

## **Evolutionary origins of membrane proteins**

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## **Abstract**

Although the genes that encode membrane proteins make about 30% of the sequenced genomes, the evolution of membrane proteins and their origins are still poorly understood. Here we address this topic by taking a closer look at those membrane proteins the ancestors of which were present in the Last Universal Common Ancestor, and in particular, the F/V-type rotating ATPases. Reconstruction of their evolutionary history provides hints for understanding not only the origin of membrane proteins, but also of membranes themselves. We argue that the evolution of biological membranes could occur as a process of co-evolution of lipid bilayers and membrane proteins, where the increase in the ion-tightness of the membrane bilayer may have been accompanied by a transition from amphiphilic, pore-forming membrane proteins to highly hydrophobic integral membrane complexes.

## Introduction

The origins of membrane proteins are inextricably coupled with the origin of lipid membranes. Indeed, membrane proteins, which contain hydrophobic stretches and are generally insoluble in water, could not have evolved in the absence of functional membranes, while purely lipid membranes would be impenetrable and hence useless without membrane proteins. The origins of biological membranes - as complex cellular devices that control the energetics of the cell and its interactions with the surrounding world (Gennis 1989) - remain obscure (Deamer 1997; Pereto et al. 2004).

The traditional approach that is employed to reconstruct the early evolution of a particular cellular system is to compare the complements of its components in bacteria and archaea, the two domains of prokaryotic life (Koonin 2003). The conservation of a set of essential genes between archaea and bacteria leaves no reasonable doubt in the existence of some version of Last Universal Common Ancestor (LUCA) of all cellular organisms (Koonin 2003; Glansdorff et al. 2008; Mushegian 2008). The comparison of particular cellular systems in bacteria and archaea yielded informative results, especially, in the case of the translation and the core transcription systems (Harris et al. 2003; Koonin 2003). However, comparison of bacteria and archaea does not shed light on the origin of biological membranes because they fundamentally differ in these two domains of prokaryotic life (Wächtershäuser 2003; Boucher et al. 2004; Pereto et al. 2004; Koonin and Martin 2005; Koga and Morii 2007; Thomas and Rana 2007). The dichotomy of the membranes led to the proposal that the Last Universal Common Ancestor (LUCA) lacked a membrane organization (Martin and Russell 2003; Koonin and Martin 2005). However, the nearly universal conservation of the key subunits of complex membrane-anchored molecular machines, such as general protein secretory pathway (Sec) system (Cao and Saier 2003) and the F/V-type ATP synthase (Gogarten et al. 1989; Nelson 1989), indicates that LUCA did possess some kind of membrane (Koonin and Martin 2005; Jekely 2006).

The universal conservation of the key subunits of the F/V-type ATPases/synthases (hereafter F/V-ATPases) - elaborate, rotating molecular machines that couple transmembrane ion transfer with the synthesis or hydrolysis of ATP (see (Boyer 1997; Walker 1998; Perzov et al. 2001; Müller and Gruber 2003; Weber and Senior 2003; Yokoyama and Imamura 2005; Beyenbach and Wieczorek 2006; Dimroth et al. 2006; Forgac 2007; Mulkidjanian et al. 2009) for reviews) - is particularly challenging, since this enzyme complex is apparently built of several modules (Walker 1998) and therefore is anything but primitive. Therefore F/V-type ATPases, together with the related bacterial

flagella, make one of the main exhibits of today's proponents of "Intelligent Design". The F-type and V-type ATPases are also remarkable as being one of the few cases, outside the translation and core transcription systems, where the classic, "Woesian" phylogeny (Woese 1987) is clearly seen, with the primary split separating bacteria from the archaeo-eukaryotic branch that splits next (Gogarten et al. 1989; Nelson 1989). F/V-type ATPases are more "demanding" than the Sec system – they require perfect, ion-tight membranes for proper functioning. Hence understanding the evolution of the F/V-type ATPases might share light on the evolution not only of membrane proteins but also of membranes proper.

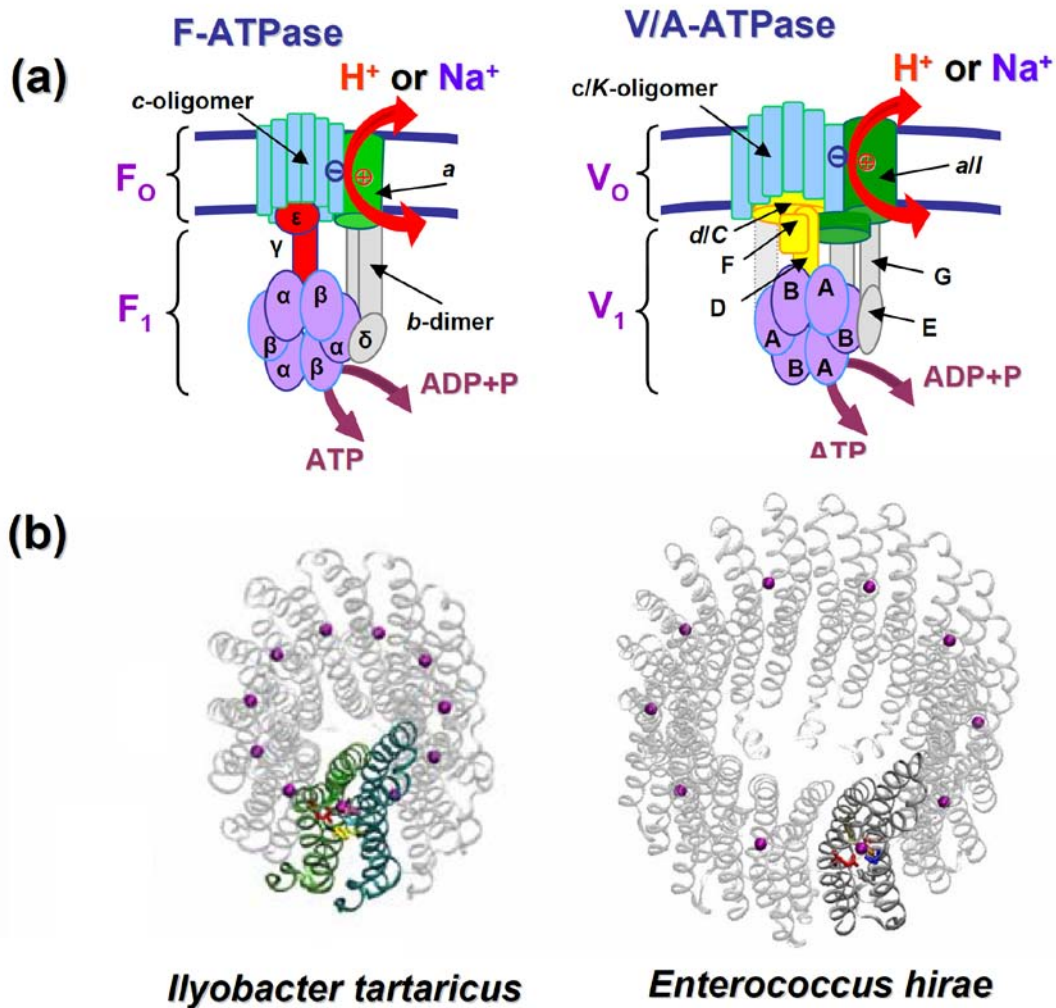
Recently, by combining structural and bioinformatics analyses, we addressed the evolution of the F/V-type ATPases by comparing the structures and sequences of archaeal and bacterial members of this class of enzymes (Mulkidjanian et al. 2007; Mulkidjanian et al. 2008a; Mulkidjanian et al. 2008b; Mulkidjanian et al. 2009). Here we survey these findings and explore their implications for the origin and the earliest evolution of membranes. We argue that the history of membrane enzymes was essentially shaped by the evolution of membranes themselves. In addition, we discuss the mechanisms of an evolutionary transition between the primitive replicating entities and the first membrane-encased life forms, as well as the role of mineral compartments of hydrothermal origin in this transition.

