface of haloarchaea and are associated with negatively charged sulfated glycolipids located in the outer leaflet (see text). (B) The proton circulation in *E. coli* according to: co-localization of respiratory complexes, represented by complex I, IV and ATP synthase (B_1); and aggregation of single components of the electron transport chain in microdomains (B_2). According to Erhardt et al. [50] and Llorente-Garcia et al. [51] high numbers of molecules per microdomain patch are observed (e.g. 70-180 and 40-60 molecules of cytochrome bd-I complex and ATP synthase), potentially creating powerful electrochemical poles. (C) The thylakoid membranes of cyanobacteria and plants. Delocalization of photosystem II and ATP synthase, respectively in grana and stroma membranes (cf. [149] and references therein). In active chloroplasts the luminal pH is around 5, whereas the stromal pH is around 8 [150]. This latter aspect might drive the asymmetrical distribution of polar lipids in thylakoid membranes, with an exclusive localization of diglycosidic headgroups on the luminal side and the presence of monoglycosidic headgroups on both sides [52,152]. (D) Mitochondrial inner membrane and co-localization of complexes I, III and IV at flat regions of the mitochondria inner membrane cristae cf. Vogel et al. [154]. Ribbons of ATP synthase dimers localized in curved regions of the cristae [155, 156] might act as electrochemical poles for long distance proton transport in mitochondrial inner membranes.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

(A)

(B)

pH	H ⁺ in cytoplasm	H ⁺ in hyducton
4	27581	298.0
5	2758	29.80
6	276	2.98
7	27.6	0.298
8	2.76	0.0298
9	0.276	0.00298
10	0.0276	0.000298

FIGURE 5

FIGURE 6

(A) *H. salinarum*

(B₁) *E. coli* (Supercomplexes)

(B₂) *E. coli* (Microdomains)

(C) Thylakoid membranes

(D) Mitochondrial inner membrane

APPENDIX – TABLE A1

Table A1. Polar glycerolipid composition of selected acidophilic, neutrophilic and alkaliphilic microbial taxa. Archaea taxa are marked in gray shade. Temp=temperature; Glyco-Phospho=glycosylphosphatidyl; Phospho=phosphatidyl; Glyco=glycosyl; Ref=reference (see Main Text). Reported values of temperature and pH are according to growth conditions.

Microbial taxa	pH	Temp	Glyco-Phospho	Glyco	Phospho	Ref.
<i>Thermoplasma acidophilum</i>	1.5	57	+	+	+	157
<i>Acidiplasma aeolicum</i>	1.5	45	+	+	+	158
<i>Methanococcus jannaschii</i>	6	85	+	+	+	159
<i>Methanopyrus kandleri</i>	6.5	98	+	+	+	160
<i>Pyrococcus furiosus</i>	7	96	+	+	+	160
<i>Methanothermus fervidus</i>	6.5	78	+	+	+	160
<i>Thermococcus kodakarensis</i>	6.5	85	+		+	161
<i>Sulfolobus acidocalcarius</i>	2.5	82.5		+	+	160
<i>Halobacterium salinarum</i>	7	30		+	+	162
<i>Halarchaeum salinum</i>	5.3	42		+	+	163
<i>Nitrosopumilus maritimus</i>	7.1	28		+	+	31
<i>Natromonas</i>	8.5	37			+	164
<i>Methanosarcina acetivorans</i>	7	35			+	165
<i>Thermus</i>	7.75	72.5	+	+	+	166,167
<i>Thermodesulfobacterium commune</i>	7	70		+	+	159
<i>Thermoanaerobacter ethanolicus</i>	8	69	+		+	168
<i>Hydrogenothermus marinus</i>	6	65		+	+	169
<i>Aciditerrimonas ferrireducens</i>	3	50	+		+	170
<i>Acidiphilum</i>	3	20			+	171
<i>Alkalimonas delamerensis</i>	10.25	37			+	172
<i>Maritimibacter alkaliphilus</i>	10	30			+	173
<i>Halomonas desiderata</i>	9.5	37			+	174
<i>Methylobacter alcaliphilus</i>	9.25	30			+	175
<i>Alcalilimnicola halodurans</i>	9	37			+	176
<i>Halomonas campaniensis</i>	9	37			+	177
<i>Dietzia natronolimnaios</i>	9	30			+	178
<i>Alkalibacterium olivoapovliticus</i>	8.5	29.5			+	179
<i>Escherichia coli</i>	7	37			+	33
<i>Salmonella typhimurium</i>	7	37			+	33
<i>Bacillus subtilis</i>	7	30		+	+	34
<i>Bacillus vulcani</i>	6	60	+		+	180
<i>Bacillus acidocalcarius</i>	3	60		+	+	181
<i>Bacillus acidicola</i>	4.5	30		+	+	182
<i>Bacillus alcalophilus</i>	10.5	30			+	34

<i>Bacillus firmus</i>	10.5	31		+	34
<i>Leuconostoc fructosum</i>	6.5	30	+	+	183
<i>Leuconostoc ficulneum</i>	6.75	30	+	+	183
<i>Wenxinia marina</i>	7.75	36	+	+	184
<i>Woodsholea maritima</i>	7	28		+	185
<i>Sulfurospirillum arcachonense</i>	7.2	26		+	186
<i>Jannaschia helgolandensis</i>	7.5	27.5		+	187
<i>Pseudogracilibacillus auburnensis</i>	7.5	29	+	+	188
<i>Chloroflexus aurantius</i>	8	59	+	+	189
<i>Anacystis nidulans</i>	7.5	34.5	+	+	190
<i>Rhodobacter sphaeroides</i>	6.8	32	+	+	191
<i>Spirulina</i> strain "pantelleria"	9.25	30	+	+	192
<i>Synechococcus</i> sp. Y-7c-s	5.5	52.5	+	+	189
<i>Prochlorococcus</i> MED4	7.5	21	+	+	35
<i>Gloeobacter violaceus</i>	7.5	26	+	+	193