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How rotating ATP synthases can modulate membrane structure

Víctor Almendro-Vedia [a,b], Paolo Natale [a,b], David Valdivieso González [a,b], M. Pilar Lillo [c], Juan L. Aragones [d], Iván López-Montero [a,b,*]

[a] Departamento Química Física, Universidad Complutense de Madrid, Avda. Complutense s/n, 28040, Madrid, Spain
[b] Instituto de Investigación Hospital Doce de Octubre (imas12), Avenida de Córdoba s/n, 28041, Madrid, Spain
[c] Departamento Química Física Biológica, Instituto de Química-Física "Rocasolano" (CSIC), Serrano 119, 28006, Madrid, Spain
[d] Departamento de Física Teórica de la Materia Condensada, Instituto Nicolás Cabrera and Condensed Matter Physics Centre (IFIMAC), Universidad Autónoma de Madrid, E-28049, Madrid, Spain

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ABSTRACT

F_1F_o-ATP synthase (ATP synthase) is a central membrane protein that synthetizes most of the ATP in the cell through a rotational movement driven by a proton gradient across the hosting membrane. In mitochondria, ATP synthases can form dimers through specific interactions between some subunits of the protein. The dimeric form of ATP synthase provides the protein with a spontaneous curvature that sustain their arrangement at the rim of the high-curvature edges of mitochondrial membrane (*cristae*). Also, a direct interaction with cardiolipin, a lipid present in the inner mitochondrial membrane, induces the dimerization of ATP synthase molecules along *cristae*. The deletion of those biochemical interactions abolishes the protein dimerization producing an altered mitochondrial function and morphology. Mechanically, membrane bending is one of the key deformation modes by which mitochondrial membranes can be shaped. In particular, bending rigidity and spontaneous curvature are important physical factors for membrane remodelling. Here, we discuss a complementary mechanism whereby the rotatory movement of the ATP synthase might modify the mechanical properties of lipid bilayers and contribute to the formation and regulation of the membrane invaginations.

1. Introduction

Cells are out of equilibrium. The myriads of enzymatic reactions that sustain life are mainly fuelled by the biochemical energy ATP (for adenosine triphosphate). This small molecule contains the equivalent of 13 k_BT upon hydrolysis (Gibbs free energy of ATP hydrolysis varies from −28 to −34 kJ/mol) and it is used in essential processes such as DNA synthesis, protein translation or cell division and motility. The synthesis of ATP is a very highly conserved metabolic process that occurs both in the membrane of prokaryotic cells and in eukaryotic organelles such as mitochondria or chloroplasts. Most of ATP is synthetized through the metabolic pathway called oxidative phosphorylation (OXPHOS). During OXPHOS, a set of four membrane protein complexes (complexes I–IV) and transporters, such as coenzyme Q and cytochrome *c*, transfer electrons from electron donors to electron acceptors via redox reactions in a process that requires oxygen consumption (cell respiration). These redox reactions couple the electron transfer with the translocation of protons across the OXPHOS hosting membrane, which creates an electrochemical proton gradient that ultimately drives the synthesis of ATP by the rotating complex V (F_1F_o-ATP synthase or ATP synthase) [1].

In eukaryotic cells, the OXPHOS system is located in mitochondria. Mitochondria are highly dynamic organelles with a structural hallmark: a double membrane that encloses their lumen. In general, the inner membrane is manifold invaginated (*cristae*) and extends deeply into the mitochondrial lumen (matrix). Observing the plethora of shapes of mitochondria found in nature [2] (Fig. 1), very simple questions rise about why and how mitochondria tune and control *cristae* size and shape. From a bioenergetic point of view, it is intuitive to think about the advantages of creating an excess of surface area through membrane folding to maximize the number of copies of the respiratory complexes in the limited volume of mitochondria. Indeed, the *cristae* membranes host most of the fully assembled complexes of the OXPHOS system and the surface area of *cristae* positively correlates with the amount of ATP produced by OXPHOS in various tissues [3]. Moreover, alterations and remodelling of inner membrane structures are described in numerous human disorders that present bioenergetic imbalances [4].

* Corresponding author. Departamento Química Física, Universidad Complutense de Madrid, Avda. Complutense s/n, 28040, Madrid, Spain.
E-mail address: ivanlopez@quim.ucm.es (I. López-Montero).

