Review

Co-evolution of primordial membranes and membrane proteins

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Studies of the past several decades have provided major insights into the structural organization of biological membranes and mechanisms of many membrane molecular machines. However, the origin(s) of the membrane(s) and membrane proteins remains enigmatic. We discuss different concepts of the origin and early evolution of membranes with a focus on the evolution of the (im)permeability to charged molecules such as proteins, nucleic acids and small ions. Reconstruction of the evolution of F-type and A/V-type membrane ATPases (ATP synthases), which are either proton- or sodium-dependent, might help us to understand not only the origin of membrane bioenergetics but also of membranes themselves. We argue that evolution of biological membranes occurred as a process of co-evolution of lipid bilayers, membrane proteins and membrane bioenergetics.

Membrane evolution and the last universal common ancestor

A topologically closed membrane is a ubiquitous feature of all cellular life forms. This membrane is not a simple lipid bilayer enclosing the innards of the cell: far from that, even in the simplest cells, the membrane is a biological device of a staggering complexity that carries diverse protein complexes mediating energy-dependent (and tightly regulated) import and export of metabolites and polymers [1]. Despite the growing understanding of the structural organization of membranes and molecular mechanisms of many membrane proteins, the origin(s) of biological membranes remains obscure [2–5].

The conservation of a set of essential genes between two major domains of life, archaea and bacteria, leaves no reasonable doubt to the existence of some version of 'last universal common ancestor' (LUCA), the prototypic organism that led to the three branches of cellular life, namely, bacteria, archaea and eukarya [6]. The standard model of evolution places the primary division of cellular life forms between archaea and bacteria (e.g. Ref. [7]). Rooting the 'tree of life' is an extremely difficult problem, and alternatives to the standard model were proposed including rooting within the bacteria [8] or between prokaryotes and eukaryotes [9]. Here, we stick to the standard model that we consider to be the most plausible, on the weight of the totality of evidence, especially, the fundamental differences between the DNA replication systems [10] and the membrane structure and biogenesis pathways (see later) of archaea and bacteria.

Under the standard model, the approach of choice for the reconstruction of the early evolution of a particular cellular system is to systematically compare its components in bacteria and archaea [11]. This approach yields informative results, especially in the case of the translation and the core transcription systems that are, to a large extent, conserved but also show substantial differences between archaea and bacteria [11]. This approach, however, fails to shed much light on the origin and early evolution of biological membranes because the chemical compositions and biogenesis pathways of archaeal and bacterial membranes are fundamentally different [4,5,12]. The glycerol moieties of the membrane phospholipids in all archaea and bacteria are of the opposite chiralities. With a few exceptions, the hydrophobic chains also differ, being based on fatty acids in bacteria and on isoprenoids in archaea; furthermore, in bacterial lipids the hydrophobic tails are usually linked to the glycerol moiety by ester bonds, whereas archaeal lipids contain ether bonds [4,5,12]. The difference extends beyond the chemical structures of the phospholipids to the evolutionary provenance of the enzymes involved in membrane biogenesis that are either non-homologous or distantly related but not orthologous in bacteria and archaea [4,5,12,13]. The dichotomy of the membranes and their biogenesis led to a proposal that the LUCA was not a typical, membrane-bounded cell but rather a consortium of replicating genetic elements that might have dwelled in networks of inorganic compartments ('bubbles') that exist at hydrothermal vents [13,14]. However, the nearly universal conservation of complex, membrane-embedded molecular machines, such as general protein secretory pathway elements [15] and the F- and A/V-type ATP synthases [16] in modern cellular life forms, strongly indicates that the LUCA did possess some kind of membranes although not necessarily a full-fledged cellular organization [13,17].

The chemical nature of the primeval membranes remains a matter of debate. It has been argued that fatty acids are the simplest amphiphilic molecules that could form abiogenically to be subsequently recruited by first organisms [3,18,19]. Alternatively, it was proposed that the first membranes consisted of polyprenyl phosphates,

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which are related to the membrane components of the modern archaea; it has been demonstrated that polyprenyl phosphates can form vesicles in the presence of sodium ions [20].

Another point of controversy is the topology of the first membranes. Some authors hypothesized that the very first life forms could recruit abiogenically formed amphiphilic molecules to form envelopes (perhaps resembling those of modern viruses that employ lipids of host cells), and that the subsequent evolution took place inside these vesicles [3,18–20]. Alternatively, 'life outside the vesicle' scenarios indicates that the first life forms could emerge at the surface of lipid vesicles (or sacks, or layers) that eventually would close up, yielding the first proto-cells [2,21,22].

A pure lipid bilayer, however, is not a practical solution for the membrane of a primordial cellular life form because it would effectively prevent exchange between the inside of a vesicle and the environment. Therefore, another unsolved question is how electrically charged compounds could be transferred across the primordial membranes. Because of the hydrophobic barrier, ions penetrate the lipid bilayer with the help of specialized membrane proteins such as channels or translocases. The membrane-embedded portions of these proteins consist, largely, of hydrophobic amino acids, and the proteins themselves are water insoluble. Hence, a chicken and egg paradox: a lipid membrane would be useless without membrane proteins but how could membrane proteins have evolved in the absence of functional membranes?

Here, we assess some of the current concepts and scenarios of the origin of membranes and the earliest stages of their evolution. The models and ideas in this field are diverse and often controversial. Therefore, in a brief review, it is impossible to present all these views in depth. We focus on the evolution of membrane (im)permeability to ions and biological polymers (proteins and nucleic acids) in conjunction with the evolution of integral membrane proteins. The evolutionary scenarios that we analyze are predicated on the standard model of cell evolution and on the related assumption that the emergence of RNA and proteins preceded the appearance of membrane-encased life forms [13]. Detailed reviews of alternative models are available including origin of life in a 'lipid world' [23] and membrane evolution in a protein-less, RNA-lipid world [24–26].

We discuss, in some detail, recent studies of the F-type and A/V-type membrane ATPases and explore their implications for the origin and the earliest stages of the evolution of membranes. It is argued that evolution of biological membranes was, actually, a process of co-evolution of (i) the lipid bilayer, (ii) membrane proteins and (iii) the membrane bioenergetics.

