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Review

# Structural and functional roles of non-bilayer lipid phases of chloroplast thylakoid membranes and mitochondrial inner membranes

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ABSTRACT

The 'standard' fluid-mosaic membrane model can provide a framework for the operation of the photosynthetic and respiratory electron transport systems, the generation of the proton motive force (pmf) and its utilization for ATP synthesis according to the chemiosmotic theory. However, this model, with the bilayer organization of all lipid molecules, assigns no function to non-bilayer lipids – while in recent years it became clear that the two fundamental energy transducing membranes of the biosphere, chloroplast thylakoid membranes (TMs) and inner mitochondrial membranes (IMMs), contain large amounts of non-bilayer (non-lamellar) lipid phases.

In this review, we summarize our understanding on the role of non-lamellar phases in TMs and IMMs: (i) We propose that for these membrane vesicles the dynamic exchange model (DEM) provides a more suitable framework than the 'standard' model; DEM complements the 'standard' model by assuming the co-existence of bilayer and non-bilayer phases and their interactions, which contribute to the structural dynamics of the membrane systems and safe-guard the membranes' high protein:lipid ratios. (ii) Non-bilayer phases play pivotal roles in membrane fusion and intermembrane lipid exchanges – essential processes in the self-assembly of these highly folded intricate membranes. (iii) The photoprotective, lipocalin-like lumenal enzyme, violaxanthin de-epoxidase, in its active state requires the presence of non-bilayer lipid phase. (iv) Cardiotoxins, water-soluble polypeptides, induce non-bilayer phases in mitochondria. (v) ATP synthesis, in mammalian heart IMMs, is positively correlated with the amount of non-bilayer packed lipids with restricted mobility. (vi) The hypothesized sub-compartments, due to non-lamellar phases, are proposed to enhance the utilization of pmf and might contribute to the recently documented functional independence of individual cristae within the same mitochondrion. Further research is needed to identify and characterize the structural entities associated with the observed non-bilayer phases; and albeit fundamental questions remain to be elucidated, non-lamellar lipid phases should be considered on a par with the bilayer phase, with which they co-exist in functional TMs and IMMs.

*Abbreviations:* CL, cardiolipin (diphosphatidylglycerol or DPG); CTI(II), cardiotoxin I(II); cyt *c*, cytochrome *c*; DANTE, delay alternating with nutation for tailored excitation; DCCD-BPF, dicyclohexylcarbodiimide-binding protein of $F_o$ ATP synthase subunit; DEM, dynamic exchange model; DGDG, digalactosyldiacylglycerol; ETC, electron transport chain; FF-EM, freeze-fracture electron microscopy; FSM, flexible surface model; IMM, inner mitochondrial membrane; LHCII, light-harvesting complex II; LPM, lateral pressure model; MGDG, monogalactosyldiacylglycerol; MICOS, mitochondrial contact site and cristae organizing system; NMR, nuclear magnetic resonance; OEC, oxygen evolving complex; OMM, outer mitochondrial membrane; OPA1, optic atrophy 1 (mitochondria-shaping protein); OXPHOS, oxidative phosphorylation system; PE(G, C, S, I), phosphatidyl -ethanolamine (-glycerol, -choline, -serine, -inositol); SQDG, sulfoquinovosyldiacylglycerol; TM, thylakoid membrane; VDE, violaxanthin de-epoxidase.

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## 1. Introduction

In the past decades it became clear that there are important capabilities of biological membranes that require the presence of non-bilayer lipids. In this review, we focus on structural and functional roles of non-bilayer lipids and non-bilayer (or non-lamellar) lipid phases of chloroplast thylakoid membranes (TMs) and the inner mitochondrial membranes (IMMs). TMs and IMMs are membrane vesicles that form highly organized structurally flexible networks; they separate the inner and outer aqueous phases and contain all components of the light-energy (TM) and the chemical-energy (IMM) transducing machineries. Brief general information about these membrane systems is provided in the Supplementary Material, focusing on some remarkable similarities of TMs and IMMs, as opposed by their strikingly different protein (and lipid) compositions and the essentially opposite-direction energy conversion.

In TMs, the major lipids are galactolipids: monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) account for about 50% and 30% of the total lipid content, respectively; they also contain sulfoquinovosyldiacylglycerol (SQDG) (~5–12%) and a phospholipid, the phosphatidylglycerol (PG) (~5–12%) [1]. As part of the adaptive response of TMs to different stress conditions, the lipid composition depends significantly on environmental factors [2,3].

IMMs contain only phospholipids (and no galactolipids). The three major phospholipids, in mammalian (rat liver) mitochondria, are phosphatidylcholine (PC, 40%), phosphatidylethanolamine (PE, 34%) and cardiolipin (CL, a diphosphatidylglycerol, 18%); as minor lipids, they also contain phosphatidylinositol (PI, 5%) and phosphatidylserine (PS, 3%) [4]. Plant cell mitochondria possess very similar lipid composition, with dominance of PC, PE and CL [5] e.g. in cauliflower mitochondria these lipids represent, respectively, 44, 34 and 11 % of the total lipid content [4].

Although the (protein and) lipid compositions of the TMs and IMMs are markedly different, there are two common features shared by these membranes. First, their lipid-to-protein molar ratios are typically between 0.25-0.30 [4,6,7]. The other common, striking feature is that their major lipid species are non-bilayer lipids – MGDG, and PE and CL, respectively. These lipid species - in contrast to the cylindrically shaped bilayer lipids (DGDG, PG and SQDG in TMs and PC, PI and PS in IMMs) - due to their conical shapes [8] - prefer not to form lamellar phases in the presence of water (Fig. 1a). Instead, they assemble into non-bilayer phases, such as cubic, isotropic or inverted hexagonal ($H_{II}$) phases These lipid phases exhibit characteristic $^{31}$P-NMR spectra (Fig. 1b).

Information on the lipid polymorphism of membrane systems can be obtained by $^{31}$P-NMR spectroscopy, which is a sensitive non-invasive technique to fingerprint the phase behavior of the bulk phospholipids *in vivo* and *in vitro* [10]. Because of their restricted mobility compared to bulk lipids, this technique is largely insensitive to annular (or shell) lipids, constituting the first lipid layer around the membrane-intrinsic proteins, and even less to structure (or non-annular) lipids that are tightly bound to the proteins, and are typically found in cavities and grooves of protein hydrophobic regions [11]. In TMs about 60% of the lipids are found in the fluid-like, bulk phase [12].

In this review, first we examine different membrane models with regard to the organization of their bulk lipid molecules: the 'standard' fluid-mosaic membrane model, which is constituted of bilayer lipids, and its different modified forms which take into account the presence of non-bilayer lipids in biological membranes. We evaluate the applicability of these models to TMs and IMMs, in which the major lipids are non-bilayer lipids and which contain non-lamellar lipid phases. We overview the presently available experimental data on lipid polymorphism of TMs and IMMs, and information concerning the origin and structural and functional roles of non-bilayer phases in these membrane systems. We also discuss future perspectives of research concerning the participation of non-lamellar phases in regulatory processes as well as in the energization of membranes and the ATP synthesis.