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Consequently, a direct link between ultrastructure and function of mitochondria is evidenced.

The formation of *cristae* is a complex out-of-equilibrium process that involves multiple proteins and lipid species. Several models focus on the role of scaffolding proteins like tBID proteins [5], mitofilin multimeric complexes [6], the MICOS machinery [7,8] or Opa1 [9]. Also, the ATP synthase has been identified as a key player in the last years. A remarkable feature of mitochondrial ATP synthases is their ability to form dimers [10–12]. Dimers, in turn, can assemble into rows, which account for the formation of highly curved ridges in mitochondrial *cristae* [13–16]. Also, the special lipid environment for optimal ATP synthesis upon protein rotation gives lipids a crucial role for mitochondria physiology. Of particular interest is the four-acyl chain lipid; cardiolipin, which is almost only found in ATP synthase-containing membranes. The special physicochemical properties of cardiolipin have been invoked in the last years to perceive this molecule as a key lipid for promoting and maintaining the mitochondrial ultrastructure [17,18]. However, a limited picture is depicted on the molecular mechanisms mediating the formation and maintenance of the membrane ultrastructures in mitochondria, which are absent on the prokaryotic membranes.

In this review we will first summarize the current view on how the specific cardiolipin-ATP synthase interactions and the ATP synthase self-interactions participate on mitochondria architecture (Section 2). However, these specific interactions might constitute only a small piece of the puzzle. Note that biological membranes can be described at the mesoscale as a two-dimensional viscous medium able to deform under external forces and thus, being shaped and remodelled. In particular, the formation of tubular and flat structures found in *cristae* needs a mechanical adaptation that can be tuned and optimized by the orchestrated action of lipids and proteins (Section 3). The OXPHOS biomembranes gather a high content of proteins whose function can significantly alter the properties of the embedding membrane, thus participating in the out of equilibrium process of membrane shaping. Specifically, the rotatory movement of ATP synthase in viscous membranes can lead to collective effects with a major impact on the self-organization properties on the embedding OXPHOS membranes. In Section 4, we discuss the biological significance for membrane remodelling by the collective effects that emerge from a set of rotating particles in fluid media. These effects have been reported both theoretically and different experimental setups built as rotating microparticles [19–24], but never discussed in the context of mitochondrial function. We conclude in Section 5 with the translation to the biological system of *cristae* the main effects of ATP synthase rotation on its ability to contribute to membrane bending, curvature generation and sensing and controlling the protein diffusive properties within the lipid bilayer.

Overall, we suggest in this review for the first time that the rotational movement of the ATP synthase, in conjunction with the specific biochemical interactions, might act as an important modulating factor for the formation and maintenance of the membrane invaginations.