Origin and evolution of the F-type and A/V-type membrane ATPases

It is our belief that insights into the co-evolution of membranes and membrane proteins can be obtained from structural and phylogenetic analyses of the F- and A/V-type ATPases (for reviews, see Refs [27–32]). These are membrane enzymes that are ubiquitous in modern cellular life forms and so were conceivably present in the LUCA

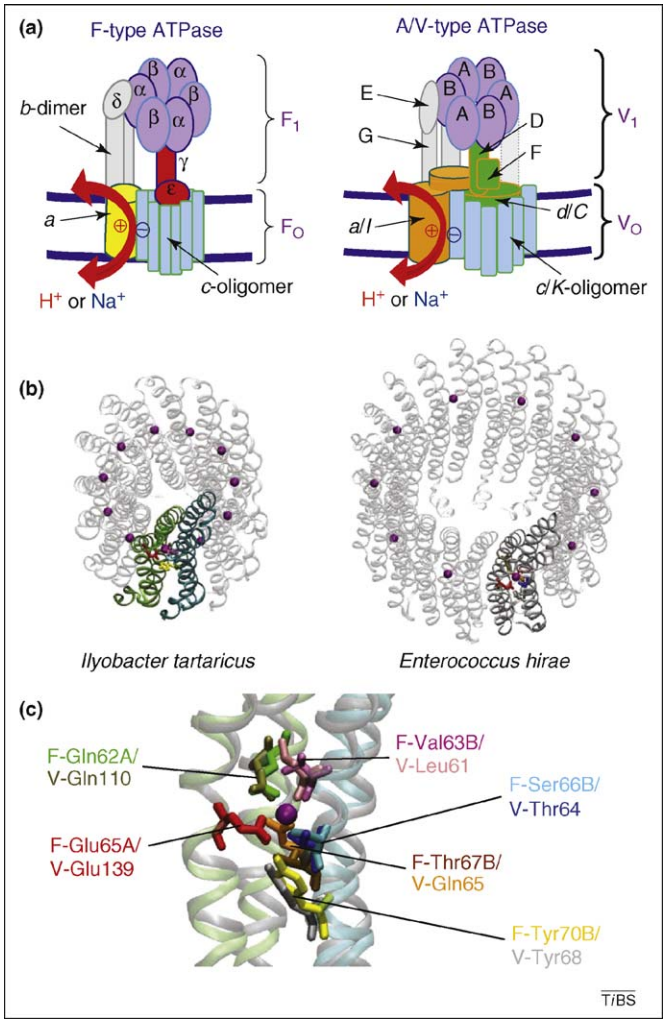

Figure 1. Structure and evolutionary relationships of F-type and A/V-type ATPases. **(a)** Modern F- and V-type ATPases; the minimal, prokaryotic sets of subunits are depicted; in the case of those V-type ATPase subunits that are differently denoted in prokaryotes and eukaryotes, double notation is used: eukaryotic/prokaryotic. The subunits that form the membrane-bound F_O and V_O parts are denoted by italic letters. The remaining subunits form the detachable, protruding F_1 and V_1 parts of the enzymes. Orthologous subunits are shown by the same colors and shapes, and non-homologous but functionally analogous subunits of the central stalk are shown by different colors and shapes. The *a* subunits that show structural similarity but might not be homologous [52] are shown by distinct but similar colors. For further details, see main text, Box 1 and Ref. [52]. **(b)** Membrane rotor subunits of the F-type and V-type, Na^+-translocating ATP synthases; left, undecamer of *c* subunits of the Na^+-translocating F-type ATP synthase of *Ilyobacter tartaricus* (PDB entry 1YCE [48]); right, decamer of K subunits of the Na^+-translocating V-type ATP synthase of *Enterococcus hirae* (PDB entry 2BL2 [49]); both rings are tilted to expose the internal pore; in *I. tartaricus,* Na^+ ions (purple) crosslink the neighbouring subunits, whereas in *E. hirae* the Na^+ ions are bound by four-helical bundles that evolved via a subunit duplication [50]. The subunits and residues that are superimposed on the panel below are highlighted, with subunit A of *I. tartaricus* shown in green and subunit B shown in blue. **(c)** Structural superposition of the Na^+-binding sites of the F-type and A/V-type ATPases; in both structures, the most important coordinating bonds to the Na^+ ion are provided by the principal ligand (Glu65A in *I. tartaricus* and Glu139 in *E. hirae*); other bonds come from a conserved glutamine (Gln32A in *I. tartaricus* and Gln110 in *E. hirae*), a hydroxy group of Ser66B in *I. tartaricus* and Thr64 in *E. hirae* and a backbone carbonyl (Val63B in *I. tartaricus* and Leu61 in *E. hirae*); in *E. hirae* a direct bond is provided by Gln65, whereas the corresponding residue in the *I. tartaricus* *c*-subunit, Thr67, apparently binds to the Na^+ ion through a water molecule (T. Meier, personal communication); the remaining, sixth bond is provided, most likely, by an unseen water molecule [50]; note the superposition, in addition to the Na^+ ligands, of non-ligating tyrosine residues (Tyr70B in *I. tartaricus* and Tyr68 in *E. hirae*) that are located beneath the Na^+ ion and stabilize the principal Glu ligand [40]. The figure was produced using the VMD software package [78].

[16]; apparently, these ATPases require ion-impermeable (ion-tight) membranes for their function. Together with two unrelated classes of proteins, the P-type ATPases and ABC transporters, the F- and A/V-type ATPases belong to a heterogeneous group of enzymes that use the energy of ATP hydrolysis to translocate inorganic cations across membranes [33]. The F- and A/V-type ATPases, however, are unique functionally in that they can efficiently operate as ATP synthases and mechanistically in that their reaction cycle is accompanied by the rotation of one enzyme part (rotor) relative to the other part (stator) (Figure 1; Box 1).

The F-type ATPases/ATP synthases are found in bacteria and eukaryotic mitochondria and chloroplasts [32], whereas the A-type ATPases/ATP synthases are found in archaea and some bacteria [34]; the latter, supposedly, got them via the lateral gene transfer from archaea [35]. The V-type ATPases are present in eukaryotic cells, specifically, in the membranes of the vacuoles; they use the energy of ATP hydrolysis to acidify cellular compartments [36–38]. The genes for homologous subunits of the A-type and V-type ATPases invariably cluster together in phylogenetic trees, to the exclusion of the F-type ATPases [16]. Thus, hereinafter, we refer to the A- and V-type ATPases, collectively, as V-type ATPases, for the sake of simplicity and after a recent suggestion [31].