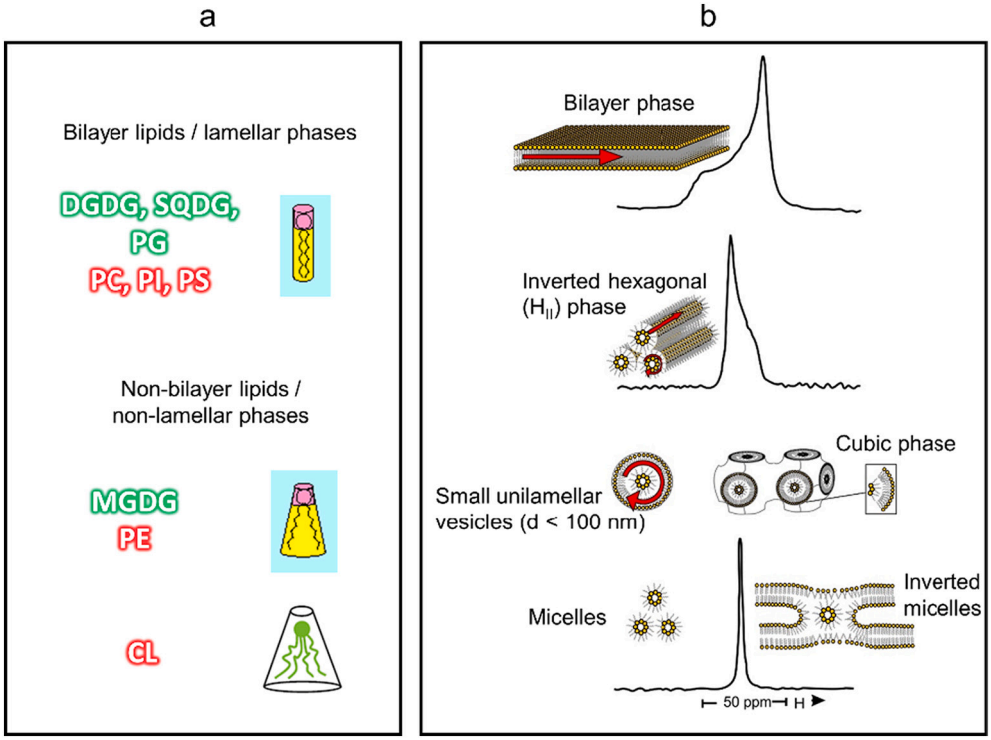


**Fig. 1.** Lipid species of chloroplast thylakoid membranes (green) and the inner mitochondrial membranes (red), which spontaneously assemble into bilayer or non-bilayer structures, according to their cylindrical or conical shapes, respectively (a) and $^{31}$P-NMR signatures of the different lipid phases according to [9] (b).

## 2. Membrane models applicable to TMs and IMMs. Two bilayers-only models and a polymorphic vesicular-membrane network model

Here, we assess the features of the 'standard' model and three membrane models which complement the 'standard' model by considering the presence of non-bilayer lipids in biological membranes. Applicability of these models to TMs and IMMs will be inspected here and in later sections.

### 2.1. The 'standard', fluid-mosaic bilayer membrane model vs. the abundance of non-bilayer lipids in TMs and IMMs

The requirement that TMs and IMMs insulate the inner aqueous phases from the outer aqueous phases can be warranted by organizing the bulk lipid molecules into bilayer structures – as in the 'standard' fluid-mosaic model [13,14] or the mosaic-like membrane model (containing highly organized protein clusters) [15]. The lipid bilayer possesses low permeability to water and most water-soluble molecules and to ions, and protons, in particular. However, within the frameworks of the 'standard' model, it is not straightforward to organize TM and IMM lipids into bilayers. This is because the 'standard' model does not take into account the occurrence of non-bilayer lipids in the membrane [13,14]. This controversy has been known for decades, since the pioneering works of V. Luzzati and coworkers in the 1960s [16]. By using small-angle X-ray diffraction – a technique capable of monitoring the long-range organization of lipids – it has been shown that the lamellar phase represents only one of the large variety of phases adopted by hydrated lipid systems, and the IMM lipids CL and PE preferentially formed $H_{II}$ phases [17–19]. As early as 1973, MGDG was also shown to adopt $H_{II}$ phase –revealed by X-ray diffraction [20]. Polymorphism of membrane lipids has been explained in terms of the molecular shapes as the basic factor determining the phases that can be adopted by a given lipid species [21]. These seminal works inspired numerous studies concerning the role on non-bilayer lipids in TMs and in different biological membranes. However, it was difficult to extrapolate the phase behavior of lipid systems to biological membranes packed with proteins, which evidently stabilized the bilayer configuration [6,22,23]. For example, for TMs, on the one hand, it was established that TM lipid mixtures at molar ratios similar to those occurring in their native membranes adopted non-bilayer phases; on the other hand, the functional state of TM was the bilayer and „non-bilayer configurations were difficult to reconcile with the need to maintain a stable semipermeable membrane system" [22]. In general, while non-bilayer lipids are present probably in all biological membranes, they are thought to be predominantly arranged as bilayers [15].

### 2.2. Lateral Pressure Model (LPM) and Flexible Surface Model (FSM)

Two models, the Lateral Pressure Model (LPM), and the Flexible Surface Model (FSM), proposed in [24,25], respectively, "challenge[d] the 'standard' model (the fluid mosaic model) found in biochemistry texts" [25]. LPM and FSM postulate that the presence of conically shaped non-bilayer lipids in the bilayer affect the structure and the functional activity of membrane-intrinsic proteins. According to LPM, non-bilayer lipids increase the lateral pressure in the hydrophobic region of the bilayer membranes and decrease it in the region of lipid headgroups. This pressure profile is proposed to "keep the [proteins] in a functional state, whereas in the absence of such force the proteins become less efficient or nonfunctional" [24]. FSM predicts that "the nonlamellar-forming tendency of the membrane lipids modulates the protein energetics" due to variations in the curvature elastic energy [25]. According to these models, non-bilayer lipids enhance the structural plasticity of membranes and lend dynamics to membrane-embedded proteins [26,27] – in line with the notion that „the bilayer must not be too stable because that would tend to limit protein dynamics" [28]. Recently, perturbation of membrane morphologies due to the presence of non-bilayer lipids has also been proposed to modify protein properties [29].

It is important to stress that in these models the occurrence of non-bilayer lipid phases in the bilayer membranes is restricted only locally and transiently (see also [27]). Neither LPM nor FSM consider persisting non-bilayer lipid phases inside the bilayer membrane or associated with it.

### 2.3. Dynamic Exchange Model (DEM)

An alternative model, the Dynamic Exchange Model (DEM) [30,31], postulates that – in membranes composed of lipid molecules of high non-bilayer propensity – bilayer and non-bilayer lipid phases coexist and are in dynamic equilibrium with each other. DEM is based on two sets of premises: (i) the ability of membrane-intrinsic proteins to force non-bilayer lipids into the bilayer – this has been documented by *in vitro* experiments using molecular macro-assemblies of purified CL and cytochrome *c* oxidase [32] and isolated MGDG and LHCII [33]; and (ii) that when large protein-free membrane patches are exposed to water, the lipid molecules readily form transient structures, which are then segregated out from the membranes [6,19,34,35]. (LHCII, light-harvesting complex II, the most abundant membrane protein of plant TMs.)

There are two additional features of DEM when applied for TMs and IMMs: (iii) TMs and IMMs are closed membrane vesicles, and (iv) that they are arranged into highly organized, extended networks.

With regard to TMs and IMMs as vesicles, (iii) it is important to stress that their inner aqueous phases are fully packed with proteins [36–39], some of which, especially those belonging to the class of lipocalins (or to lipocalin-like proteins), are capable to bind lipid molecules [40–43]. Lipocalins are also present in the outer aqueous phases, e.g. the zeaxanthin-epoxidase enzyme of TMs [43]. They are found probably in all prokaryotic and eukaryotic cells and are associated with many important biological processes [43]. These water-soluble proteins may hold (at least transiently) some of the excess lipids that are expelled from the bilayer membrane.

As to the network formation of TMs and IMMs and their structural dynamics, (iv) the roles of membrane fusion and fission, and thus the involvement of non-lamellar phases, are well established. Indeed, numerous *in vitro* and *in vivo* data have shown that non-bilayer lipids and non-lamellar lipid phases play key roles in the fusion of membranes and intermembrane exchange of lipids [19,44,45]. It has recently been shown that isolated granum and stroma TMs spontaneously form extended networks – interconnecting, via narrow channels, the protein-rich membrane domains embedded in the bilayer membrane [46].