2. ATP synthases and membrane organization

2.1. Structural aspects of ATP synthases

ATP synthase is the most important biological motor with rotational

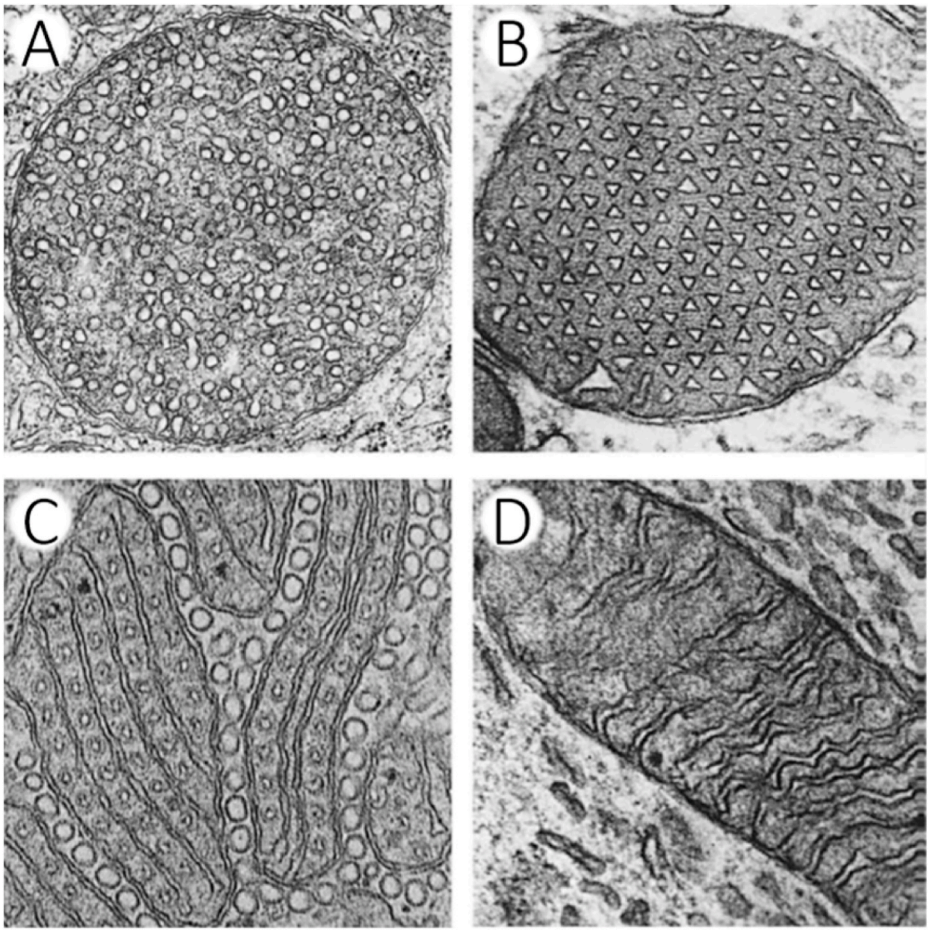


Fig. 1. Cristae structures as observed by electron microscopy within different mitochondria from various tissues: A. Adrenal cortex. B. Astrocyte. C. Fish pseudobranch. D. Ventricular cardiac muscle. Adapted with permission from Ref. [2].

movement in nature. Structurally, ATP synthase is composed of two linked multi-subunit complexes: the soluble catalytic head F_1, and the membrane-spanning rotor F_o (Fig. 2A). The electrochemical gradient of protons across F_o causes the central stalk to spin rapidly within the head inducing the synthesis of ATP in F_1. Depending on the organism, ATP synthases consists of at least eight different subunits, comprising more than 20 polypeptide chains. The water-soluble catalytic F_1 region consists mainly of five different subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$. The three subunits α and β are arranged in an alternating manner, forming the hexameric $\alpha_3\beta_3$ complex. Both subunits α and β consist of an N-terminal β-barrel domain and contain the nucleotide-binding domain. The $\alpha_3\beta_3$ complex is attached to the F_o-rotating ring through the central stalk that consists in the γδε subunits. The amino- and carboxy-terminal portions of subunit γ form an elongated coiled-coil that penetrates the centre of $\alpha_3\beta_3$ complex and upon rotation it induces conformational changes in the $\alpha_3\beta_3$ hexamer that lead to synthesis of ATP. To prevent the rotation of the F_1 head, the $\alpha_3\beta_3$ hexamer is firmly connected to the F_o part through a stator stalk (also known as peripheral stalk) that comprises different subunits that considerably vary among species. In mammals, subunits *8*, *b*, *d*, *e*, *f*, *F6* (*h* in yeast), *g*, and *OSCP* (Oligomycin sensitivity-conferring protein) comprise the stator [25].

After unpinning the rotational movement of F_1-ATP synthase in 1997 [26], the molecular aspects of protein rotation at a single molecule level gained the attention for decades [27–30]. The combination of extensive structural, biochemical, biophysical, and theoretical studies has revealed the basic properties of F_1-ATPases as motor proteins. Briefly, the stoichiometries of protons and ATP per rotation, the step rotation, the reaction schemes or the torque transmission have been described for diverse F_1-ATPases. Interestingly, the high structural homology of ATPases from different organisms makes these proteins share identical mechanisms for functioning. Readers are encouraged to visit the recent review on the molecular aspects of F_1-ATPase rotation [31].