Among the prokaryotic F-type and V-type ATPases both proton-translocating and Na^+-translocating forms were found (for reviews, see Refs [32,34,39]). The ion specificity is determined by the structure of the ion-binding sites of the membrane moieties [40] and is decisive for the nature of the bioenergetic cycle in any organism. Indeed, although the proton-motive force (PMF) and/or the sodium-motive force (SMF) can be generated by a plethora of primary sodium or proton pumps (Box 2), the F- and V-type ATPases are unique in their ability to utilize PMF and/or SMF to produce ATP [41–43]. In the absence of sodium, Na^+ ATPases can translocate protons [40,44], whereas H^+ ATPases are apparently incapable of translocating Na^+ [45]. This functional asymmetry is most likely to be due to the higher coordination number of Na^+, which usually requires six ligands [46], whereas a single ionisable group can be, in principle, sufficient for proton translocation [42,43]. Comparative analysis of the *c* subunits of Na^+-translocating and H^+-translocating ATPases identified several residues that are involved in Na^+ binding and are the principal determinants of the coupling ion specificity [32,34,47]. However, the exact modes of Na^+ binding in F-type and V-type ATPases remained obscure until the structures of the membrane-spanning, rotating *c*-oligomers of the Na^+-translocating ATP synthases of the F- and V-type were resolved (Figure 1b,c) [48,49]. Strikingly, superposition of these structures reveals nearly identical sets of amino acids involved in Na^+ binding (Figure 1c). Combined with the topology of the phylogenetic tree of F- and V-type ATPases [50], this apparent identity of the Na^+-binding sites, surprisingly, indicates that the last common ancestor of the extant F- and V-type ATPase probably possessed a Na^+-binding site. Indeed, Na^+-dependent ATPases are scattered among proton-dependent ATPases in both the F and the V branches of the phylogenetic tree [50]. Barring the extremely unlikely convergent emergence of the same set of Na^+ ligands in several lineages, these findings indicate that utilization of sodium gradient for ATP synthesis is the ancestral modality of membrane bioenergetics.

Comparisons of the F- and V-type ATPases shows that they are built of both homologous and unrelated subunits [51,52] (Figure 1a; Box 1). The subunits of the catalytic hexamer and the membrane *c*-ring are highly conserved [16]. The subunits that are thought to form the peripheral stalk(s) show structural and and also functional similarity [51], although it remains unclear whether or not they are homologous [52]. By contrast, the subunits of the rotating central stalks, which connect the catalytic hexamers with the *c*-ring (shown by dissimilar colors in Figure 1a) are not homologous as indicated by the presence of dissimilar structural folds [52].

Building on this conservation pattern, we proposed a hypothetical scenario whereby the F-type and V-type ATPases evolved from an ATP-dependent protein translocase in which the translocated protein itself occupied the place of the central stalk [52]. The catalytic hexamers of F-

Box 1. F-type and V-type ATPases

The catalytic headpieces of F-type and V-type ATPases show high similarity. In F-type ATPases, the protruding hexamers (Figure 1a) are formed from alternating three α and three β subunits (B and A subunits in V-type ATPases, respectively), with each of the β (A) subunits carrying an ATP/ADP-binding catalytic site [27–31]. The ion-translocating, membrane-spanning F_O sector of simple, bacterial F-type ATPases is a complex of the integral membrane *a* subunit, two *b* subunits and 10–15 small *c* subunits [32,79,80]. The membrane part is connected to the headpiece by two distinct stalks; the peripheral stalk consists of the protruding parts of the membrane-anchored *b* subunits that are connected to the $\alpha_3\beta_3$ hexamer via the δ subunit. The central stalk consists of the elongated γ subunit that connects the two parts of the enzyme and the globular ε subunit that performs regulatory functions [28,30,81]. The V-type ATPases, although sharing a common overall scaffold with F-type ATPases, differ from them in several structural and functional features [29,31,36,52,82]. In particular, the D and F subunits that make up the central stalk are unrelated to the subunits γ and ε of the F-type ATPases. The central stalk of the V-type ATPases contains an additional *d*/C subunit atop of the *c*-oligomer, which serves as a socket for the D and F subunits [83] and has no counterpart in F-type ATPases (Figure 1a). The number of *c*/K subunits varies, at least, between six (eukaryotic V-type ATPases [38]) and 13 (some archaeal V-type ATPases [34]). The composition of peripheral stalk(s) and their number in V-type ATPases remains ambiguous, with values of up to three being reported [82,84].

The ion current through F_O/V_O is coupled with the rotation of the central stalk together with the ring of the *c*-subunits (rotor) along the interface with the *a* subunit that is rigidly bound, via the peripheral stalk(s), to the catalytic hexamer (stator). The ion transfer results from the sequential interaction of a single, electrically charged group of the stator (Arg^+) with multiple, oppositely charged groups of the rotor (Glu^- or Asp^-) that are capable of binding to the translocated ion. The direction of rotation is determined by the sign of the ion-motive force (for the principles of the mechanism, see Ref. [41]; for recent reviews, see Refs [32,85,86]). The catalysis of ATP synthesis or hydrolysis is mediated by the interaction of the rotating central shaft with the catalytic subunits of the hexamer; three substrate molecules are processed per full rotation in conjunction with both synthesis and hydrolysis of ATP [27,30,87]. The number of ions being translocated across the membrane per full rotation is believed to be equal to the number of the ion-binding sites in the *c*-oligomer (see above).

type and V-type ATPases are homologous to hexameric helicases, specifically, the bacterial RNA helicase Rho, a transcription termination factor [53]. This relationship has previously led to the hypothesis that the ancestral membrane ATPase evolved as a combination of a hexameric helicase and a membrane ion channel [28]. Actually, the membrane parts of the F-type and V-type ATPases (F_O and V_O, respectively, in Figure 1) function not as channels but as membrane ion translocases; their ion-binding sites, which are located at the interface between the *a* subunit and the *c*-ring, are not accessible from both sides of the membrane simultaneously [32]. Furthermore, the structures have little in common with typical membrane channels. As shown in Figure 1, the *c*-oligomers are lipid-plumbed membrane pores with internal diameters of ~3 nm and ~2 nm for V_O and F_O, respectively [48,49]. Conceivably, such a pore (without the lipid plumbing) would be large enough to enable passive import and export of biopolymers in primordial cells. When combined with an ATP-driven helicase, this type of membrane pore could yield an active, energy-dependent polynucleotide translocase that subsequently could give rise to a protein translocase [52].