### **Comparative analysis of F/V-type ATPases: example of function cooption?**

Together with two evolutionarily unrelated families, the P-type ATPases and ABC transporters, the F/V ATPases belong to a heterogeneous group of enzymes that use the energy of ATP hydrolysis to translocate ions across membranes (Skulachev 1988; Gennis 1989; Cramer and Knaff 1990; Saier 2000). The F/V type ATPases, however, are unique functionally, because they can efficiently operate as ATP synthases, and mechanistically, in that their reaction cycle is accompanied by rotation of one enzyme part relative to the other (Noji et al. 1997; Imamura et al. 2005). Biochemically, F/V-ATPases are composed of membrane bound parts ( $F_o$  and  $V_o$ , respectively) and catalytic protruding segments ( $F_1$  and  $V_1$ ), which can be washed off the membrane, e.g. by  $Mg^{2+}$ -free solution (see Fig. 1a). The headpiece of the better studied F-type ATPases is a hexamer of three  $\alpha$  and three  $\beta$  subunits with each of the latter carrying an ATP/ADP-binding catalytic site (Stock et al. 2000). The hexamer, together with the peripheral stalk and the membrane anchor, make the "stator" of this enzyme

complex. The “rotor” consists of the elongated  $\gamma$  subunit that, via the globular  $\epsilon$  subunit, is connected to a ring-like oligomer of 10-15 small  $c$  subunits (see Fig. 1 and (Deckers-Hebestreit et al. 2000; Gibbons et al. 2000; Stock et al. 2000; Capaldi and Aggeler 2002; Angevine et al. 2003; Pogoryelov et al. 2005)). The sequential hydrolysis of ATP molecules by the  $\alpha_3\beta_3$  catalytic hexamer rotates the central stalk together with the ring of  $c$ -subunits relative to the stator, so that the ring slides along the membrane subunits of the stator (Boyer 1997; Noji et al. 1997; Panke et al. 2000; Itoh et al. 2004). This sliding movement is coupled to the transmembrane ion transfer and generation of membrane potential (Cherepanov et al. 1999; Mulkidjanian 2006). The enzyme also functions in the opposite direction, i.e., as an ATP synthase. In this mode, the ion current rotates the  $c$ -ring, and the ATP synthesis is mediated by sequential interaction of the rotating  $\gamma$  subunit with the three catalytic  $\beta$  subunits (Cherepanov et al. 1999; Capaldi and Aggeler 2002; Weber and Senior 2003). The V-type ATPases share a common overall scaffold with the F-ATPases but differ from them in many structural and functional features (for details see Fig. 1a and Müller and Gruber 2003; Imamura et al. 2005; Yokoyama and Imamura 2005; Drory and Nelson 2006; Mulkidjanian et al. 2007; Mulkidjanian et al. 2009).

F-type ATPases are found in bacteria and in eukaryotic mitochondria and chloroplasts, whereas the V-type ATPases are found in archaea, some bacteria, and in membranes of eukaryotic cells (Gogarten et al. 1989; Perzov et al. 2001; Nakanishi-Matsui and Futai 2006; Mulkidjanian et al. 2008b). In particular, vacuoles contain V-type ATPases that use the energy of ATP hydrolysis to acidify cellular compartments (Nelson 1989; Perzov et al. 2001; Beyenbach and Wieczorek 2006; Forgac 2007). Some authors classify the simpler, prokaryotic V-type ATPases into a separate subgroup of A-type (from archaeal) ATPases/ATP synthases (Hilario and Gogarten 1998; Müller and Gruber 2003). Others, however, prefer to speak about bacterial and eukaryotic V-type ATPases (Perzov et al. 2001; Drory and Nelson 2006; Nakanishi-Matsui and Futai 2006). In phylogenetic trees, the A-type ATPases invariably cluster together with the eukaryotic V-ATPases and separately from the F-type ATPases (Gogarten et al. 1989; Hilario and Gogarten 1993; Hilario and Gogarten 1998).



**Fig. 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; 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. The *a* subunits that show structural similarity but might not be homologous (Mulikidjanian et al. 2007) are shown by distinct but similar colours; in the case of those V-ATPase subunits that are differently denoted in prokaryotes and eukaryotes, double notation is used: eukaryotic/prokaryotic. The composition of peripheral stalk(s) and their number in V-ATPases remains ambiguous, with values of up to 3 being reported (Esteban et al. 2008; Kitagawa et al. 2008). For further details, see refs. (Mulikidjanian et al. 2007; Mulikidjanian et al. 2009). (b) membrane rotor subunits of the Na<sup>+</sup>-translocating ATP synthases; left, undecamer of *c* subunits of the Na<sup>+</sup>-translocating F-type ATP synthase of *Ilyobacter tartaricus* (PDB entry 1YCE (Meier et al. 2005)); right, decamer of *K* subunits of the Na<sup>+</sup>-translocating V-type ATP synthase of *Enterococcus hirae* (PDB entry 2BL2 (Murata et al. 2005)); both rings are tilted to expose the internal pore; in *I. tartaricus*, Na<sup>+</sup> ions (purple) crosslink the neighbouring subunits, whereas in *E. hirae* the Na<sup>+</sup> ions are bound by four-helical bundles that evolved via a subunit duplication (see also (Mulikidjanian et al. 2008b; Mulikidjanian et al. 2009)).

Among the F-type and the V-type ATPases, both proton-translocating and Na<sup>+</sup>-translocating forms are found. The ion specificity of the sodium-dependent F/V-type ATPases is, in fact, limited to the ion-binding sites of their membrane-embedded parts F<sub>O</sub> and V<sub>O</sub>, respectively (see Fig. 1 and (von Ballmoos et al. 2008)). In the absence of sodium, Na<sup>+</sup>-ATPases have the capacity to translocate protons (Dimroth 1997; von Ballmoos and Dimroth 2007). In contrast, H<sup>+</sup>-ATPases are apparently incapable of translocating Na<sup>+</sup> ions (Zhang and Fillingame 1995). This asymmetry is most likely due to the higher coordination number of Na<sup>+</sup>, which requires six ligands (Frausto da Silva and Williams 1991), while proton, in principle, can be translocated by a single ionisable group. Comparative analyses of the subunits *c* of Na<sup>+</sup>-translocating and H<sup>+</sup>-translocating ATPases identified several residues that are involved in Na<sup>+</sup>-binding and are the principal determinants of the coupling ion specificity (Zhang and Fillingame 1995; Rahlfs and Müller 1997; Dzioba et al. 2003). However, the exact modes of Na<sup>+</sup> binding in F- and V-ATPases remained obscure until the structures of the membrane-spanning, rotating *c*-oligomers of the Na<sup>+</sup>-translocating ATP synthases of the F- and V-type have been resolved (see Fig. 1b and (Meier et al. 2005; Murata et al. 2005; Meier et al. 2009)). Strikingly, the superposition of these structures reveals nearly identical sets of amino acids involved in Na<sup>+</sup> binding which almost perfectly superimpose in space (Mulkidjanian et al. 2008b). When pitted against the topology of the phylogenetic tree of F/V-type ATPases, the similarity of the Na<sup>+</sup>-binding sites in the two prokaryotic domains led to the conclusion that the last common ancestor of the extant F- and V-type ATPase, most likely, possessed a Na<sup>+</sup>-binding site (Mulkidjanian et al. 2008b). Indeed, sodium-dependent ATPases are scattered among proton-dependent ATPases in both the F- and the V-branches of the phylogenetic tree (Mulkidjanian et al. 2008b). Barring the extremely unlikely convergent emergence of the same set of Na<sup>+</sup> ligands in several lineages, these findings suggest that the common ancestor of F- and V-type ATPases contained a Na<sup>+</sup>-binding site.