The F_o *c*-ring typically consists of 8 identical subunits in mammals [32] and higher symmetries have been found in yeast [33] and other organisms [34]. Together with subunit a, the c-ring [35,36] shuttles protons across the membrane. During ATP synthesis, proton translocation from the intermembrane space to the matrix through the F_o region, at the interface of subunit *a* and the *c*-ring, drives rotation of the central rotor subcomplex (which includes the γδε subunit and the *c-ring*). The recent advances of electron microscopy have unveiled the structural basis of the membrane ring that sustain the molecular mechanisms for the electrochemical transduction that results in the mechanical rotation of mitochondrial F_o-ATPases. The discovery of four long, horizontal membrane-intrinsic α-helices, arranged in two hairpins at an angle of approximately 70° relative to plane of the membrane, allows the formation of two hydrophilic cavities that direct the proton pathway from the lumenal side to the matrix [37]. Together with the conserved arginine in the stator *a*-subunit and the proton translocation through the *c*-subunit glutamates, the relative location of the hydrophilic cavities drives the unidirectional rotation of F_o by a two-state Brownian ratchet mechanism [38–40]. Note that the molecular motor is reversible and an excess of ATP provokes a rotation in the opposite direction and a reverse flux of protons [26].

2.2. Biochemical interactions supporting ATP synthase dimerization in mitochondria

The structural composition of the mitochondrial F_o ring is more complex than that of bacterial or chloroplasts homologue. In yeast, in addition to the mitochondrial F_o core subunits *a*, *b* and *c*, the supplementary subunits *d*, *e*, *f*, *g*, *h* (*F6* in mammals), *i/j*, *k*, *l*, and *8* (*8 AND A6L* in mammals) can be found [41]. In contrast, the mammalian F_o rotor contains the additional subunits *DAPIT* (diabetes associated protein in insulin-sensitive tissue) [42] and *k* (former *6.8 PL* (6.8 kDa Proteolipid)) [43–45] and misses subunits *i/j*, and *l* present in yeast [46]. Some of these domains (*d*, *f*, *h*, and *8*) are involved in the ATP synthase activity and, more interestingly, ATP synthase dimer formation that directly depends on subunits *e*, *g* and *i/j* and the core domains *a* and *b* (Fig. 2B). The supramolecular rearrangement of ATP synthase as a dimer was first shown by gel electrophoresis [47,48] and confirmed years later by other techniques such as simulations or electron microscopy [10–12]. In particular, subunits *e*, *g*, and the N terminus of subunit *b* combine to form a domain adjacent to the core subunits *a*, *b*, and *c*. The structure and position of this domain appears to be responsible for the accumulation of ATP synthase in the highly curved regions, which is necessary for *cristae* formation. The two dimer interfaces formed by subunits a and *i/j* form a nearly 90° wedge shape dimer inducing the local bending of the mitochondrial membrane [10–12,49–51] (Fig. 2C).

Deletion of F_o interactions abolishes the ATP synthase dimerization, and, remarkably, mitochondria exhibit altered morphology, suggesting that biogenesis of mitochondrial *cristae* depends on formation of the F_1F_o dimer [52]. More recently, using electron cryo-tomography of mitochondrial membranes, it has been proved that wild-type mitochondria ATP synthase dimers are found in rows along the highly curved *cristae* ridges, and appear to be crucial for membrane morphology. Strains deficient in the dimer-specific subunits lack both dimers and lamellar *cristae*. Instead, *cristae* are either absent or balloon-shaped, with ATP synthase monomers distribute randomly in the membrane [11]. More