Emergence of integral membrane proteins

In the preceding section, we proposed that the common ancestor of the *c*-oligomers in the F- and V ATPases could initially function as a membrane pore. Such pores that could be required to enable passive exchange of ions, small molecules and even polymers between protocells and their environment [46,54] also might represent a transition state in the evolution of integral membrane proteins. Integral membrane proteins contain long stretches of hydrophobic amino acid residues. By contrast, in water-soluble, small globular proteins the distribution of polar and non-polar amino acids in the polypeptide chain is quasi-random [55]. Assuming that the quasi-random distribution pattern is ancestral, a gradual transition from soluble proteins to membrane proteins with long hydrophobic stretches must be envisaged. Furthermore, modern membrane proteins are cotranslationally inserted into the membrane by the

Box 2. Basics of membrane bioenergetics

The textbook proton cycle that was defined by Mitchell [88] and operates in mitochondria, chloroplasts and most prokaryotes includes the generation of the proton-motive force (*PMF*) by membrane H^+ pumps and its utilization for ATP synthesis, solute transport, motility and other processes, with *PMF* being defined as:

$$PMF = \Delta\psi - 2.3RT/F(pH_{in} - pH_{out}) = \Delta\psi - 2.3RT/F \cdot \Delta pH \quad \text{[Equation I]}$$

where $\Delta\psi$ is the transmembrane difference of the electric potential, and the membrane topology corresponds to one of a prokaryotic cell where H^+ ions are pumped outwards [41–43].

However, certain bacteria and archaea (thermophilic anaerobes, marine bacteria and some bacterial pathogens) use Na^+ as a coupling ion in addition to H^+ or even instead of it [39,41,44,70,74]. Similarly to the H^+ cycle, the Na^+ cycle includes Na^+ pumps that produce sodium motive force (*SMF*), Na^+-translocating ATP synthase, Na^+-dependent membrane transporters and a Na^+-dependent flagellar motor.

Figure I schematically shows typical pumps that translocate Na^+ (left panel) and H^+ ions (right panel), respectively. The 'sodium world' as defined by Skulachev [41] utilizes Na^+-specific versions of proton-translocating enzymes, such as F- and A/V-type ATPases or membrane pyrophosphatases, in addition to three classes of Na^+-translocating pumps that are not present in the 'proton world'. These include Na^+-transporting oxaloacetate decarboxylase and similar biotin-dependent membrane-bound enzymes [44,89], Na^+-translocating N^5-methyltetrahydromethanopterin:coenzyme M methyltransferase [90] and two closely related enzymes, Na^+-translocating NADH:ubiquinone oxidoreductase [91] and Na^+-translocating ferredoxin:NAD oxidoreductase [92].

In turn, proton-tight membranes contain proton pumps that do not have counterparts in the sodium world. These is a well-studied variety of redox-driven H^+ translocases that are embedded in the membrane and can chemically couple proton pumping steps with thermodynamically favorable redox reactions [41–43]. Some of these proton pumps are depicted in Figure I. A full-fledged electron transfer chain, for example that of α-proteobacteria, can cover the redox span of ~1.2 eV from organic substrates to oxygen, with all this redox energy available for the transmembrane proton transfer [41–43].

As shown in Figure I, the sodium-transporting redox pumps can use only a modest difference in redox potentials of ~0.4 V from NADH to quinones. Why, then, do some extremophilic bacteria rely on sodium bioenergetics despite its energetic inefficiency as compared with the proton-dependent bioenergetics? Whether an organism relies on Na^+ or H^+ as the coupling ion might depend on a trade-off between the amount of potentially available free energy and the intensity of ion leakage across the coupling membrane. The leakage of biological membranes to protons is 10^5–10^7 times higher than that to sodium ions and increases with temperature [73]. Under mesophilic conditions, bacteria routinely select the more efficient proton energetics and cope with proton leaks. However, in some obligate anaerobes, thermophils and alkaliphils, of which the energy budget cannot cover the losses caused by proton leaks, Na^+ energetic, apparently, became the favored one [70].

A further complication of proton bioenergetics is that proton concentrations outside and inside of a prokaryotic cell are often similar, so that the *PMF* (calculated by applying Equation I) might be insufficient to drive ATP synthesis. Especially dramatic is the case of alkaliphilic bacteria that thrive at high pH; here, the calculated *PMF* can drop almost to zero, indicating that so-called local coupling mechanisms could operate [93]. One group of such mechanisms implies that the effective concentration of protons on the external surface of proton-expelling cells might be higher than that in the bulk [94,95]. This mechanism might rely on the electrostatic barrier that separates the negatively charged membrane surface from the bulk water phase; the barrier is high enough to keep the pH value at the external surface of metabolizing prokaryotes neutral even when the surrounding medium is strongly alkaline [96]. Therefore, in the general case, the surface-to-surface pH difference upon estimating the *PMF* should be considered:

$$PMF = \Delta\psi - 2.3RT/F \cdot \Delta pH_{surface} \quad \text{[Equation II]}$$

Structural analysis of prokaryotic proton pumps has revealed that their periplasmic surfaces are rich in aspartic acid and glutamic acid residues that are likely to facilitate proton transfer along the membrane–water interface between the respiratory H^+ pumps and ATP synthases [96]. A direct intramembrane transfer of H^+ from the respiratory complex to the F_O portion of the ATP synthase occurring via protein–protein interaction has also been postulated [93] and, eventually, experimentally validated [97]. The two mechanisms of local coupling are complementary. Underneath the surface layer of negatively charged acidic side chains, the prokaryotic proton pumps possess a buried plexus of arginines and lysines that could operate as proton buffers or sponges [96]. Protons can be transferred between the sponges of two neighboring enzymes either directly inside the membrane or via surface acidic groups. In addition, the buried plexus of arginine and lysine residues, owing to its net positive charge [98], should prevent proton leakage through membrane proteins and along the protein–lipid interfaces.