The ion specificity of the F/V-type ATPases, however, is decisive for the nature of the bioenergetic cycle in any organism. Although proton-motive force (*PMF*) and/or sodium motive force (*SMF*) can be generated by a plethora of primary sodium or proton pumps, F/V-type ATPases are unique in their ability to utilize *PMF* and/or *SMF* to produce ATP (Cramer and Knaff 1990). Owing to its nearly ubiquitous presence, the proton-based energetics has been generally viewed as the primary form of biological energy transduction (Deamer 1997; von Ballmoos and Dimroth 2007). By contrast, the ability of some prokaryotes to utilize sodium gradient for ATP synthesis has been usually construed as

a later adaptation to survival in extreme environments (Konings 2006; von Ballmoos and Dimroth 2007). The results of our analysis indicated that the sodium-based mechanisms of energy conversion preceded the proton-based bioenergetics.

However unexpected it might be (but, see (Skulachev 1988; Dibrov 1991; Häse et al. 2001)), the evolutionary primacy of sodium bioenergetics seems to find independent support in membrane biochemistry. As argued in more detail elsewhere (Mulkidjanian et al. 2008b; Mulkidjanian et al. 2009), creating a non-leaky membrane that can maintain a *PMF* sufficient to drive ATP synthesis is a harder task than making a sodium-tight membrane. The conductivity of lipid bilayers for protons is by 6-9 orders of magnitude higher than the conductivity for Na<sup>+</sup> and other small cations (Deamer 1987; Haines 2001; Konings 2006). This difference is based on the unique mechanism of transmembrane proton translocation: whereas the conductivity for other cations depends on how fast they can cross the membrane/water interface (Deamer 1987; Nagle 1987; Tepper and Voth 2006), the rate of proton transfer across the membrane is limited not by the proton transfer across the interface, but by the “hopping” of protons across the highly hydrophobic midplane of the lipid bilayer (Deamer 1987; Haines 2001). Hence, proton leakage can be suppressed by decreasing the lipid mobility in the midplane of the bilayer and/or increasing the hydrocarbon density in this region. Accordingly, proton tightness can be achieved, for example, by branching the ends of the lipid tails and/or incorporating hydrocarbons with a selective affinity to the cleavage plane of the bilayer (Haines 2001).

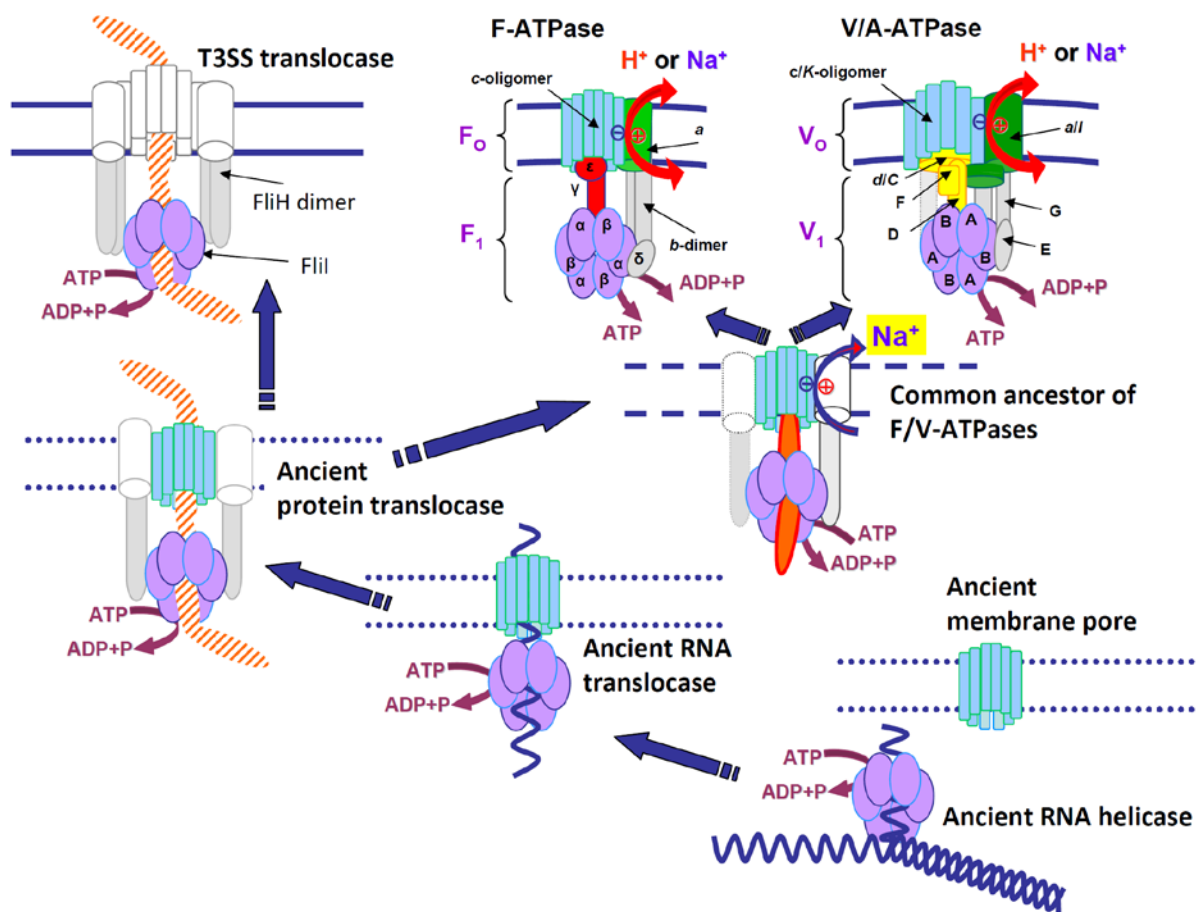
In agreement with the hypothesis on independent emergence of proton-based energetics in different lineages, representatives of the three domains of life employ distinct solutions to make their membranes tighter to protons, namely, the mobility of side chains is restricted in distinct ways and different hydrocarbons are packed in the midplane of the H<sup>+</sup>-tight membranes (see (Haines 2001; Konings et al. 2002; Konings 2006; Mulkidjanian et al. 2008b) for details). This fact supports the suggestion on the independent transition from the sodium to proton bioenergetics in different lineages.

Where did the first, apparently, sodium-translocating F/V type ATPases come from? The comparison of the F- and V-type ATPases shows that they are built of both homologous and unrelated subunits (see Fig. 1 and (Mulkidjanian et al. 2007)). The subunits of the catalytic hexamer and the membrane *c*-ring are highly conserved (Gogarten et al. 1989; Nelson 1989; Gogarten et al. 1992; Lapiere et al. 2006). The subunits that are thought to form the hydrophilic parts of the peripheral



stalk(s), also appear to be homologous, despite low sequence similarity (Supekova et al. 1995; Pallen et al. 2006). The membrane parts of the peripheral stalks show structural and functional similarity as well (Kawasaki-Nishi et al. 2001; Kawano et al. 2002), although it remains unclear whether or not they are homologous. By contrast, the subunits of the rotating central shafts, which couple the catalytic hexamers with the *c*-ring (shown by dissimilar colours in Fig. 1), are not homologous (Nelson 1989) as substantiated by the presence of dissimilar structural folds (Mulkidjanian et al. 2007).