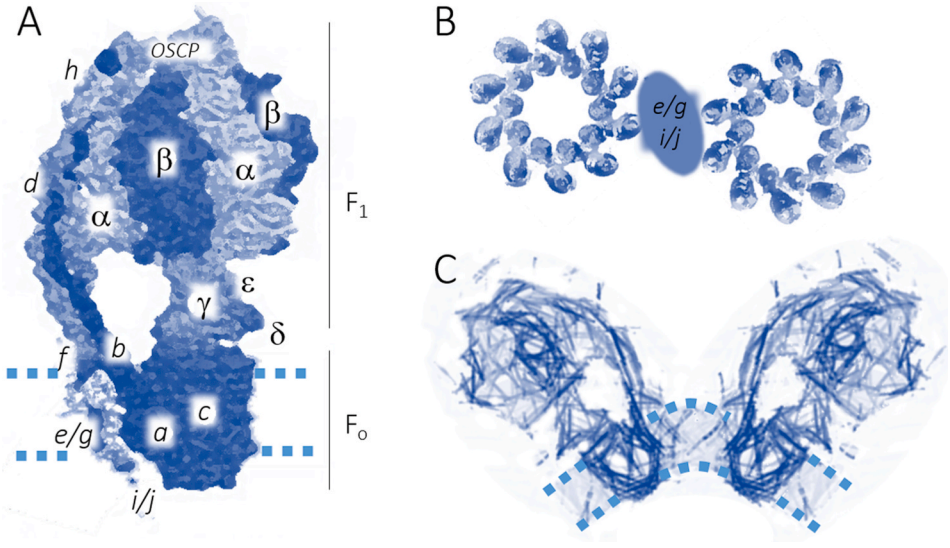


Fig. 2. A. Side view of a surface representation of the F_1F_o-ATP synthase from yeast [46] within a membrane (dashed lines). F_1 comprises the αβ catalytic domain that binds to the *c*-ring though the central stalk (composed of subunits δγε). The peripheral stalk connects the upside of F_1 (OSCP) to the *c*-ring through subunits *h*, *d*, *f*, *b* and *a*. Subunits *e*, *g*, *i* and *j* are responsible for protein dimerization. B. Top view of two c_{10}-ring from adjacent ATP synthases. Interfacial interactions are mediated by *e*, *g*, *i* and *j* subunits. C. Schematic representation of an ATP synthase dimer within a lipid membrane. The tilt angle between monomers varies considerably and depends on the organism. Dimers impose a spontaneous curvature to the membrane.

important, there are strong evidences that ATP synthases cluster as discrete domains that reorganize with the energy requirements of the cell [3].

2.3. Specific interaction between cardiolipin and the F_o rotor

The function of transmembrane proteins is often upregulated by specific interactions with particular lipids that are in tight contact with specific regions of the protein [53]. Interestingly, the hosting biomembranes of F_1F_o ATP synthase share very similar lipid composition. In particular, the bacterial cell membranes and the eukaryotic inner mitochondrial membranes have a high content of cardiolipin of around 10 and 20% of the total lipid content [54,55], whereas this lipid that is almost absent within other biological membranes. Cardiolipin is an unusual phospholipid due to its unique diphosphatidylglycerol structure that provides this phospholipid with four acyl chains, endorsing a high stability on PC/CL mixtures containing up to 20% mol of CL [56]. There is a growing number of studies suggesting a direct link between the function of ATP synthase and cardiolipin [57]. A direct evidence for a transient but direct interaction of CL with the F_o membrane rotor was firstly reported by Duncan et al. [58]. Through coarse-grained simulations, the authors monitored the presence of three to four cardiolipins bound to conserved amino acids in the F_o region at any given instant. More recently, the presence of multiple cardiolipin molecules associated to F_o protein have been identified by cryo-EM to 2.8 Å resolution of an ATP synthase dimer from mitochondria of *Euglena gracilis* [59]. The cardiolipin molecules provide the transmembrane segments of the protein with a lipid microenvironment which has been suggested for distinct functional roles.

It has been established, for instance, that cardiolipin promotes the dimerization of ATP synthase molecules in the *cristae* [17,60] mediating subunit interactions between the transmembrane helices of ATP synthases [59]. Also, cardiolipin supports the organization of ATP synthase dimers into ribbonlike structures along the high-curvature edges of mitochondrial *cristae* [17]. Mutants deficient in cardiolipin were found to lack these supramolecular structures in their mitochondria, and consequently, the oxidative phosphorylation was slower in these mutants [61]. The gathering effect of cardiolipin might lay on the ability of these lipid to be sorted into membrane regions with high curvature. This ability was firstly reported in bacterial membranes [62] and very recently quantified in model systems [63]. Finally, cardiolipin is involved in the supramolecular organization of other proteins and supercomplexes participating in oxidative phosphorylation [64,65] as well as ADP/ATP carriers [66]. The crucial physiological role of cardiolipin in the IMM is evidenced by severe diseases associated with a cardiolipin deficiency, such as the Barth syndrome [67].