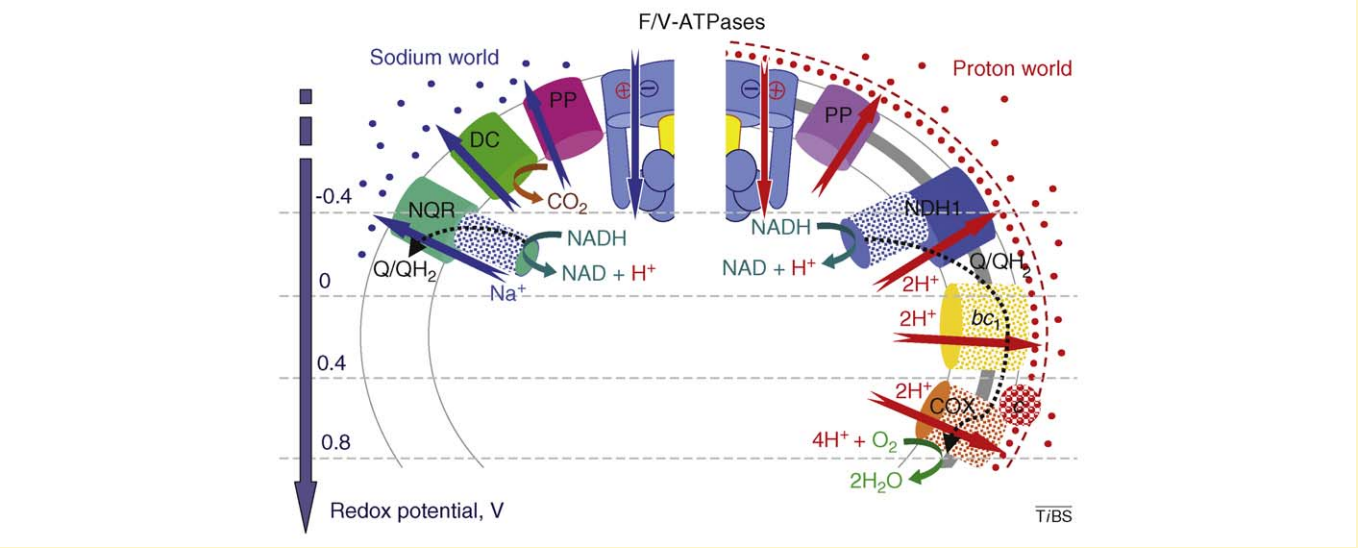

Figure I. Proton-dependent bioenergetics compared with sodium-dependent bioenergetics. Two halves of bacterial cells are shown with elements of sodium bioenergetics depicted to the left and elements of proton bioenergetics shown to the right. Blue spheres denote sodium ions, and red spheres denote protons. Blue arrows indicate sodium transfer steps, red arrows indicate proton transfer steps and dashed black arrows indicate electron transfer steps. The numbers of translocated H^+ or Na^+ ions are given per one electron. Patterned shapes denote redox modules. A dashed red line outside the proton-tight membrane indicates the interfacial electrostatic barrier for protons that confines them to the membrane surface; the checked strip indicates the higher atom density in the midplain of a proton-tight membrane. The scale of redox potentials (left) emphasizes that the Na^+ pump NQR uses a redox span of only ~0.4 V, whereas the full-fledged chain of redox-driven proton pumps can use the whole biochemically relevant redox span of 1.2 V. Abbreviations: PP, membrane pyrophosphatase; DC, membrane Na^+-transporting decarboxylase; NQR, Na^+-translocating NADH:ubiquinone oxidoreductase; NDH1, NADH:ubiquinone oxidoreductase of type 1; bc_1, cytochrome bc_1 complex; c, cytochrome *c*; COX, cytochrome oxidase; Q, ubiquinone; QH_2, ubiquinol.

translocon machinery – a membrane-embedded protein complex [56] that could not exist before membrane proteins evolved. In the absence of the translocon, a hydrophobic protein, even if occasionally synthesized, would remain stuck in the ribosome. Therefore, any scenario of membrane-protein evolution must also address the evolution of mechanisms of protein insertion into the membrane.

One tentative mechanism of translocon-independent protein insertion might be an 'inside-out' transition of a water-soluble protein after its adsorption on a membrane. This hypothesis exploits the idea that membrane proteins are 'inside-out' versions of globular proteins, as discussed for the specific case of bacteriorhodopsin [57]. Indeed, the cores of globular proteins are formed by packed hydrophobic residues; if a globule is large enough, it can even accommodate a long stretch(es) of hydrophobic amino acid residues in its core [55]. Thus, the first membrane-anchored proteins could evolve via attachment of water-soluble proteins to membranes, their 'inside-out' turning, and insertion of their internal hydrophobic regions into the membrane [2]. The proposed ancient mechanism of spontaneous protein membrane anchoring via the 'inside-out' transition is employed by some extant proteins, for instance by the tail domain of vinculin [58].

The anchoring mechanism, however, hardly can explain the emergence of integral membrane proteins that are almost completely embedded in the lipid bilayer. A global analysis of such proteins led to the conclusion that their evolution proceeded from nonspecific oligomeric channels, which were built of small proteins with only a few transmembrane segments, towards larger, specific membrane translocators that emerged by gene duplication [59]. However plausible with regard to the subsequent evolution of membrane proteins, this model does not explain their ultimate origin. The commonly discussed scenario whereby a stand-alone hydrophobic α-helix yields increasingly complex membrane proteins via multiple duplications [60] seems unlikely because a solo, water-insoluble α-helix could hardly leave a ribosome in the absence of a translocon complex. Physically more plausible are models that start from amphiphilic α-helices. One such scenario was recently developed and tested by molecular dynamics simulations [61]. It was shown that spontaneous, unassisted insertion of amphiphilic α-helices into a lipid bilayer is physically sustainable provided that the helices dimerize on the membrane surface and then oligomerize in the membrane, making pores. The formation of a water-filled pore helps to stabilize the polar residues of the α-helices,

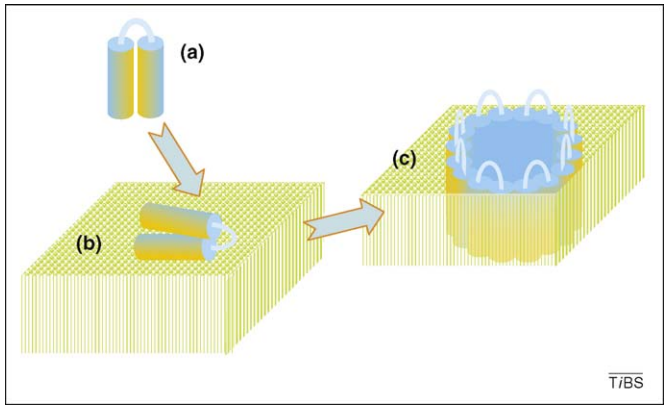

Figure 2. Insertion of a folded water soluble, α-helical hairpin into the membrane via an 'inside-out' transition. Blue denotes hydrophilic surfaces of α-helices; yellow denotes their hydrophobic surfaces. **(a)** A soluble α-helical hairpin. **(b)** The α-helical hairpin spreads on the membrane surface by interacting with the lipid bilayer. **(c)** The proteins turn 'inside-out', aggregate and insert into the membrane forming a pore.

whereas the non-polar residues interact with the lipid phase [61]. A weakness of this model is that a single α-helix does not comprise a thermodynamically stable fold, so the starting point of the scenario remains dubious.