Building on this conservation pattern, we suggested that the common ancestor of the F-type and V-type ATP was not an ion-translocating ATPase but rather an ATP-dependent protein translocase in which the translocated protein itself occupied the place of the central stalk (Mulkidjanian et al. 2007). Indeed, the catalytic hexamers of F-type and V-type ATPases are homologous to hexameric helicases, specifically, the bacterial RNA helicase Rho, a transcription termination factor (Patel and Picha 2000). This relationship led to the earlier hypothesis that the ancestral membrane ATPase evolved as a combination of a hexameric helicase and a membrane ion channel (Walker 1998). However, the structures of the membrane segments of the F/V-ATPases ( $F_o$  and  $V_o$ , respectively, see Fig. 1) have little in common with membrane channels or transporters which are usually formed by bundles of alpha-helices (von Heijne 2006). As shown in Fig. 1b, the *c*-oligomers are wide, lipid-plumbed membrane pores with internal diameters of ~ 3 nm and ~ 2 nm for  $V_o$  and  $F_o$ , respectively (Meier et al. 2005; Murata et al. 2005). Conceivably, such a pore (without lipid plumbing) was large enough to allow passive import and export of biopolymers in primordial cells. When combined with an ATP-driven RNA helicase, this type of membrane pore could yield an active RNA translocase that subsequently would give rise to a ATP-driven protein translocase, as depicted in Fig. 2. Then it is not surprising that a direct homologous relationship exists between the F/V-ATPases and those subunits of the bacterial flagellar motors and Type III secretion system (T3SS) that are responsible for the ATP-driven export of flagellin or secreted proteins by these machines. This relationship can be traced through the catalytic subunits (Vogler et al. 1991) and the subunits of the peripheral stalk of the F/V-ATPases (Pallen et al. 2006).



**Figure 2. The proposed scenario of evolution from separate RNA helicases and primitive membrane pores, via membrane RNA and protein translocases, to the ion-translocating membrane ATPases.** The color code is as in Figure 1; ancient/uncharacterized protein subunits are not colored. The striped shapes denote the translocated, partially unfolded proteins. The presence of two peripheral stalks in the primordial protein translocase and the flagellar/T3SS systems is purely hypothetical and based on the consideration that a system with one peripheral stalk would be unstable in the absence of the translocated substrate. The involvement of two FliH subunits in each peripheral stalk is based on the ability of FliH dimers to form a complex with one FliI subunit (Minamino and Namba 2004; Imada et al. 2007).

As discussed in more detail elsewhere (Mulikidjanian et al. 2007), there is a plausible path for the transition from a protein translocase to an ion-translocating machine. The key to the transition is decrease of the pore conductivity, possibly, as a result of several amino acid replacements in the c-subunit, which would cause translocated proteins to get stuck in 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, otherwise, keep together the membrane subunits. Given that the structural requirements for a central stalk are likely to be minimal (Mnatsakanyan et al. 2009), this scenario naturally incorporates independent recruitment of unrelated and even structurally dissimilar proteins as central stalks in ancestral archaea and bacteria. The transition from a protein translocase to an ATP-driven ion translocase would be complete with the recruitment of the central stalk subunits, i.e., inclusion of their genes in the operons of the F-type and V-type ATPases, respectively (Mulkiđjanian et al. 2007).

### **Emergence of integral membrane proteins**

In the previous section we have noted that the common ancestor of the c-oligomers in the F- and V ATPases could initially function as a membrane pore. As argued by several authors (Frausto da Silva and Williams 1991; Szathmary 2007), such pores could be needed to enable exchange of small molecules and even polymers between proto-cells and their environment. At the same time, they could represent a transition state towards the first integral membrane proteins. Integral membrane proteins contain long stretches of hydrophobic amino acid residues. In contrast, in water-soluble globular proteins, the distribution of polar and non-polar amino acids in the polypeptide chain is quasi-random (Finkelstein and Ptitsyn 2002). Assuming that the quasi-random distribution pattern is an ancestral trait, a gradual, multi-step transition from soluble proteins to membrane proteins with long hydrophobic stretches has to be envisaged. Furthermore, modern membrane proteins are co-translationally inserted into the membrane by the translocon machinery that ensures proper protein folding in the membrane (White and von Heijne 2008). The translocon itself is a membrane-bound protein complex that could not have existed before the membrane proteins evolved. In the absence of the translocon, a hydrophobic protein, if even occasionally synthesized, would remain stuck to a primeval ribosome. Therefore, a scenario of the membrane evolution must enclose an evolutionary scenario for the emergence of integral membrane proteins.

The global evolutionary analysis of integral membrane proteins by Saier and co-workers led to the conclusion that the evolution went from non-specific oligomeric channels, which were built of peptides with only a few transmembrane segments, towards larger, specific membrane translocators that emerged by gene duplication (Saier 2003), see also the chapter by Saier et al. in this volume. Still, the widespread notion that a stand-alone hydrophobic  $\alpha$ -helix could, via multiple gene duplication,

yield increasingly complex membrane proteins (see e.g. (Popot and Engelman 2000)) does not appear plausible: a solo, water-insoluble  $\alpha$ -helix could hardly leave the ribosome in the absence of a translocon complex.

Physically more plausible are the scenarios that start from amphiphilic  $\alpha$ -helices (Pohorille et al. 2003; Mulkidjanian et al. 2009). The simplest  $\alpha$ -helical protein fold is an  $\alpha$ -helical hairpin (*long alpha-hairpin* according to the SCOP classification (Andreeva et al. 2008)). These hairpins are stabilized via hydrophobic interaction of the two  $\alpha$ -helices. Since such stabilization is unlikely to be particularly strong, a hairpin, upon an eventual interaction with a membrane, might spread on its surface and then reassemble within the membrane in such a way that the non-polar side chains would interact with the hydrophobic lipid phase. The hairpins, then, should tend to aggregate, leading to the formation of water-filled pores, inside which the polar surfaces of  $\alpha$ -helices would be stabilized. This arrangement seems to be partially retained by the c-ring of the F-ATPase that is built up of  $\alpha$ -helical hairpins (see Fig. 1b) and is sealed 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 segment(s) of the  $\gamma$ -subunit and water (Pogoryelov et al. 2008). The described mechanism of spontaneous protein insertion into the membrane, which does not require translocon machinery, is still used by certain bacterial toxins and related proteins. Those proteins are monomeric in their water-soluble state, but oligomerize in the membrane with the formation of pores (see (Parker and Feil 2005; Anderluh and Lakey 2008) and references therein).

Membrane pores could be formed, in principle, not only by many small hairpins - which themselves could result from multiple duplication events, as inferred for the c subunit of the F/V-type ATPase (Davis 2002) – but also by larger amphiphilic proteins that, after binding to membranes, might undergo “inside-out” rearrangements (see also (Engelman and Zaccai 1980)) with the formation of a water-filled pore in the middle of a helical bundle. This kind of protein architecture is exemplified by SecY (Van den Berg et al. 2004), another ubiquitous membrane protein besides the c-subunit of the F/V-ATP synthase. Starting from the pores that were built up of amphiphilic stretches of amino acids, integral membrane proteins could then evolve via the combined effect of (i) multiple replacements of polar amino acids by non-polar ones and (ii) gene duplications, ultimately yielding multi-helix hydrophobic bundles (Saier 2000; Saier 2003). Concomitantly, some membrane proteins would form

the first translocons, enabling controlled insertion of these hydrophobic bundles into the membrane (White and von Heijne 2008).