DEM was proposed to maintain the homeostasis of the energy-converting membranes [30,31] – via a mechanism based on the capability of their lipids to enter (forced in) the membrane (i) and to (spontaneously) segregate from it (ii). By this means the lipid-to-protein ratio of TMs and IMMs can be self-regulated [30]. Dilution of these membranes, i.e. the presence of excess lipids in the bilayer, could easily hamper cooperative interactions between proteins and protein clusters and would probably seriously perturb the migration of the excitation energy in TMs and the operation of the photosynthetic and respiratory electron transport chains (ETCs) [47]. On the other hand, the lack of sufficient amounts of lipids in the bilayer membrane could easily impede the mobility of the quinone molecules [48]. It can be inferred from protein crowding data and theoretical considerations [49] that TMs operate close to the percolation threshold of membrane proteins and near the diffusion limit of small lipophilic molecules like plastoquinone and xanthophyll pigments. (For the macroorganization of proteins in TMs and IMMs, see Supplementary Information.)

Exchange of lipid molecules also occurs between the bilayer membrane and plastoglobuli which bud from the outer leaflet of TMs and "actively participate in thylakoid function from biogenesis to

senescence" [50]. Trafficking of lipids between TMs and plastoglobuli might participate in maintaining a constant lipid:protein ratio in TMs [51] – probably as part of a long-term regulatory mechanism. However, plastoglobuli are highly unlikely to contribute to the lipid polymorphism of TMs – because they contain only trace amounts of TM lipids [52].

## 3. Structural roles of non-bilayer lipids and non-lamellar lipid phases in thylakoid membranes

It has been thoroughly documented that non-lamellar lipid phases can relatively easily be induced not only in model systems using isolated TM lipids (reviewed in [6]) but also in different TM preparations. Freeze-fracture electron microscopy (FF-EM) data have shown that the extrusion of lipids from isolated TMs – instigated by high concentrations of co-solutes such as sugars or betaine [53] or by storing the isolated membranes for days at non-cryogenic low temperature [54] – leads to the formation of an $H_{II}$ phase. Similar, highly ordered tubular structures were observed in TM preparations isolated from spinach leaves grown at low light intensity [55]. Non-bilayer lipid phases were also induced in lyophilized and reconstituted TMs, and in TMs at high temperatures and upon removing their oxygen evolving complex (OEC) – these phase transitions were revealed by $^{31}$P-NMR spectroscopy [56]. Although PG is a minor lipid of TMs (see Introduction), it can be used to monitor the phase behavior of TMs because it is laterally evenly distributed in the membrane [57]. Also, molecular dynamics simulations of thylakoid lipids have shown "a well-mixed system in both the lamellar and inverted hexagonal state" [35].

In fully functional isolated TMs, the presence of a non-bilayer, isotropic phase, in addition to the bilayer, and interacting with it, was first demonstrated in [58] using $^{31}$P-NMR spectroscopy. Recent, high-resolution $^{31}$P-NMR spectroscopy on freshly isolated TMs, as well as on granum and stroma TM fractions, have revealed the co-existence of the bilayer phase with three non-lamellar phases, an $H_{II}$ phase and (at least) two isotropic phases [59,60] (Fig. 2a). $^{31}$P-NMR spectroscopy data, the integrated areas associated with different lipid phases, show that only about 40% of the bulk lipids are found in the bilayer phase and the rest of them are located in non-bilayer phases, with largest (>~40%) contribution from the $H_{II}$ phase (Dlouhy et al. 2021a; see also Figure 2a). The polymorphic lipid-phase behavior of isolated TMs has also been substantiated by time-resolved fluorescence spectroscopy using TMs stained with merocyanine 540 [60–62].

Regarding the origin of the different lipid phases, we emphasize that the presently available data allow only tentative assignments [60] (Fig. 2b). The $H_{II}$ phase was proposed to originate from membrane-extruded lipid molecules – in accordance with the above cited works. However, in freshly isolated TMs, the lipid molecules appeared not to form large, ordered tubular assemblies like those observed earlier on different samples with FF-EM [53]. In isolated digitonin-fragmented granum and stroma TMs, which also exhibited sizeable $H_{II}$ phases, detected by $^{31}$P-NMR spectroscopy [59], some loosely ordered lipid assemblies were identified using FF-EM and electron tomography [63]. These assemblies could be responsible for the $H_{II}$ phase but, unlike purified MGDG [33], displayed no small-angle X-ray scattering signature [46]. This suggests that the extruded lipid molecules do not assemble into large tubular structures with long-range order (cf. [65]). In this context it is to be noted that formation of large, ordered lipid aggregates are spatially hindered in the lumen because its narrow width and also because this aqueous phase is fully packed with proteins [36,66].

As to the isotropic phases, they were proposed to be given rise by (i) membrane fusions and (ii) lipocalin:lipid molecular assemblies in the aqueous phases. In more detail: (i) Fusion of membranes has been thoroughly documented to involve non-bilayer phases via hemifusion stalk (Fig. 2c, d). In fact, TM lipids have been shown to readily form stalks (Fig. 2e, f) [35]; also, plant TM systems contain regions where membranes appear to fuse together, e.g. at the junction of the granum and stroma TMs as well as in the regions where adjacent stroma TMs (belonging to neighboring grana) are jointed [67] (see also Supplementary Material, Fig. S2); fusion of isolated subchloroplast TM particles has also been demonstrated [46]. (ii) As far as lipocalin:lipid assemblies are concerned, the activity of the lipocalin-like lumenal enzyme, violaxanthin de-epoxidase (VDE) has been shown to require the presence of non-bilayer lipid phase – both *in vitro* and *in vivo* [63,68–70] (see also 5.1). VDE plays key role in photoprotection of the photosynthetic machinery of plants and algae [70–74]. Similar to VDE, *in vitro* assay shows the association of thylakoid lipids with the plastid lipocalin LCNP (Jingfang Hao and Alizée Malnoë, personal communication). LCNP is located in the lumen and participates in qH, the sustained non-photochemical quenching of the excess excitation energy [40,75]. This lipocalin protects thylakoid lipids against stress-induced peroxidations [41].

Different water-soluble proteins have also been shown to closely interact with the TM lipids. The 30 kDa inner membrane-associated protein (IM30; aka vesicle-inducing protein in plastids 1, VIPP1), is tightly bound to lipids of TMs and is crucially involved in the biogenesis, stabilization and maintenance of TMs of cyanobacteria, algae and higher plants [76,77]. Further, heat-shock proteins have been proposed to