One of the simplest protein folds is an α-helical hairpin (long α-hairpin according to the Structural Classification of Proteins [SCOP] [62]). The hairpins are stabilized via hydrophobic interaction of the two α-helices (Figure 2a). However, this stabilization is unlikely to be particularly strong, so upon interaction with the membrane a hairpin might spread on its surface (Figure 2b) and then reassemble within the membrane such that the non-polar side chains would interact with the hydrophobic lipid phase (Figure 2c). The hairpins, then, would aggregate yielding water-filled pores inside which the polar surfaces of α-helices would be stabilized (Figure 2c). This arrangement seems to be partially retained by the *c*-ring of the F-type ATPase that consists of α-helical hairpins (Figure 1b) and is plumbed by lipid only from the periplasmic side of the membrane; from the cytoplasmic site, the cavity is lined by polar residues and is apparently filled with water and segment(s) of the γ-subunit [63]. This mechanism of spontaneous protein insertion into the membrane, which does not require translocon machinery, is used by diverse bacterial toxins and related proteins. These proteins are monomeric in their water-soluble state but oligomerize in the membrane where they form pores [64,65].

Starting from pores that were built of amphiphilic α-helical proteins, the integral membrane proteins could then gradually evolve via multiple gene duplications and replacements of polar amino acids by non-polar ones, ultimately yielding tight, multi-helix, hydrophobic bundles such as the widespread 12-helix fold of membrane transporters [33]. Concomitantly, some membrane proteins would join to form the first translocons, enabling controlled insertion of these bundles into the membrane.

Co-evolution of membranes and membrane bioenergetics

Szathmáry [54] recently put forward a scenario of co-evolution of membranes and metabolism, in which evolution proceeded through progressive sequestration of protocells from the environment. Under this model, the gradual build-up of enzymatic pathways inside the proto-