### **Emergence of lipid membranes**

The first membrane proteins required lipid membranes. What were their origins? The comparison of bacteria and archaea can hardly help to clarify the origins of lipid membranes because, as already noted, they are fundamentally different in these two domains (see (Boucher et al. 2004; Pereto et al. 2004; Thomas and Rana 2007) for reviews). In both prokaryotic domains phospholipids are built of glycerol phosphate (GP) moieties to which two hydrophobic hydrocarbon chains are attached. The GP moieties, however, are different: while bacteria use *sn*-glycerol-1-phosphate (G1P), archaea utilize its optical isomer *sn*-glycerol-3-phosphate (G3P). The hydrophobic chains, with a few exceptions, differ as well, being based on fatty acids in bacteria and on isoprenoids in archaea. In bacterial lipids, the hydrophobic tails are linked to the glycerol moiety by ester bonds whereas archaeal lipids contain ether bonds. The difference extends beyond the chemical structures of the phospholipids, to the evolutionary provenance of the enzymes involved in synthesis of phospholipids - they are either non-homologous or distantly related but not orthologous in bacteria and archaea (Boucher et al. 2004; Pereto et al. 2004; Koonin and Martin 2005; Koga and Morii 2007).

The evolutionary stage when the first lipid membranes could emerge is also uncertain. The “lipids early” models suggest that the first life forms, presumably RNA-based, were enclosed in lipid vesicles from the very beginning (see e.g. (Segre et al. 2001; Deamer 2008)), whereas the “lipids late” models suggest that lipid membranes could be preceded by emergence and evolution of simple, virus-like, RNA/protein life forms (see e.g. (Martin and Russell 2003; Koonin and Martin 2005; Koonin 2006)).

Several lines of evidence support the “lipids late” schemes.

a) The “lipid early” schemes imply that the first lipids were recruited from the available abiogenically synthesized compounds. Although amphiphilic molecules such as fatty acids are found in meteorites (Deamer and Pashley 1989) and could be present on the primeval Earth, it is unlikely that they all had uniformly long hydrophobic tails, which is a pre-condition for the formation of a stable bilayer. In contrast, the enzyme-synthesized amphiphilic molecules can be expected to be more homogenous.

b) It is generally accepted that a pure lipid bilayer is not a practical solution for a primeval organism because it would prevent any exchange between the interior and the environment. Therefore the “lipids early” models suggest that the first membranes were leaky, enabling the exchange of low-molecular compartments with the surrounding milieu (Deamer 2008). The existence of the first life forms should, however, also depend on their ability to exchange genes and to share enzymes (Koonin and Martin 2005; Szathmáry 2007). The known machines for the translocation of biological polymers across the membrane are made of proteins, which implies a co-evolution of membrane proteins and lipids.

c) Table 1 contains the list of ubiquitous genes that are likely to be present in the LUCA. Only 2 of these ca. 60 entries, namely the above discussed c-subunit of the F/V-ATPases and the SecY pore subunit, belong to membrane proteins. This under-representation of membrane proteins suggests that the emergence of membrane proteins (and membranes) may have followed the emergence of RNA/protein organisms.

d) The existence of a pre-cellular RNA/protein world is supported by finding of viral hallmark genes shared by many groups of RNA and DNA viruses – but missing in cellular life forms. The inhabitants of this world might have been virus-like particles enclosed in protein envelopes (Koonin 2006; Koonin et al. 2006).

A really strong argument in favor of the “lipids early” models is that the lipid vesicles, by separating the first replicating entities, may have enabled their Darwinian selection (see e.g. (Monnard and Deamer 2001)). The primeval compartmentalization, however, could have been achieved even without lipid vesicles. Russell and co-workers have hypothesized that the early stages of evolution may have taken place inside iron-sulfide bubbles that formed at warm, alkaline hydrothermal vents (Russell and Hall 1997; Martin and Russell 2003; Russell and Hall 2006). It has been suggested that iron-sulfide “bubbles” could encase LUCA consortia of small, virus-like replicating entities (Koonin and Martin 2005; Koonin et al. 2006). Such entities could share a common pool of metabolites and genes, so that each interacting consortium, e.g. inhabitants of one inorganic “bubble” at a hydrothermal vent, would comprise a distinct evolutionary unit. Such a scheme, with an extensive (gene) exchange between the members of one consortium but not between different, mechanistically separated consortia solves a major conundrum between the notion of extensive gene mixing that is considered a

major feature of early evolution (Woese 1998) and the requirement of separately evolving units as subjects of Darwinian selection (Koonin and Martin 2005; Mulkidjanian et al. 2009).

This “inorganic” solution of the compartmentalization problem is further exploited in the recent “Zinc world” scenario according to which the life on Earth emerged, powered by solar radiation, within photosynthetically active precipitates of zinc sulfide (ZnS) (Mulkidjanian 2009; Mulkidjanian and Galperin 2009). Honeycomb-like ZnS precipitates are widespread at the sites of deep sea hydrothermal activity (Takai et al. 2001; Hauss et al. 2005; Kormas et al. 2006; Tivey 2007). Here, the extremely hot hydrothermal fluids leach metal ions from the crust and bring them to the surface (Kelley et al. 2002; Tivey 2007). Since hydrothermal fluids are rich in H<sub>2</sub>S, their interaction with cold ocean water leads to the precipitation of metal sulfide particles that form “smoke” over the “chimneys” of hydrothermal vents (Kelley et al. 2002; Tivey 2007). These particles eventually aggregate, settle down, and, ultimately, form sponge-like structures around the vent orifices. The sulfides of iron and copper precipitate promptly (Seewald and Seyfried 1990), their deposition starts already inside the orifices of hydrothermal vents (Kormas et al. 2006). The sulfides of zinc and manganese precipitate slower (Seewald and Seyfried 1990) and can spread over, forming halos around the iron-sulfur apices of hydrothermal vents (see (Tivey 2007) for a recent review). The Zn world model suggests that under the high pressure of the primeval, CO<sub>2</sub>-dominated atmosphere, very hot, Zn-enriched hydrothermal fluids could reach even the sub-aerial, illuminated environments, so that ZnS could precipitate within reach of UV-rich solar beams (nowadays such hot fluids can discharge to the continental surface only as steam geysers). Zinc sulfide is a very powerful photocatalyst; it can reduce CO<sub>2</sub> to formate with a quantum yield of up to 80% (Henglein 1984; Henglein et al. 1984; Kanemoto et al. 1992; Eggins et al. 1993), can produce diverse other organic compounds from CO<sub>2</sub> (Fox and Dulay 1993; Eggins et al. 1998), including the intermediates of the Krebs cycle (Zhang et al. 2007; Guzman and Martin 2009), and can drive various transformations of carbon- and nitrogen-containing substrates (Yanagida et al. 1985; Kisch and Kunneth 1991; Kisch and Lindner 2001; Marinkovic and Hoffmann 2001; Ohtani et al. 2003). In the illuminated environments, the UV light, serving as a selective factor, may have favored the accumulation of RNA-like polymers as particular photostable (Mulkidjanian et al. 2003; Sobolewski and Domcke 2006). A direct contact of the first RNA-based life forms with the surfaces of porous ZnS compartments should be of key importance: these surfaces, besides catalyzing abiogenic photosynthesis of useful metabolites and serving as templates for the synthesis of longer biopolymers

from simpler building blocks, could prevent the first biopolymers from photo-dissociation by absorbing from them the excess radiation (Mulkiđjanian 2009). The idea that the first RNA molecules may have been shaped by ZnS surfaces is supported by an almost perfect match of the distances that separate the positively charged Zn<sup>2+</sup> ions at the ZnS surface (Dinsmore et al. 2000) with the distances between the phosphate groups in the RNA backbone (0.58–0.59 nm (Saenger 1984)). In addition, Zn<sup>2+</sup> ions showed an exclusive ability to catalyze the formation of naturally occurring 3'-5' linkages upon abiogenic polymerization of nucleotides (Bridson and Orgel 1980; Van Roode and Orgel 1980).