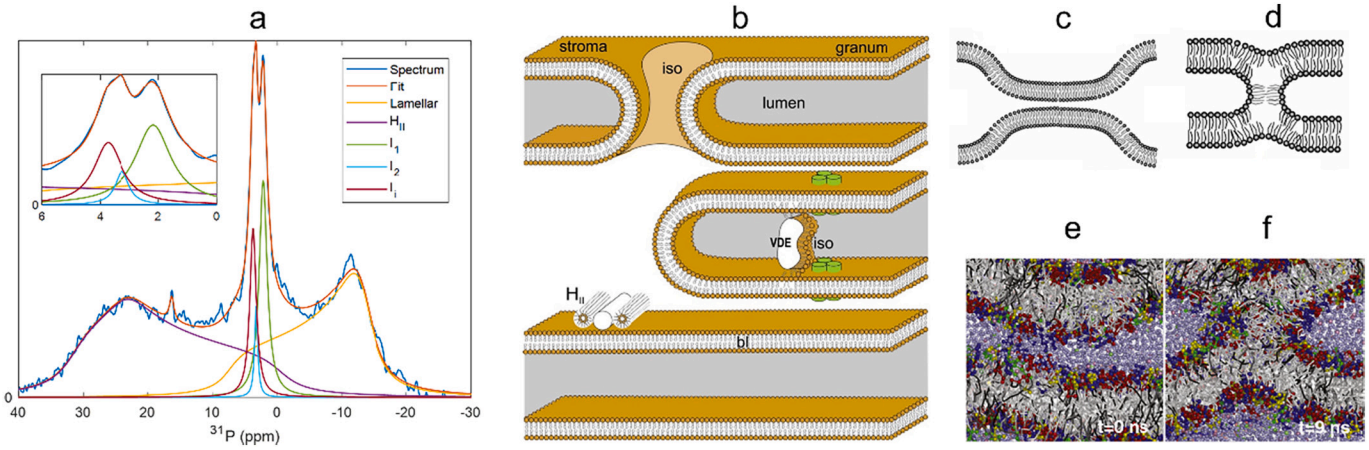


**Fig. 2.** Typical $^{31}$P-NMR spectrum of isolated spinach thylakoid membranes and their spectral components arising from different lipid phases; inset shows the isotropic region (a); schematic model of thylakoid membranes with tentative assignments of the different lipid phases (b); scheme of the formation of hemifusion stalk (c, d); and a molecular dynamic model of the spontaneous transition of thylakoid lipids from bilayer state to a stalk (e, f). In Panels (a) and (b), the isotropic phases are marked as $I_1$, $I_2$ and $I_i$ and Iso, respectively; in Panel (b): bl, bilayer, lamellar phase; VDE, violaxanthin de-epoxidase. Panels (a) and (b) were copied from [63,60], respectively. Panels (c) and (d), and (e) and (f) were cropped from [64,35], respectively.

stabilize the membrane via counteracting the formation of thermally-induced non-bilayer structures [78,79]. In general, membrane lipids have been shown to be involved in thermo-sensing in different organisms [80,81]. Recently, thermo-sensing of plants was proposed to depend on lipid phase transitions [82]. Our observations – the strong temperature dependence of the polymorphic phase behavior of isolated plant TMs [58,63] and the enhanced thermally-induced segregation of lipid molecules from the bilayer in *dgd1* mutant TMs with high MGDG/DGDG ratio [83] – support this hypothesis.

Non-bilayer lipids and non-lamellar lipid phases play pivotal roles in chloroplast differentiation; most particularly during the ontogeny of thylakoid network from a paracrystalline tubular prolamellar body in etioplasts to a mature TM system [84,85]. The role of delicate balance between the bilayer and non-bilayer lipids is also clearly demonstrated by the facts that galactolipid deficiencies disturb the TM ultrastructure [86].

With regard to the possible regulatory roles of non-bilayer lipids in the bilayer phase, it is interesting to point out that the profoundly different protein composition of granum and stroma TMs not only do not bring about different lipid compositions in these membrane fractions [57] but they display very similar lipid polymorphisms [46]. This, according to LPM, would indicate that the same pressure profile is suited to PSII and PSI and the ATP synthase. (Granum TMs contain PSII (Photosystem II) and LHCII, which are assembled into macrodomains with long-range, often semi-crystalline order; in contrast, PSI and the ATP synthase are found in the stroma TMs [87,88], see also Supplementary Material). However, non-bilayer lipids in the bilayer are unlikely to affect the structure and function of PSII and PSI supercomplexes; these highly organized multisubunit protein assemblies possess compact structures that are largely retained even after detergent solubilization [89,90]. The same reasoning might not apply to detached or loosely attached LHCII, the photophysical properties of which depend strongly on the physico-chemical environment of complexes [91], and possibly also on the local pressure variations in the TMs [92]. Indeed, as revealed by *in vitro* experiments, MGDG stabilizes the trimeric form of LHCII, which can be explained by "steric matching of conically formed MGDG and the hourglass shape of trimeric LHCII" [93]. In general, this type of negative-curvature stress has been proposed to play a key role in protein folding in the membrane [94]. Hence, we propose that curvature forces and lateral pressure variations in TMs, due to the presence of non-bilayer lipids in the bilayer [24,25], play roles in the assembly of the photosynthetic protein (super)complexes and the ontogeny of TMs – as also indicated by the facts that MGDG plays central role in the biogenesis of the TMs and the self-assembly of the 3D TM system [95–97]. However, these factors are unlikely to control the activity of the PSII and PSI supercomplexes in mature TMs.

## 4. Structural roles of non-bilayer lipids and non-lamellar lipid phases in mitochondrial membranes

### 4.1. Role of non-bilayer lipids in stabilization of protein complexes

In mitochondria, non-bilayer lipids play specific roles in the assembly and structural dynamics of the respiratory system [98]. The two non-bilayer phospholipids, CL and PE, greatly affect not only the membrane morphology but also the functional activities of IMM-embedded proteins. CL and PE create asymmetrical mechanical stress in the membrane, which thus requires less energy for creating folds in IMM and for inducing tubular membrane invaginations [99].

Although both PE and CL are required for a full activity of the mitochondrial ETC and the efficient generation of membrane potential [100], there are differences in their effects. CL is needed for the structural stability of the respiratory chain supercomplexes, the proper activity of cytochrome *c* oxidase, and the stable membrane potential [100]. Indeed, CL can be used as a marker for inner mitochondrial membranes in plants [5]. Lack of PE decreases the membrane potential of IMM and the activity of cytochrome *c* oxidase. However, in the absence of CL, PE has been reported not to stabilize but to destabilize the respiratory-chain supercomplexes [100]. CL is needed for the dimerization of the ATP synthases and for promoting the ribbon-like assembly of ATP synthase dimers – affecting the lateral organization and morphology of cristae membranes [101]. CL tightly binds not only to proteins of the ETC but also to cyt *c* and transporter proteins, including the ADP-ATP, pyruvate and phosphate carriers [102]. (Cyt *c*, cytochrome *c* is a water-soluble protein found in the intermembrane aqueous space, which transfers electrons from the cytochrome *b/c1* complex, ComplexIII (CIII) to CIV of the mitochondrial ETC. See Supplementary Material, Fig. S1b.) CL deficiency negatively impacts the activity and efficiency of these proteins while PE deficiency does not have the same effect [102]. It has been suggested that the role of PE primarily lays in preserving membrane integrity in the curved areas of IMM while CL serves in maintaining tight associations of proteins in the respiratory chain supercomplexes (Respirasomes), and ATP-synthase dimers and oligomers [100]. The two phosphate groups, the high flexibility of the four alkyl chains of CL and its conical shape facilitate the formation of a tight but flexible bond between proteins of the dimeric and oligomeric forms of the ATP synthase [103,104], and possibly proteins of Respirasomes, in the highly curved IMMs.

### 4.2. Role of non-bilayer lipids in mitochondrial ultrastructure, membrane fusion and fission

CL is important for maintaining mitochondrial dynamics and remodeling, which occur via fission, fusion, shape transition, and intermembrane lipid exchange [105,106]. Efficient inner membrane fusion, one of the multiple steps in mitochondrial shape transitions, is promoted by close interactions of short and long OPA1 isoforms with CL, but not PE [105,106]. It seems that the higher non-bilayer propensity of CL compared to PE makes CL in the bulk phase an ideal phospholipid for supporting mitochondrial remodeling.

Mitochondria are highly dynamic organelles. The dynamic exchanges between OMM and IMM, i.e. between the outer and the inner mitochondrial membranes, have been known for decades. A great diversity in the cristae architecture adapted for each type of cells, tissues, metabolic conditions, energy demands, and the states of health and diseases have been demonstrated in recent years [107,108]. For a long time, cristae were deemed as interconnected entities with conserved and static structure but recently it was discovered that they are independent bioenergetic units – with different ATP synthesis rates, and ETC activities different from that in different parts of the IMM [109] (see Supplementary Material, Fig. S2d).