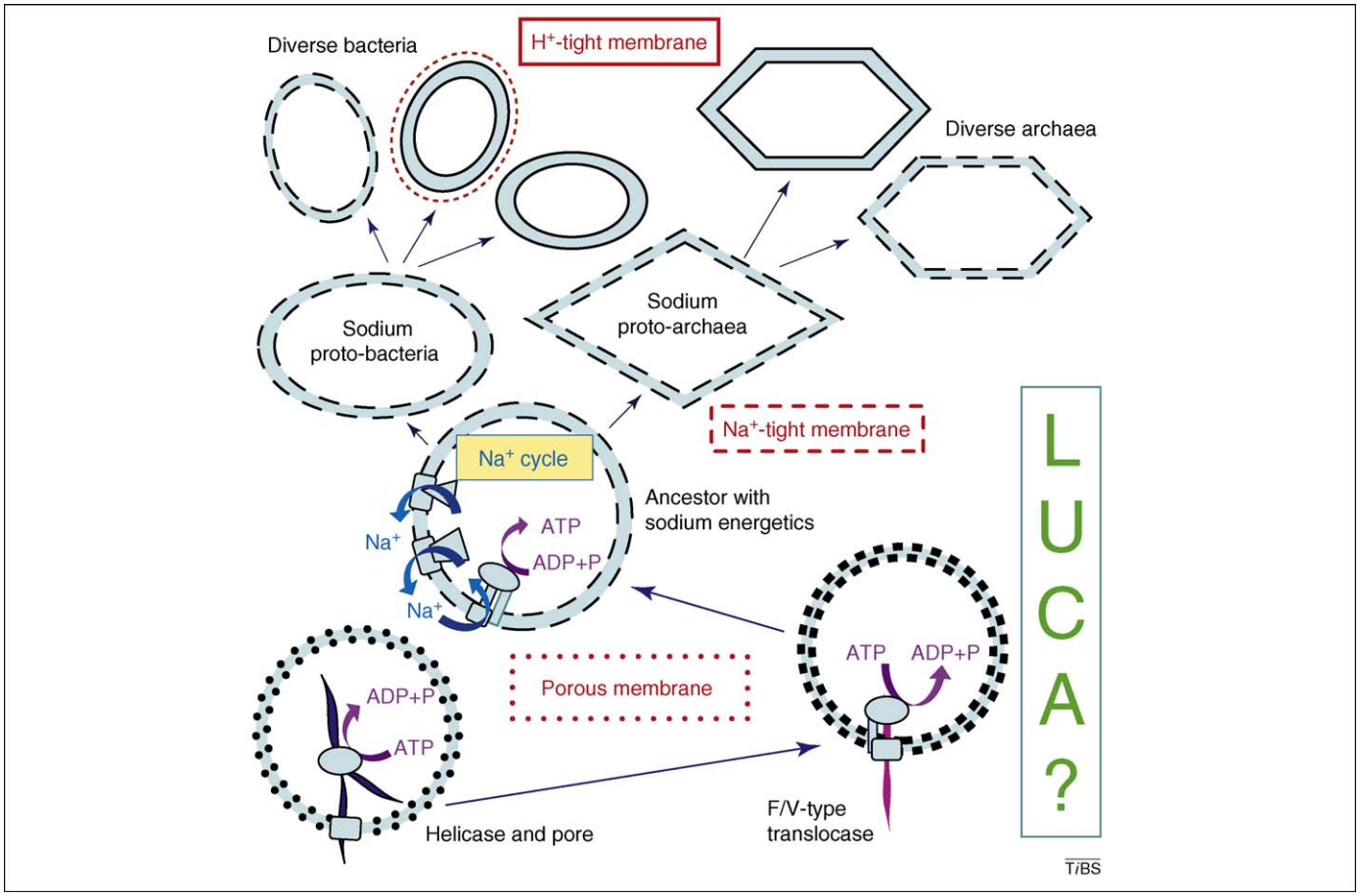

Figure 3. The proposed scenario for the evolution of membranes and membrane enzymes from separate RNA helicases and primitive membrane pores via membrane RNA and protein translocases to the F- and V-type ATPases. Reproduced, with permission, from Ref. [50]. The scheme shows the proposed transition from primitive, porous membranes that were leaky both to Na^+ and H^+ (dotted lines) via membranes that were Na^+ tight but H^+ leaky (dashed lines) to the modern-type membranes that are impermeable to both H^+ and Na^+ (solid lines). The brown dashed contour around one of the modern bacteria emphasizes that the membrane pores in the outer membranes of Gram-negative bacteria, although formed not by α-helices but by β-barrels, can be considered as a recapitulation of the primordial membrane architecture. The common ancestor of the F- and V-type ATPases possessed a Na^+-binding site, the structure of which can be inferred from the superposition shown in Figure 1c. The question mark indicates the ambiguity of the placement of the LUCA on the scheme. Regardless of whether LUCA was a modern-type cell or a consortium of replicating, membrane-bounded entities, it either had porous membranes so that the common ancestor of the F- and V-type ATPases operated as a polymer translocase, with Na^+ ions performing a structural role, or had membranes that were tight to sodium but permeable to protons; in this case, the LUCA could possess sodium-dependent energetics (for details, see main text and Ref. [50]).

cell should be accompanied by a decrease in membrane permeability. Membrane-coupled energy-conversion reactions that collectively comprise the membrane bioenergetics (Box 2) are an essential part of cell metabolism; unlike other metabolic reactions, these processes are impossible without ion-tight membranes. Here, within the general framework of the Szathmáry's model and building upon our previous analysis of the evolution of membrane ATPases [50,52], we propose a scenario of co-evolution of membranes and membrane bioenergetics in which the gradual decrease in membrane permeability enables the emergence of new energy-converting enzymes (Figure 3).

As discussed earlier, ATP-driven biopolymer translocases could evolve from a combination of a helicase and a membrane pore (Figure 3). The primordial translocase could employ sodium cations to crosslink and stabilize the hairpin subunits of the pore (as in *Ilyobacter tartaricus*; Figure 1b,c), preventing its destruction by the translocated polymer. Thus, even at the stage of the RNA or protein translocase, when the porous, primordial membranes would be leaky to both Na^+ and H^+, there could have been a mechanistic demand for Na^+ binding and, accordingly, selection for the corresponding set of amino acid ligands. This scenario seems to be supported by experiments demonstrating a dramatic destabilization of *c*-oligomers of Na^+-translocating F-type ATPases from *I. tartaricus* and *Propionigenium modestum* in the absence of Na^+ [66].

Because the concentration of negatively charged proteins and polynucleotides inside a (proto)cell should be higher than it is outside, even the porous primeval membranes, as argued by Fraústo da Silva and Williams [46], would maintain transmembrane electric potential difference owing to the Donnan effect (up to 50 mV, negative inside [1]). This potential could shape the 'positive-inside' mechanism of protein insertion into the membrane [67] and also promote the emergence of voltage sensitivity in membrane proteins.

The next stage of evolution is envisaged as selection for tighter membranes that would maintain the ionic homeostasis of the evolving cells, a task of ever-increasing importance considering the growing ocean salinity. The primordial ocean emerged from condensation of water vapor [68] and initially should have a low sodium level. However, the sodium concentration in the ocean was high already ~3.5 Gyr ago, as judged from the chemical composition of geologically trapped sea water [68]. The sodium concentration inside all known cells is, on the contrary, low, possibly because modern cells, in line with the trend of chemistry conservation [69], strive to maintain the internal sodium level as low as it was at the emergence of life. The need to keep the internal sodium concentration low should strongly favor evolution of sodium-tight membranes and membrane pumps capable of expunging Na^+ out of the cell; selection for mechanisms to keep sodium out could be the driving force behind the aforementioned transition from a protein translocase to an ion-translocating membrane ATPase. The key to the transition could be the decrease in the conductivity of the membrane pore. Amino acid replacements leading to increased hydrophobicity on the inside of the pore might cause translocated proteins to get stuck within the translocase. Then, the torque from ATP hydrolysis, transmitted by the stuck substrate polypeptide, would cause rotation of the *c*-ring relative to the ex-centric membrane stator. This rotation could eventually be coupled with transmembrane ion translocation along the contact interface via membrane-embedded, charged amino acid side chains that kept the membrane subunits together (Box 1). The transition to the ion translocase could be completed by the ultimate recruitment of unrelated and even structurally dissimilar proteins as central stalks in ancestral archaea and bacteria, as a result of the inclusion of the genes encoding the respective proteins into the operons of the F- and V-type ATPases [52].

Unlike the other Na^+ pumps (Box 2), the common ancestor of the F- and V-type ATPases, owing to its rotating scaffold, would be potentially able to translocate Na^+ ions in both directions. Upon further increase in the ocean salinity, reversal of the rotation would result in the Na^+-driven synthesis of ATP by this primordial rotary machine. Already in the Archean era, the concentration of Na^+ in the ocean water was approximately 1 M [68], as compared with ~0.01 M inside the cell; that is, the Na^+ gradient, together with the transmembrane voltage, could be powerful enough to cause the switch of the rotary machine from the hydrolysis to the synthesis of ATP. The advent of the ion-gradient-driven ATP synthesis can be considered to be the birth of membrane bioenergetics: together with the ancient outward Na^+ pumps, the ancestral F- and V-type ATP synthases would complete the first, sodium-dependent bioenergetic cycle in a cell membrane [50,70] (Figure 3).

The emergence of the energy-converting, sodium-tight membranes should put constraints on the ion tightness of membrane-embedded translocation systems. Conceivably, simple pores that might have been common at the early stages of membrane evolution failed to pass this evolutionary bottleneck and were supplanted by gated ion channels and ion-tight machines of the general protein secretion pathway. The survivors of the primordial machinery seem to be, in addition to the F- and V-type ATPases with their pores plumbed by lipid, the ATP-driven type III protein-secretion systems and the closely related flagellin-secretion systems of bacterial flagella [51,52]. Both the catalytic subunits and the subunits of the peripheral stalk of the F-type and V-type ATPases are homologous to the corresponding subunits in these protein-secretion systems [51]. As discussed previously [52], although the extant type III secretion systems are limited to bacteria in their spread, they might be direct descendants of the primordial protein translocases that also gave rise to the F- and V-type ATPases.