As the ZnS-mediated photosynthesis is accompanied by the release of Zn<sup>2+</sup> ions (Henglein 1984; Kisch and K nneth 1991), it should yield a steadily Zn-enriched milieu within ZnS compartments. A Zn-rich milieu is geologically unusual; the equilibrium concentration of Zn in the anoxic primeval waters was estimated as 10<sup>-15</sup> – 10<sup>-12</sup> M (Zerkle et al. 2005; Dupont et al. 2006; Williams and Frausto da Silva 2006). If the LUCA consortia indeed dwelled within photosynthesizing ZnS compartments, then Zn<sup>2+</sup> ions could be preferably recruited as metal cofactors by the proteins and RNA molecules of the LUCA. This prediction is easily testable. Table 1 exemplifies that the ubiquitous proteins - which are likely to be present in the LUCA - show notable preference for Zn as compared to other transition metals (see refs. (Mulkiđjanian 2009; Mulkiđjanian and Galperin 2009) for further details on the Zn world scenario).

The photosynthesizing Zn world, however, could exist only as long as the pressure of the CO<sub>2</sub> dominated atmosphere was high enough to enable delivery of very hot, Zn-enriched hydrothermal fluids at illuminated settings. When the atmospheric pressure dropped below ca. 10 bar, the continental hydrothermal fluids should cool down and become gradually depleted of Zn ions, so that fresh ZnS surfaces could no longer form in sub-aerial settings, but only deeply at the sea floor. The organisms would have found alternative ways to reduce CO<sub>2</sub> and should have learned to deal with Fe<sup>2+</sup>, the dominating transition metal ion in primordial sea (with a estimated content of 10<sup>-5</sup>-10<sup>-6</sup> M (Zerkle et al. 2005; Dupont et al. 2006; Williams and Frausto da Silva 2006). Iron, unlike zinc, can generate harmful hydroxyl radicals and is therefore detrimental for RNA (Meares et al. 2003; Cohn et al. 2004; Luther and Rickard 2005; Cohn et al. 2006). Lipids can prevent the damaging action of iron-containing minerals on RNA (Cohn et al. 2004), so that the need to protect biopolymers from iron-containing surfaces could have prompted the transition from surface-confined replicators to lipid-encased life forms.



**Table 1.** Products of ubiquitous genes and their association with essential divalent metals

(the table is taken from ref. (Mulikidjanian and Galperin 2009))

Protein function	EC number (if available)	Functional dependence on metals	Metals in at least some structures
<b>Products of ubiquitous genes, according to (Koonin 2003)</b>			
<b>Translation and ribosomal biogenesis</b>			
Ribosomal proteins (33 in total)		Mg	Mg, Zn
Seryl-tRNA synthetase	6.1.1.11	Mg, Zn	Mn, Zn
Methionyl tRNA synthetase	6.1.1.10	Mg, Zn	Zn
Histidyl tRNA synthetase	6.1.1.21	Mg	No metals seen
Tryptophanyl- tRNA synthetase	6.1.1.2	Mg, Zn	Mg
Tyrosyl- tRNA synthetase	6.1.1.1	Mg	No metals seen
Phenylalanyl- tRNA synthetase	6.1.1.20	Mg, Zn	Mg
Aspartyl- tRNA synthetase	6.1.1.12	Mg	Mg, Mn
Valyl-tRNA synthetase	6.1.1.9	Mg	Zn
Isoleucyl-tRNA synthetase	6.1.1.5	Mg, Zn	Zn
Leucyl-tRNA synthetase	6.1.1.4	Mg	Zn
Threonyl-tRNA synthetase	6.1.1.2	Mg, Zn	Zn
Arginyl-tRNA synthetase	6.1.1.19	Mg	No metals seen
Prolyl-tRNA synthetase	6.1.1.15	Mg, Zn	Mg, Zn, Mn
Alanyl-tRNA synthetase	6.1.1.7	Mg, Zn	Mg, Zn
Translation elongation factor G	3.6.5.3	Mg	Mg
Translation elongation factor P/ translation initiation factor eIF5-a			Zn
Translation initiation factor 2			Zn
Translation initiation factor IF-1			No divalent metals
Pseudouridylate synthase	5.4.99.12	Mg, Zn	No metals seen
Methionine aminopeptidase	3.4.11.18	Mn, Zn, or Co	Mn or Zn or Co
<b>Transcription</b>			
Transcription antiterminator NusG	-	-	No metals seen
DNA-directed RNA polymerase, subunits $\alpha$ , $\beta$ , $\beta'$	2.7.7.6	Mg	Mg, Mn, Zn
<b>Replication</b>			
DNA polymerase III, subunit $\beta$	2.7.7.7	Mg	Mg
Clamp loader ATPase (DNA polymerase III subunit $\gamma$ and $\tau$ )	2.7.7.7.	Mg	Mg, Zn

Topoisomerase IA	5.99.1.2	Mg	No metals seen
<b>Repair and Recombination</b>			
5'-3' exonuclease (including N-terminal domain Poll)	3.1.11.-	Mg	Mg
RecA/RadA recombinase	-	-	Mg
<b>Chaperone function</b>			
Chaperonin GroEL	3.6.4.9	Mg	Mg
O-sialoglycoprotease/ apurinic endonuclease	3.4.24.57	Zn	Mg, Fe
<b>Nucleotide and amino acid metabolism</b>			
Thymidylate kinase	2.7.4.9	Mg	Mg
Thioredoxin reductase	1.8.1.9	-	No metals seen
Thioredoxin		-	Zn
CDP-diglyceride-synthase	2.7.7.41	Mg	No entries
<b>Energy conversion</b>			
Phosphomannomutase	5.4.2.8	Mg	Mg, Zn
Catalytic subunit of the membrane ATP synthase	3.6.1.34	Mg	Mg
Proteolipid subunits of the membrane ATP synthase	3.6.1.34	-	No metals seen
Triosephosphate isomerase	5.3.1.1	-	No metals seen
<b>Coenzymes</b>			
Glycine hydroxymethyltransferase	2.1.2.1	Mg	No metals seen
<b>Secretion</b>			
Preprotein translocase subunit SecY	-	-	Zn
Signal recognition particle GTPase FtsY	-	-	Mg
<b>Miscellaneous</b>			
Predicted GTPase	-	-	No metal ligands in the structure
<b>Additional ubiquitous gene products from ref. (Charlebois and Doolittle 2004)</b>			
DNA primase (dnaG)	2.7.7.7	-	Zn
S-adenosylmethionine-6-N',N'-adenosyl (rRNA) dimethyltransferase (KsgA)	2.1.1.48	Mg	No metals seen
Transcription pausing, L factor (NusA)	-	-	No metals seen

The lists of ubiquitous genes were extracted from refs. (Koonin 2003; Charlebois and Doolittle 2004). The data on the dependence of functional activity on particular metals were taken from the BRENDA database (Chang et al. 2009). According to the BRENDA database, the enzymatic activity of most Mg-dependent enzymes could be routinely restored by Mn. As concentration of Mg<sup>2+</sup> ions in the cell is ca. 10<sup>-2</sup> M, whereas that of Mn<sup>2+</sup> ions is ca. 10<sup>-6</sup> M, the data on the functional importance of Mn were not included in the table. The presence of metals in protein structures was as listed in the Protein Data Bank (Henrick et al. 2008) entries. See ref. (Mulikidjanian and Galperin 2009) for further details and references.