Cristae have also been shown to be capable of dynamic remodeling in response to changes in the energy demands and physiological states of cells and tissues in a timescale of seconds [107,108,110,111]. As shown by electron tomography cristae membranes are not simply extended invaginations of the IMM but are 'sub-organelles' connected to the inner boundary membrane via slit-like structures, called crista junctions [112,113] (see Supplementary Material, Fig. S2c). Cristae and cristae junctions dynamically interact with each other. Remodeling of cristae membranes – their disappearance and formation of new cristae – can take place in a few seconds, as revealed by advanced optical microscopy techniques [107,111,114]. Cristae dynamics impacts the membrane potential of an individual crista, triggering significant effects on oxidative phosphorylation, $Ca^{2+}$ homeostasis and apoptosis [110,111].

Remodeling of cristae and formation of cristae junctions depend largely on MICOS, OPA1, ATP synthase and the lipid microenvironment, which is predominantly composed of CL [108,111,115,116]. Imbalance in remodeling leads to a wide range of diseases [110,115,117]. The non-bilayer propensity of CL also facilitates the formation of new cristae [110,116,117]. Membrane-bending activity of cristae is controlled by subunits of the MICOS complex (MIC60 and MIC10), which are located at cristae junctions and are surrounded by CL [108]. The width of cristae

is regulated by OPA1 bound to CL, while short and long forms of OPA1, also bound to CL, keep cristae junctions tight. It is believed that the two phosphate groups of CL are largely involved in keeping proteins in cristae junctions tight. ATP-synthase dimers, surrounded primarily by CL, along with the CL-rich lipid microenvironment in the inner leaflet of the cristae membrane, induce positive membrane curvature at the cristae tip [108]. Decline in CL concentration drastically changes not only the cristae architecture but also the landscape of OXPHOS complexes [118], leading to neurodegeneration, cardiovascular pathologies and cancer [107,119]. The loss of mitochondrial CL in the cardiac muscle cells leads to highly interconnected or abnormally swollen cristae [120,121]. This causes substantial defects in skeletal and heart musculature [120].

We would like to stress that remodeling of cristae architecture is a dynamic process that involves membrane fusion and fission [122–125]. This is also evident from the formation of numerous cristae-membrane interconnections and their multiple junctions to the inner boundary membrane (see Supplementary Material, Fig. S2c). Disruptions in the mitochondrial fusion and fission dynamics, triggered by decreased levels of CL, caused by aging and diseases, leads to neurological and cardiovascular disorders and cancer [120,125]. Recent data also revealed that a reduced CL content of the IMM diminishes the coupling efficiency of the respiratory ETC to the ATP synthase, which can be restored by CL enrichment of the isolated mitochondria [126].

In the course of over three decades of research studies, conducted by one of the authors of this review paper, on membrane fusion and fission in model membrane systems mimicking the lipid composition of IMM, it was revealed that membrane fusion and fission are driven by CL-mediated bilayer to non-bilayer transitions [127–136]. Non-bilayer phospholipid structures in the model IMM systems were identified by $^{31}$P-NMR spectroscopy, detecting lipid motions on the timescale of $10^{-2}$ to $10^{-4}$ s. These data strongly suggested that the high propensity of CL to form non-bilayer structures in IMM was a key factor behind the processes of the rapid cristae remodeling occurring in a timescale of seconds. An excellent review on minimal model systems of cristae membranes also stresses the prominent role of non-bilayer lipids in fusion and fission of cristae membranes and cristae-shape dynamics [137].

In broad terms, these conclusions and data pointing to the role of CL in promoting mitochondrial fusion are in harmony with the mechanisms of membrane fusion via non-bilayer intermediates [19,44,45] (see also 3.1.).

### *4.3. Stimulation of bilayer to non-bilayer polymorphic phase transitions by special proteins*

The first studies on phase transitions of phospholipids in mitochondrial samples were commenced more than four decades ago by employing $^{31}$P-NMR spectroscopy [138]. $^{31}$P-NMR spectra from aqueous dispersions of rat liver mitochondrial PE revealed bilayer-to-$H_{II}$ phase transitions in the 10–37 °C temperature range. Under the same conditions, isolated PC molecules retained their bilayer organization, whereas in the total lipid mixtures both bilayer and isotropic phases were present. It was also observed, probably for the first time, that in intact rat liver mitochondria phospholipids at 37 °C co-existed in two phases: bilayer and non-bilayer. A fraction of bovine liver mitochondrial proteolipids, predominantly composed of $F_o$ protein subunits of the ATP synthase and tightly bound CL, was isolated more than a decade later [139]. The line shape of the $^{31}$P-NMR spectrum taken from the aqueous fraction of these mitochondrial proteolipids had a somewhat broad symmetrical signal with a resonance peak shifted by 6 ppm to the high-field side (hereafter referred to as the 6 ppm signal). It was suggested that this signal originated from non-bilayer packed CL molecules partially immobilized by binding to the $F_o$ subunit; this was later corroborated by the results of computer modeling [103,104]. One more decade later, cobra venom cardiotoxins CTI and CTII, which phenocopied the ability of C8 subunit of $F_o$ sector in bovine mitochondrial ATP synthase to form lipid-protein oligomers by binding strongly to CL [135], were used to probe phospholipid packing in cauliflower mitochondria. The $^{31}$P-NMR spectrum of intact cauliflower mitochondria at 18 °C indicated that the phospholipids were arranged in bilayer structures but treatments with CTI and CTII induced two additional, non-bilayer peaks superimposed on the bilayer signal [135]. The non-bilayer signal peaking at 0 ppm could be assigned to phospholipids displaying rapid isotropic motion and the other peak, the 6 ppm signal, to non-bilayer arranged phospholipids with restricted molecular mobility.

It should be noted here that non-bilayer phases have been observed in mitochondria not only by NMR spectroscopy. Small-angle neutron scattering technique also showed non-lamellar hexagonal lipid packing in rat heart mitochondria under conditions of osmotic swelling; and highly ordered hexagonal structures were detected in some mitochondria using electron microscopy technique [140].

To study structural details of the interaction of cardiotoxins with cauliflower mitochondria and with model membranes mimicking these mitochondria, a set of powerful physical methods – including $^{1}$H-NMR, $^{2}$H-NMR, EPR (electron paramagnetic resonance) of spin probes in oriented phospholipid films and differential scanning calorimetry – were used in combination with $^{31}$P-NMR spectroscopy [135]. It was concluded that the 6 ppm signal arises from phospholipids, predominantly CL, found in the intermembrane junctions between the OMM and the IMM. It should be noted that the mobility of these lipid molecules differs from the mobility of annular lipids, which move slower than the $^{31}$P-NMR time scale of $10^{-2}$–$10^{-4}$ s and which thus remain silent.

The above findings suggested that the 6 ppm non-bilayer lipid assembly could be induced by different mitochondrial proteins with structural motifs shared with the cardiotoxins CTI and CTII. The best studied potent cardiotoxin-like protein is the dicyclohexylcarbodiimide-binding protein (DCCD-BPF), which is part of the C8 rotor subunit in mammalian mitochondrial ATP synthase. This hydrophobic C8 rotor subunit of the $F_o$ sector is embedded in IMM and is directly involved in proton shuttling through the $F_o$ sector [141]. It has indeed been shown that DCCD-BPF and CTII share similar membranotropic properties [103,104,142]. Both DCCD-BPF and CTII triggered the formation of the 6 ppm signal when reconstituted separately in multilamellar dispersions mimicking the phospholipid composition of bovine heart mitochondria. Further, it was shown that low concentrations of CTI and CTII stimulated the ATP synthesis parallel with the generation of the 6 ppm phase [143]. It is interesting to note that the C8-ring is proposed to be part of the mitochondrial permeability transition pore (MPTP) [144]. Hence, the ability of the DCCD-BPF to induce non-bilayer structures may be central in the process of inner and outer membrane fusion during the MPTP formation.