The final evolutionary step in the present scenario is the transition to proton-tight, elaborate membranes [50]. The proton-based bioenergetics is more lucrative than the sodium-based bioenergetics because proton transfer can be chemically coupled to redox reactions, especially those of oxygen and diverse quinones, thus enabling the advent of efficient redox-driven generators of PMF (Box 2). However, because of the much higher conductivity of lipid bilayers to protons compared with sodium ions (Box 2), creation of a non-leaky membrane capable of maintaining a PMF sufficient to drive ATP synthesis is a harder task than

creation of a sodium-tight membrane; representatives of the three domains of life employed distinct solutions to this problem. Protons, unlike sodium ions, easily enter water clusters that are nested between lipid hydrocarbon chains, so that the rate-limiting step of transmembrane proton transfer is proton 'hopping' from one water cluster to another when these clusters collide [3,71,72]. Thus, proton leakage can be suppressed by decreasing the probability of such hopping by (i) restricting the lipid mobility and/or (ii) increasing the hydrocarbon density in the midplane of the bilayer [71]. Different organisms utilize radically different means to achieve proton tightness of their membranes. For instance, in some archaea, phytanyl chains of two diether lipids are fused to form single C40 membrane-spanning lipid molecules. In many bacteria, membrane fatty acids have branched termini or terminate with cyclohexane or cycloheptane, resulting in additional molecular crowding at the midplane of the bilayer. In addition, different organisms pack different hydrocarbons in the midplane of their H^+-tight membranes [50,71,73]. The diversity of the mechanisms that ensure proton tightness of membranes is compatible with the aforementioned hypothesis of independent transitions from sodium to proton bioenergetics in multiple lineages. In addition, the energy-converting enzymes had to develop structural traits that enabled the use of PMF for ATP synthesis by facilitating proton transfer between the generators of PMF and the ATP synthase (Box 2). Thus, the transition from the sodium-dependent to the proton-dependent energetics would require substantial 'upgrades' to both the lipid bilayer and the energy-converting membrane enzymes.

After such 'upgrades' were completed, the more energetically efficient and versatile proton-based bioenergetics could spread over [50,70]. Once the membranes could maintain PMF and the first proton pumps emerged, the sodium-binding sites of the F- and V-type ATPases became obsolete and, apparently, deteriorated independently on multiple occasions [50]. The ancestral, less effective sodium bioenergetics persisted in anaerobic thermophiles and alkaliphiles that cannot benefit from proton energetics and in some marine and parasitic bacteria and archaea that exist in high-sodium environments [70] (Box 2). However, apparent traces of the primordial Na^+-based bioenergetics are still seen in the universal distribution of Na^+ gradients and Na^+-dependent systems of solute transport in almost all known cell types. In particular, plasma membranes of animal cells are 'sodium membranes' [41] and, with some exceptions [37], cannot maintain a proton gradient.

Owing to its nearly ubiquitous presence, proton-based energetics is usually viewed as the primary form of biological energy transduction [3,40,44]. By contrast, the ability of some prokaryotes to use the sodium gradient for ATP synthesis is usually construed as a later adaptation to survival in extreme environments [40,73]. The scenario of the origin of proton-driven ATP synthases from a helicase and a simple membrane pore, via the sequential intermediate stages of RNA and then protein translocases and sodium-driven ATPase, respectively (Figure 3), implies (perhaps, counter-intuitively but in accordance with some previous ideas [39,41,74]) that membrane bioenergetics, especially in its modern version that is centered around transmembrane proton gradients, is a relatively late innovation in the evolution of life.

Conclusions and outlook

The present scenario describes co-evolution of (i) lipid bilayers (from leaky to proton-tight), (ii) membrane proteins (from amphiphilic, pore-forming ones to highly hydrophobic integral membrane proteins) and (iii) membrane bioenergetics (from the relatively simple, sodium-dependent form to the sophisticated proton bioenergetics). The scenario favors the primitive 'porous' membranes as an intermediate step between membrane-less pre-cellular life forms and modern cells that are bounded by ion-tight membranes. Such porous membranes could house various protein and polynucleotide translocases, favoring horizontal gene transfer, gene mixing and sharing of enzymes and their products between the first life forms, which are features that are considered to be essential for the early stages of the evolution of life [13,75]. An attractive, albeit speculative, possibility is that the primitive membranes encased replicating moieties in which the ancient translocases mediated RNA transfer similarly to the way such molecular devices function in some extant viruses [76]. Recent modeling studies indicate that a consortium of such replicating moieties could remain viable if connected via a metabolic network, with different replicators contributing different metabolites to a common pool [77]. Sharing a common pool of metabolites and genes, each interacting consortium, for instance, inhabitants of one inorganic 'bubble' at a hydrothermal vent [13,14], would comprise a distinct evolutionary unit. The precipitation of sulfides at hydrothermal vents should lead to a continuous formation of new, empty compartments so that more competitive consortiums could overcome others by 'moving in' first. Such a scheme, with an extensive (gene) exchange via membrane pores between the members of one consortium but not between dwellers of different, physically discrete inorganic compartments, solves a major conundrum between the notion of extensive gene mixing that is considered to be a major feature of early evolution [75] and the requirement of separately evolving units as the agency of Darwinian selection.

Comparative analysis can be powerful for the reconstruction of events that occurred during evolution after the LUCA, but the potential for the reconstruction of the earlier stages of life evolution is limited. In particular, it remains uncertain whether the LUCA was a loose consortium of replicating entities [13,75] or a more complex organism resembling modern prokaryotes [6,8]. Our analysis, however, strongly suggests that the LUCA lacked proton-tight membranes and proton-based energetics but, rather, had porous or only sodium-tight membranes [50,70] (Figure 3). Much uncertainty remains also regarding the chemical nature of membranes in the LUCA and earlier. The scenario discussed here predicts specific properties of primeval membranes with respect to their tightness to protons and sodium ions. Therefore, different stages of membrane evolution delineated here are, in principle, amenable to experimental tests and reconstruction, for

example by studying ion conductivity of bilayers formed of primitive lipids [3,18–20].

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