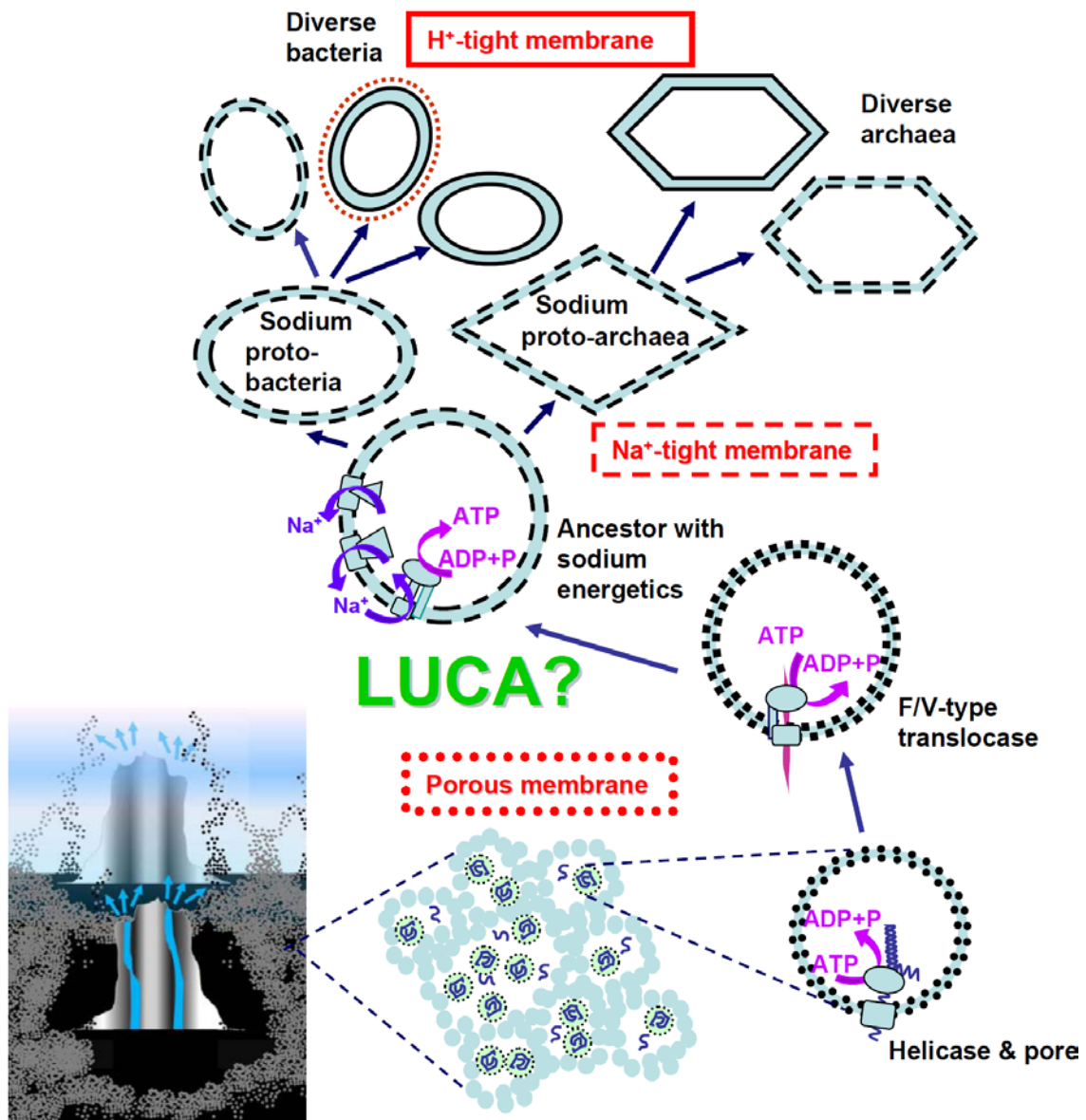
Why then are the lipid membranes of modern archaea and bacteria so different? Several hypotheses were suggested to explain the aforementioned usage of different GP enantiomers by archaea and bacteria. Koga has suggested that the first GP moieties were racemic because of their abiogenic origin; only later the enzymes for the synthesis of G1P and G3P separately evolved in archaea and bacteria, respectively (Koga et al. 1998; Koga and Morii 2007). Wächtershäuser has suggested that membranes of pre-cells were built of lipids that contained racemic GPs units that were synthesized by a primitive non-stereospecific enzyme. The further segregation of the G1P and G3P-containing lipids was suggested to be physico-chemical, so that lipids that carried the same GP enantiomers clustered together and eventually yielded subpopulations of organisms enriched in either enantiomeric phospholipid. It was suggested further that the higher stability of “homochiral” over “heterochiral” membranes could favor the emergence of different enzymes for stereospecific synthesis of different GP enantiomers in archaea and bacteria, respectively (Wächtershäuser 2003). Pereto and co-workers have hypothesized that G1P and G3P were initially synthesized in a non-specific way, as byproducts of two different dehydrogenases already present in the cenancestor, and that specific enzymes for synthesis of G1P and G3P separately evolved from these two dehydrogenases in archaeal and bacterial lineages, respectively (Pereto et al. 2004).

All these hypotheses are based on the assumption that the phospholipids of the LUCA (or pre-cells, or cenancestor) contained GP moieties that, as in modern membranes, linked two lipid “tails” together. In fact, there is no evidence that the very first membranes were built in this way. Even the modern membranes contain, besides GP-containing two-tailed phospholipids, also single-tailed fatty acids and four-tailed cardiolipin molecules. The concept of gradual, multistep membrane evolution, as outlined in previous sections, is better compatible with a scenario where the first lipids could be simple and single-tailed. As argued above, the function of first, supposedly porous, membranes was limited to occluding biological polymers while enabling the exchange of small molecules and ions. The experiments with simple amphiphilic compounds have shown that vesicles made either of fatty acids (Deamer and Dworkin 2005; Deamer 2008) or of phosphorylated isoprenoids (Nomura et al. 2002; Gotoh et al. 2006; Streiff et al. 2007) can entrap polynucleotides and proteins. Isoprenoids were likely to be present at the stage of LUCA: their enzymatic synthesis is simple, and they are found in all domains of life, unlike fatty acids that, most likely, have emerged in the bacterial lineage (Smit and

Mushegian 2000). Hence, one can speculate that the leaky membranes of LUCA were simple, being built of e.g. phosphorylated isoprenoids, To attain ion-tight membranes, the first cells, however, had to stabilize the membrane/water interface and increase the thickness of the membrane, since the permeability of lipid bilayer to small ions (with exception of protons, see above) is limited by ion penetration across the membrane/water interface and depends on the membrane thickness (Deamer 1987; Nagle 1987; Tepper and Voth 2006). A pairwise linking of hydrophobic tails by GP moieties seems to be the chemically simplest way to solve both tasks: the membrane interface becomes less leaky to ions and the thickness of the bilayer increases by ca. 0.6 nm. In addition, the phosphate moiety of GP ensures the amphiphilicity of the bilayer and an eventual binding of a headgroup. Bacteria and Archaea may have found this simple solution independently, by using different GP enantiomers and unrelated enzymes. In Bacteria this transition may have been accompanied by the recruitment of fatty acids; the isoprenoid derivatives, however, were retained by bacterial membranes, in particular, as hopanoids and single-tailed quinones (Haines 2001; Hauss et al. 2005).