Another protein with membranotropic behavior similar to DCCD-BPF or CTII might be cyt *c*. Cyt *c,* having large positive charge, electrostatically interacts with the negatively charged CL phosphate groups and is known to stimulate the opening of toroidal lipid pores [145] and the formation of non-bilayer phase in model systems [146,147]. Cyt *c* is also known to form nanospheres with CL, which possess lipoperoxidase activity [148]. Taking into account that cyt *c* peroxidase is activated by reactive oxygen species [149], the formation of non-bilayer structures of cyt *c* with CL may play key role in mitochondrial stress signaling and apoptosis [150]. An additional potential phase-transition stimulating protein is the intermembrane protein creatine kinase. Its C-terminal lysines lead to preferential interaction with CL and stimulate lipid separation [151–153]. There are other natural and artificial substances, which may possess chemico-physical features dramatically affecting the lipid packing. It is possible that Szeto–Schiller CL-targeting peptides also phenocopy surface areas of DCCD-BPF, which may explain rejuvenation of mitochondrial functions by these peptides [154].

## 5. Functional roles of non-bilayer lipid phases in TMs and IMMs

Although our understanding about the physiological significance of the non-lamellar lipid phases in TMs and IMMs is still rudimentary, in recent years several important bioenergetic functions have been identified.

### 5.1. Structural flexibility of TMs; modulation of the activity of a water-soluble enzyme

The polyphasic lipid-phase behavior of TMs displayed large variations upon changing the temperature and the physico-chemical environment (pH, osmotic and ionic strengths) of the membranes [60,62]. It has been observed that gradually increasing the temperature leads to the gradual destabilization of the bilayer phase and the increase of the isotropic phases and, as expected, the permeability of membranes [155]. The reversible temperature- and low-pH induced enhancements of the isotropic lipid phases of isolated TMs were correlated with the reversible enhancements of the activity of VDE [63]. Hence, these data provided evidence that, similar to what had been established earlier in model (PC/PE) membranes [69], the activity of this water-soluble enzyme in its native TM also depends on the presence of (a) non-bilayer lipid phase(s). At the same time, surprisingly, the activity of VDE negatively correlated with the ability of TMs to hold the transmembrane ΔpH and ΔΨ [63], despite the well-established fact that its activation requires the acidification (<pH 5.8) of the lumen [70,71].

The role of non-bilayer lipid phases in the energization of membranes as a function of temperature is controversial. Upon increasing the temperature from 5 to 25 °C both the electron transport rate and the rate of ATP synthesis increase [156,157]. Under the same conditions, however, the bilayer phase is destabilized, and the isotropic phases are enhanced, and the membranes become more permeable to ions, as reflected by the acceleration of the decay of ΔpH and ΔΨ [63], see also [158]. The accelerated decays evidently act against the utilization of $\Delta\mu_{H}^{+}$ for ATP synthesis. This controversy might be resolved, and the membrane permeability for ions might become less relevant, if – similar to cristae membranes – ATP synthesis would be occurring according to the model of local (or kinetic) coupling, e.g. when $H^+$ ions are transmitted directly from proton pumps to ATP synthase along the surface [159,160] or inside the membrane [161]. These problems, i.e. the localized versus delocalized proton gradients and their roles in the ATP synthesis in TMs, have been reviewed by R. Dilley [162]. It was proposed that TMs switch between the localized and delocalized membrane-energization mechanisms and postulated that under certain conditions protons can be localized in membrane-sequestered domains formed by the lumen-exposed parts of thylakoid proteins which, e.g. LHCII proteins, might be part of the local $H^+$ diffusion pathways. In this context, we note that the extended ordered arrays of PSII-LHCII supercomplexes might constitute the structural basis for such proton-conducting protein domains from PSII (in the granum) to the ATP synthase (in the stroma region) (see Supplementary Material, section S3.).

### 5.2. Role of non-bilayer lipid phases in mitochondrial ATP synthesis

A refined mitochondria preparation procedure, in which phosphate contaminants of non-phospholipid nature were removed, produced fully functional mitochondria for $^{31}$P-NMR studies that allowed to assess phospholipid concentrations in various lipid phases with high accuracy [142]. This made it possible to relate the polymorphic transitions of mitochondrial phospholipids directly to ATP synthase activity. It was shown that the gradual increase in the temperature in bovine heart mitochondria from 15 to 40 °C resulted in gradual increase in the percentage of the non-bilayer 6 ppm signal. Application of a DANTE (delay alternating with nutation for tailored excitation) train of saturation pulses at the high-field peak of the lamellar spectrum has shown that these non-bilayers do not exchange with lamellar phospholipids in the $^{31}$P-NMR time scale (Fig. 3 inset). Most importantly, the increase in the percentage of these immobilized non-bilayers was proportional to the increase of mitochondrial ATP production [142]. Additional experiments – using CTI, CTII and phospholipase $A_2$ [103,104,142] – provided further evidence for the correlation between the amount of non-bilayer, partially immobilized phospholipids in mitochondrial membranes and the rate of ATP production (Figure 3). To rationalize the strong dependency of ATP synthesis on the partially immobilized non-bilayer lipid phase, in the following paragraphs we overview and evaluate literature data on the mechanisms and effects of cristae remodeling and membrane compartmentalizations in relation to OXPHOS pathways.

In the current view, isolated cristae compartments may serve as the platform for oligomerization of OXPHOS proteins and for proton retention on the membrane surface [160]. The clustering of OXPHOS oligomers is mediated by the membrane raft-like structures with the high membrane surface curvature, which is triggered by interaction of CL with the ATP synthase and possibly with proteins of the ETC. Oligomeric clusters of OXPHOS in the apex of cristae that are capable of transferring protons along the membrane surface are optimal structures to provide kinetic coupling of the respiratory chain with ATP synthase [159,163]. The concept of $H^+$ ions transmitted along the chain from complex to complex, without the formation of proton gradient between the bulk phases, was first proposed by R. Williams [164] and then confirmed on the octane-water model [165]. Now, it is clear that protons do not diffuse immediately to bulk water [166,167] but diffuse short-distance laterally along membrane surface from proton pumps to ATP synthases in model systems [168] and mitochondria [160,169,170].

Attraction of protons to phosphate groups of CL also increases the conical shape and inclination of CL to form non-bilayer structures. Hence, the extent of CL protonation, which depends on ATP synthase and Respirasome activity, may be the autoregulatory factor, controlling mitochondrial ultrastructure and thus OXPHOS efficiency. Similar effect on CL inclination to form non-bilayer structures can be exerted by calcium ions [171], which, on the other hand, are known activators of the

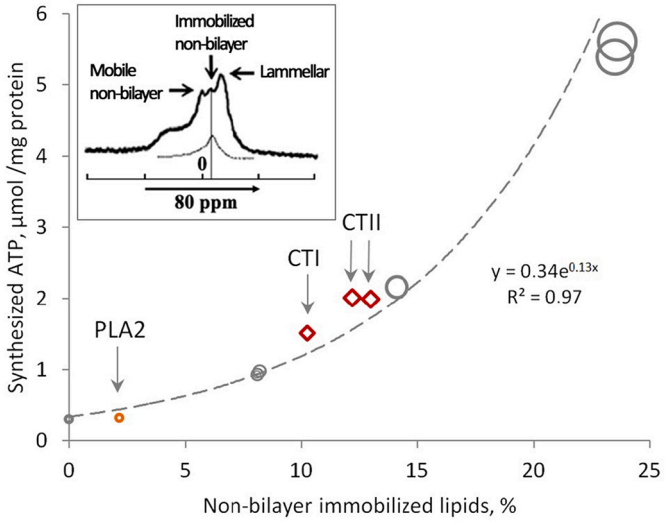


**Fig. 3.** Dependence of ATP synthesis on the percentage of immobilized non-bilayer lipids in mitochondria samples. The percentage of these lipids was varied by changing the temperature or by adding CTI, CTII or phospholipase $A_2$ (PLA2). Immobilized lipids were observed in $^{31}$P-NMR spectra at 6 ppm; this signal was retained after applying a DANTE train of saturation pulses at the high-field peak of the lamellar spectral component [142]. The ATP levels, expressed as µmol ATP synthesized per mg of mitochondrial proteins, were monitored by taking measurements on aliquots from the $^{31}$P-NMR sample tubes. The sizes of the markers overlap the error values. Data points were obtained from [142,143]. Typical $^{31}$P-NMR spectra are presented in the inset (the thin line below the main spectrum shows the 6 ppm signal remaining after DANTE train).