### **Scenario for the origin and evolution of membranes and membrane proteins**

Apparently, the central theme in the early cellular evolution was the increasing tightness of cell envelopes. Indeed, the emergence of such a complex device that is the modern biological membrane could proceed only via many intermediate stages. Szathmary and co-workers have recently developed and modeled a set of evolutionarily scenarios that exemplified the crucial importance of the interaction and exchange between the primeval replicating entities for the stability of their populations (Szathmary 2006; Szathmary 2007; Konnyu et al. 2008; Branciamore et al. 2009). According to Szathmary, an increase in the complexity of pro-cells should be accompanied by their progressive sequestering from the environment, so that the gradual build up of enzymatic pathways inside the pro-cells would be accompanied by decrease in membrane permeability (Szathmary 2007). Fig. 3 depicts a tentative scenario of a co-evolution of membranes and membrane proteins where the gradual decrease in membrane permeability, on one hand, enables the emergence of new enzyme systems that demand tight membranes and, on the other hand, leads to expunction of “leaky” membrane proteins.



**Figure 3. The proposed scenario for the evolution of membranes and membrane enzymes.** The scheme suggests the emergence of first replicating entities within honeycomb-like ZnS precipitates of hydrothermal origin. Note that FeS and ZnS particles (black and grey dots, respectively) precipitate at different distances from the hot spring (the picture is based on data from (Seewald and Seyfried 1990; Takai et al. 2001; Kelley et al. 2002; Kormas et al. 2006; Russell 2006)). The evolution of membranes is shown as a transition from primitive, porous membranes that were leaky both to Na<sup>+</sup> and H<sup>+</sup> (dotted lines), via membranes that were Na<sup>+</sup>-tight but H<sup>+</sup>-leaky (dashed lines) to the modern-type membranes that are impermeable to both H<sup>+</sup> and Na<sup>+</sup> (solid lines). As the common ancestor of the F- and V-ATPases possessed a Na<sup>+</sup>-binding site (Mulkiđjanian et al. 2008b; Mulkiđjanian et al. 2009), the LUCA (regardless of whether it was a modern-type cell or a consortium that included replicating, membrane-surrounded entities) either had porous membranes so that the common ancestor of the F- and A/V ATPases operated as a polymer translocase, with Na<sup>+</sup> 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 energetic (see main text, and (Mulkiđjanian et al. 2008b; Mulkiđjanian 2009; Mulkiđjanian and Galperin 2009; Mulkiđjanian et al. 2009) for details).

The scenario starts from simple replicating entities that may have dwelled in honeycomb-like mineral compartments, which, in the framework of the Zn world scenario (Mulkiđjanian 2009; Mulkiđjanian and Galperin 2009), could help to (photo)select the first RNA organisms, provide a shelter and nourish them. At this stage, the first replicators could survive only by sharing metabolites, enzymes and genes. Gradually, however, the first life forms may have attained protecting envelopes that initially could be built predominantly of proteins. The subsequent transition to the predominantly lipid membranes should be accompanied by the emergence of primitive membrane pores that might resemble the c-rings of the F-type and V-type ATPases. The requirement for horizontal gene transfer and gene mixing should, however, drive the emergence of active, ATP-driven RNA and protein translocases, giving rise, in particular, to the ancestor of the F/V-type ATPases, which, apparently, was a chimera of a (former) RNA-helicase and a membrane pore.

The next stage of evolution is envisaged as selection for tighter membranes that would maintain the ionic homeostasis of the evolving cells. According to the principle of chemistry conservation (see e.g. (Mulkiđjanian and Galperin 2007)), primordial cells would strive to keep their internal chemistry similar to the chemical compositions of the brine in which the first life forms had emerged. Besides the need to maintain a high internal Zn concentration (after the supposed dramatic shift of the Zn/Fe ratio in their habitats, see also (Mulkiđjanian and Galperin 2009)), the first cells should be also challenged by growing sodium content in the sea water. Since the cytoplasm of all cells contains more potassium than sodium, and the translation systems specifically require  $K^+$  for functioning (Bayley and Kushner 1964; Spirin et al. 1988), the first life forms were likely to emerge in  $K^+$ -rich environments (Natochin 2007; Mulkiđjanian 2009). The concentration of  $Na^+$  in the sea water should, however, increase with time (DeRonde et al. 1997; Foriel et al. 2004; Pinti 2005), affecting the  $Na^+/K^+$  ratio inside the pro-cells. These challenges should strongly favor evolution both of ion-tight membranes and of ion pumps, in particular those capable of expunging  $Na^+$  ions out of the cell. This requirement could be behind the transition from a protein translocase to the precursor of a  $Na^+$ -translocating membrane ATPase.

Most likely, the ancestral rotating ATPases would pump  $Na^+$  along with other  $Na^+$ -pumps, such as the  $Na^+$ -transporting pyrophosphatase (Malinen et al. 2007) and  $Na^+$ -transporting decarboxylase (Dimroth 1997), which are present in both bacteria and archaea and appear to antedate the divergence of the three domains of life. Unlike the other  $Na^+$  pumps, the common

ancestor of the V/F-ATPases, owing to its rotating scaffold, would be potentially able to translocate  $\text{Na}^+$  ions in both directions. Upon further increase in the ocean salinity, reversal of the rotation would result in  $\text{Na}^+$ -driven synthesis of ATP by this primordial rotary machine. Already in Archaean, the concentration of  $\text{Na}^+$  in the ocean water was approx. 1M (DeRonde et al. 1997; Foriel et al. 2004; Pinti 2005), i.e. it was high enough for the rotary machine to switch from the ATP hydrolysis to the ATP synthesis mode. This event marked the birth of membrane bioenergetics: together with the ancient  $\text{Na}^+$  pumps, the ancestral V/F-type ATP synthases would complete the first, sodium-dependent bioenergetic cycle in a cell membrane, as shown in Figure 3.

The final evolutionary step in the present scenario is envisaged as transition to proton-tight, elaborate membranes that provided better protection to the cells. These membranes, in addition, were more lucrative from the point of view of energetics: proton transfer can be chemically coupled to redox reactions, especially those of water and diverse quinones, thus enabling the advent of efficient redox- and light-driven generators of *PMF*, such as cytochrome *bc<sub>1</sub>* complex (Mulkidjanian 2007), cytochrome oxidase (Brzezinski 2004) or water-splitting photosystem II (Junge et al. 2002). Therefore, 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 deteriorated independently in multiple lineages. Ancestral, less effective sodium bioenergetics persisted in anaerobic thermophiles and alkaliphiles that cannot benefit from proton energetics, as well as in some marine and parasitic bacteria and archaea that exist in high-sodium environments (Mulkidjanian et al. 2008a). Further traces of  $\text{Na}^+$ -based bioenergetics are seen in the universal distribution of  $\text{Na}^+$  gradients and  $\text{Na}^+$ -dependent systems of solute transport in virtually all known cell types. In particular, plasma membranes of animal cells remained proton-leaky, “sodium membranes” (Skulachev 1988) inasmuch as they, although with some exceptions (Wieczorek et al. 1999), cannot maintain  $\text{H}^+$  gradient.

In conclusion, we would like to submit that the evolution of membrane proteins should be considered together with the evolution of the membrane lipids since the specific physical properties of lipid bilayers, in particular, their permeability, control the functions that membrane proteins can perform.

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