mitochondrial bioenergetic machinery [172]. The property of CL to increase the membrane curvature on calcium binding may be a potential mechanism of known $Ca^{2+}$ stimulating action on the ATP synthase, which is not yet fully resolved. The increase of the membrane curvature leads to electrostatic charge redistribution, pushing protons into the region of maximum curvature where ATP synthase dimers are located. This might be the reason why ATP synthases must be dimerized for maximum efficiency. It should be noted that clustering and degree of tightness of proteins in the OXPHOS changes dynamically in response to changes in the physiological state. When mitochondria respire actively, cristae membranes do not leak protons as the OXPHOS is tightly clustered; in contrast, in resting mitochondria, cristae membranes leak protons as the OXPHOS proteins are more diffusely dispersed. This leakage is not considered as a waste, but a protection mechanism against the generation of reactive oxygen species [173].

The initial step in creation of compartments in the intra-cristae space is facilitated by intermembrane junction, which is driven by attraction of CL molecules on the internal sides of parallel membranes of cristae to a cationic peptide on the surface of opposite membrane of cristae (cf. [143,174]). The potency of CTII and similar proteins to stimulate membrane fusion was described above in detail. CTII is situated in the center of the junction and is predominantly surrounded by CL molecules forming inverted micelle. The center of inverted micelle is the initial point of intermembrane contact – the result of intermembrane attraction between cardiotoxin and CLs. Alkyl chains of inverted micelle are shielded from aqueous environment by two layers of phospholipids on both sides of the inverted micelle. CL molecules are found on the surface of phospholipid layers with the highest curvature, which may serve not only for creation of intermembrane junctions in intra-cristae space but also for cristae-membranes' network interconnectivity (Fig. 4). The intermembrane junction in intra-cristae space could be created between two areas of maximum curvature protruding into the intra-cristae space.

The resulting bridges (junctions) between adjacent cristae membranes, which are likely to be made of non-bilayer packed phospholipids that surround cationic proteins like cyt *c*, creatine kinase or even misfolded DCCD-BPF (not incorporated into ATP-synthase c-rings), may prevent cristae adjacent membranes from tight packing (Fig. 4). This is a very important feature because nucleotide transport in mitochondria occurs in the intra-cristae space, where ATP is translocated from the matrix side by ATP/ADP carrier (AAC, ANT). In intra-cristae space creatine kinases transfer phosphate group from part of ATP molecules to the more light-weighted and mobile creatin molecules. The resulting ADP returns to the mitochondrial matrix for oxidative phosphorylation, while creatine phosphate serves as intermediate carrier of energized bond to the cytosol. Enough space is needed between the cristae membranes to support efficient nucleotide and creatine diffusion, which seems to be a limiting factor affecting mitochondrial maximal activity [176].

The membrane curvature on a side of intra-cristate space caused by ATP-synthase dimers increases the density of protons near the $F_o$

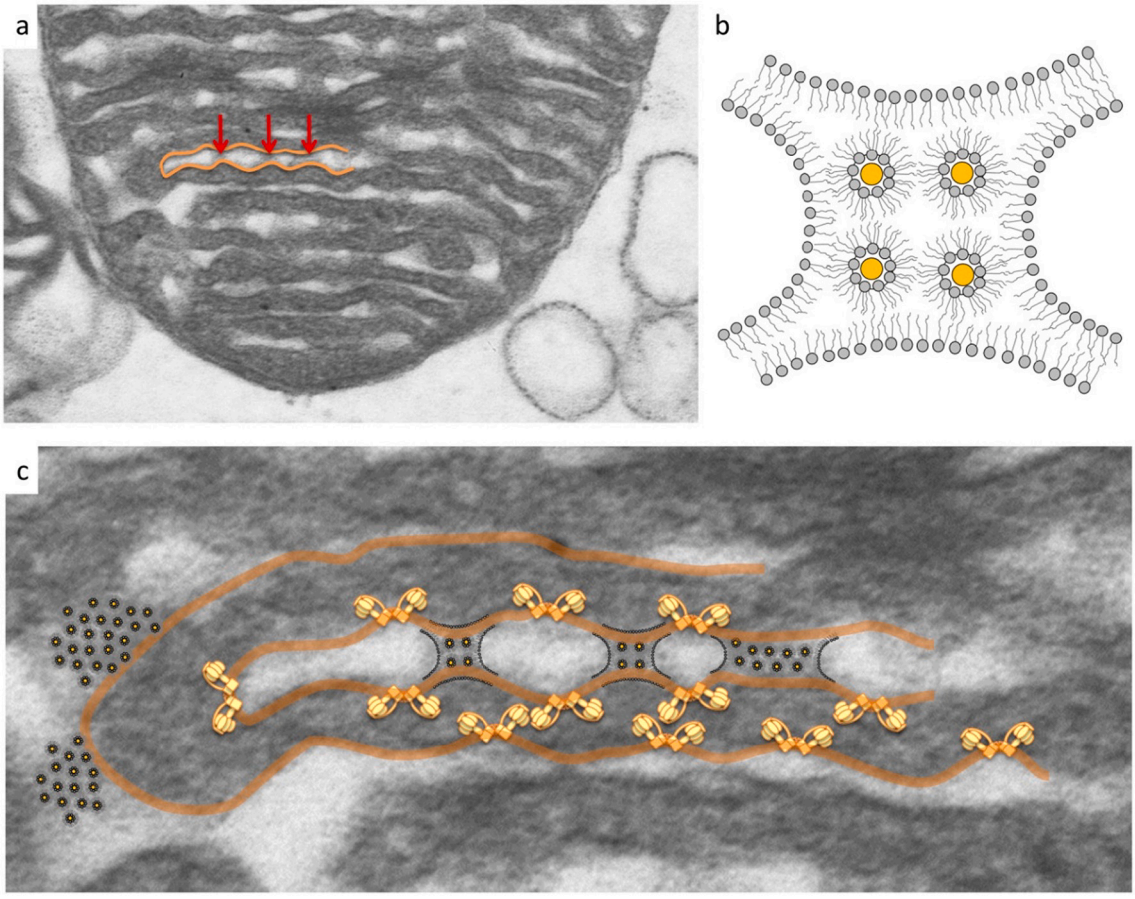


**Fig. 4.** Stabilization of cristae interconnections by non-bilayer junctions. Red arrows indicate the unidentified regions in the intra-cristae space which may represent intermembrane junctions with non-bilayer packed lipids as described in [143,174]. (a) An example of electron microphotography of rat heart mitochondria ultra-thin slice. The image is from the same data set part of which was previously published by coauthors of this review [175]. Mitochondria are fixed with glutaraldehyde and contrasted with osmium tetroxide. Orange lines outline the borders of the bilayer. (b) Simplified model of an intermembrane junction slice containing non-bilayer, inverted hexagonal ($H_{II}$) or inverted micellar structure. The non-bilayer phase, stabilizing cardiotoxin-like proteins, are shown in orange. Panel (c) displays the magnified fragment of cristae from Panel (a) and with overlayed proposed positions of the ATP synthase dimers and the intermembrane junctions. The model is schematic, the scale and positions are not strictly sustained.

subunits as charges gather on the inner surface at a point of maximally curved membrane. In addition, the formation of compartments between two junctions in the intra-cristae space 'squeeze' $H^+$ ions along the inner interface surface of compartments, which further increases the local concentration of protons on the membrane surface and promotes greater transport of protons via the $F_o$ subunit of the ATP synthase [165]. This creates an additional capacity on the interface, which is used to meet the increased demand in ATP production or temporary substrate deficiency.

The multilayer structures of ions and counterions on the interfaces of porous electrodes are known to have extremely high electrical capacities (so-called ionistors or supercapacitors). We propose that mitochondrial lipid phases, which embed the ETC complexes generating the electrochemical potential gradient, possess the properties similar to those of supercapacitors (for more information about supercapacitors see [177]). Taking into account the previously reported ability of ATP synthase to use excess protons from the interface for the synthesis of ATP in the absence of a membrane and transmembrane gradient [165], it is evident that the interfacial protons but not the protons in the bulk phase are the intermediate energy carriers for ATP synthesis. Thus, when kinetic coupling of OXPHOS is realized, the surface capacity of the mitochondrial membrane fulfills the same buffering function as the well-sealed lumen of TMs, most probably performing according to the chemiosmotic model. This agrees well with mitochondria not having fully isolated compartments or high buffer capacity of intermembrane space to implement efficient ATP synthesis and to support efficient transport of the reaction products such as substrates and nucleotides in bulk phases and protons on the interface.

It should be noted that the non-bilayer junctions in the intra-cristae space are highly dynamic structures, which continuously undergo bilayer to non-bilayer transitions in response to changes in proton concentration on the membrane interface following the mechanism of reversible reactions. (Note that, in fact, reversible low-pH induced changes in the lipid phase behavior of TMs have been observed [63]). With the increase in local concentration of protons on the interface, conical shape of CL increases, leading to the formation of non-bilayer junctions, while a decrease in the local concentration of protons on the interface leads to the disappearance of non-bilayer junctions via transformation of non-bilayer junction to bilayer. Due to reversibility of the process, intermembrane junctions in intra-crista space do not interfere with the transport of substrates and nucleotides in the bulk phase and protons on the interface. It may be also proposed that under certain conditions non-bilayer junctions induce fusion of cristae membranes, forming a channel connecting mitochondrial matrix on opposite sides of the crista. Such a process can occur during the transition from the lamellar structure of the cristae to the tubular structure.

Overall, the presently available data related to IMM phospholipids with high non-bilayer propensity and the presence of non-bilayer structures in IMM allow us to conclude that non-bilayer lipids, and CL in particular, play very important roles in regulating the bioenergetics of mitochondria – via their involvement in the structural dynamics and remodeling of cristae and of the whole mitochondria, and in the stabilization of the Respirasomes and the dimeric and oligomeric forms of ATP synthases. It becomes more and more obvious that non-bilayer lipid structures are essential in mitochondrial energy transduction – as they do not only facilitate mitochondrial remodeling through fusion and fission of cristae and IMM and OMM but also facilitate mitochondrial ATP synthesis via increasing the area of the proton-retaining interface and via preventing tight compression of cristae membranes to facilitate nucleotide diffusion. In addition, the main non-bilayer lipid of mitochondria – CL – serves as a "lubricant" for the work of the ATP synthase rotor [178], while its phosphate groups increase the negative charge of the membrane contributing to the accumulation and lateral diffusion of protons on the lipid-water interface.

## 6. Conclusion and perspectives

In this review we analyzed the role of non-bilayer lipids and the polymorphic phase behaviors of TMs and IMMs in the functions and molecular architectures of these two fundamental energy-converting membranes. Compared to the fact that the main lipid species of these membranes are non-bilayer lipids, relatively little attention has been paid to the structural and functional roles of the lipid polymorphisms of these membranes. This might be explained by the immense success of the 'standard' fluid-mosaic membrane model [13,14], which offers explanation for the most important properties of biological membranes. This model is based on the bilayer organization of the bulk lipid molecules in the membranes and provides the simplest framework for the energy conversion mechanism according to the chemiosmotic theory. However, it does not take into consideration the presence of non-bilayer lipids, while by today, compelling evidence shows that fully functional TMs and IMMs contain non-bilayer lipid phases.

Concepts concerning the roles of the non-bilayer phases that are present in TMs and IMMs are just about beginning to take shape. For a long time, it was assumed that the high concentration of integral proteins in energy-converting membranes is enough to stabilize the lamellar membrane structure [179]. This is indeed partially true, and has been documented both for CL [32] and MGDG [33] (see Section 2.3). However, as revealed mainly but not exclusively by NMR spectroscopy lamellar structures co-exist with non-bilayer structures both in TMs and in IMMs.

While numerous questions remain open, including the identity and plasticity of TM and IMM domains that are distinct from the bilayer membrane sections embedding the energy-converting machineries, it is safe to draw a few conclusions: (i) Non-bilayer lipids participate in the assembly and stabilization of pigment-protein complexes (most likely via exerting lateral pressure-variations in the membrane) and the ontogeny of TMs. (ii) Non-bilayer lipid phases are instrumental in fusion processes and junction formations, which play key roles in the self-assembly and remodeling of TMs and IMMs. (iii) In plant TMs, the functional activity of the water-soluble lumenal enzyme VDE is regulated by the availability of non-bilayer isotropic lipid phase(s). (iv) In mitochondria, non-bilayer phases can be triggered by cardiotoxins, water-soluble polypeptides. (v) In IMMs (at least in mammalian heart IMM) ATP synthesis positively correlates with the percentage of a non-bilayer lipid phase with restricted mobility.

Lipid polymorphism in TMs and IMMs are best described within the frameworks of DEM. This model is based on the ability of lipid mixtures with high non-bilayer propensity to segregate out and (re)enter the bilayer. By this mechanism, non-bilayer lipids can self-regulate the membrane homeostasis, safe-guarding their optimal, remarkably high protein-to-lipid ratio of TMs and IMMs. DEM explains the lipid polymorphisms of TMs and IMMs by taking into account that TMs and IMMs are membrane vesicles and that they form highly organized, structurally flexible networks – thereby, DEM predicts and rationalizes the (putative and observed) occurrence of non-bilayer phases in the inner and outer aqueous phases of vesicles and in domains responsible for membrane fusion and junctions. By these means, non-bilayer lipids contribute significantly both to the stability and to the structural plasticity of TMs and IMMs.

In this work, we also state a number of hypotheses that should be tested more rigorously in the future. We propose that subcompartmentalizing of TMs and IMMs by junctions, formed with the involvement of non-bilayer lipid phases may lead to localization of pmf to smaller regions. In fact, such subcompartments of IMMs, largely independently operating individual cristae, have been documented [109] and might be suspected also for granum-stroma TM assemblies, which are interconnected merely via narrow channels [67]. Non-bilayer junctions may additionally stabilize subcompartments ensuring optimal space for substrate diffusion. At the same time, isotropic lipid phases increase the surface of the lipid-water interface and thus the capacity of

the protons for ATP synthesis, which can be intermediately accumulated.

Overall, data listed in this review show that non-bilayer structures play pivotal roles in many processes in TMs and IMMs and should not be ignored. Their further study might provide new understanding of important processes in these fundamental bioenergetic systems.

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## Author contributions

The work was initiated, and the first draft of the manuscript was written by G.G. and E.S.G.; L.S.Y. and S.V.N., and V. Š. and O.D. contributed with covering important additional aspects regarding the structure, functions and energization of the inner mitochondrial and the thylakoid membrane systems, respectively. All authors participated in the final editing of the manuscript.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plipres.2022.101163.

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