Cardiolipin has been also allocated to function as a proton trap to optimize the ATP synthase activity [68]. Cardiolipin has two phosphate ester groups able to exchange protons with their environment. Each of these groups is characterized with a different pK: $pK_1 < 4.0$ and $pK_2 > 8.0$. Consequently, at physiological pH, cardiolipin might have only one negative charge as the most acidic phosphodiester bond should remain protonated. However, several studies suggest that cardiolipin carries two negative charges at neutral pH [69,70]. The deprotonated bonds are thus supposed to participate in proton exchange, acting as a buffer. Thus, the cardiolipin pool around ATP synthase [71] might act as a sink, capturing protons and providing the mechanoenzyme with nearby protons available to transport across the membrane and produce ATP. The specific binding of cardiolipins to the horizontal membrane-intrinsic α-helices of subunit *a* [59] with their acyl chains extending towards the rotor-stator interface allocates cardiolipins a double role, thereby acting concomitantly as a seal for proton leakage at the protein-lipid interface and as the moiety for proton translocation in the rotor-stator interface. The lateral distance between a proton source and ATP synthase is essential for the ATP-synthesis rate [72].

Finally, a lubricating role for the cardiolipin belt around the ATP synthases has been recently suggested [58]. Due to their flexible backbone, cardiolipin molecules can bind the convex surface of F_o *c*-ring. Cardiolipin has been shown to bind selectively but transiently to fully methylated and conserved residues K43, Q44, Q45 and S47 in the inner leaflet, and residue K7 in the outer leaflet. A similar interaction has been found for algae-specific subunits forming a peripheral F_o subcomplex [59]. Fluid film lubrication occurs when opposing bearing surfaces are completely separated by a lubricant film, i.e. the cardiolipin belt. The lubricant film prevents energy losses as a direct consequence of friction and wear produced during the relative interfacial motion. As for cardiolipin, the lubricating hypothesis provides this lipid with a shielding role for the ATP synthase, thus reducing the friction between the protein and other lipids, allowing the enzyme to spin more efficiently. A tribological demonstration of this effect remains open.

3. Mechanics of membrane shaping

Beyond the fundamental role of a particular lipid, the biomembrane embedding the transmembrane proteins is considered as a two-dimensional fluid characterized by different mechanical parameters. As for any other material, these parameters define the compliance of the membrane to be shaped when exposed to different modes of deformation [73]. Here, we are introducing only those that allow for a simple description of vesicle shapes and tubulation at equilibrium, i.e. the bending modulus κ, which provides the basic energy scale for bending deformations, the spontaneous curvature c_0, which represents the preferred curvature of the membrane under unconstrained conditions and the surface tension, σ, which provides the energy cost for increasing the surface area (see Box 1).

3.1. Membrane bending rigidity, surface tension and spontaneous curvature in different membrane shapes

3.1.1. Vesicles

The simplest view for describing vesicle shapes was given by Helfrich [74,75]. For deflated vesicles, where the volume to surface ratio, v, is smaller than the corresponding value for a sphere ($v_{sp} = 1/3R$), the vesicle is easily deformable and the equilibrium shapes are dictated by the enclosed volume (i.e. or the excess of area), the bending modulus and the difference between the spontaneous curvature and the actual curvature of the equilibrium shape. The equilibrium shape minimizes the total curvature energy (Eq. (1)) at given constant surface area, *S* and constant volume, V.

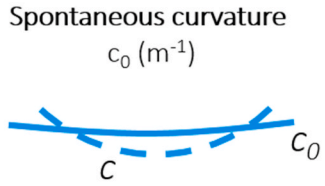
$$\triangle E_{bending} = \frac{\kappa}{2}\int dA(c - c_0)^2 \quad (1)$$

As κ is usually on the order of 10^{-20} J/m^2 for lipid bilayers [76], the shape of the vesicle is essentially determined by the spontaneous curvature. In addition, for tensionless membranes (surface tension, $\sigma < 10^{-3}$ mN/m [77]); stretching is not required for vesicles to adopt non-spherical shapes. So, the beauty of the Helfrich theory lays on the ability to describe a plethora of shapes, including those resembling erythrocytes, with such a small number of parameters. Later, other models included the bilayer structure of lipid membranes and the density/area difference between leaflets to accurately describe different shapes [78,79]. Note however that one can include into the spontaneous curvature the general trend of certain lipids prone to accumulate into highly curved regions [80,81] or an asymmetrical composition leading to produce curved membranes. Changes in c_0 (through a change in the density/area difference between leaflets) or v (through an unbalanced osmotic pressure across the vesicle) can produce changes in the equilibrium shape [82,83].

3.1.2. Lipid tubes

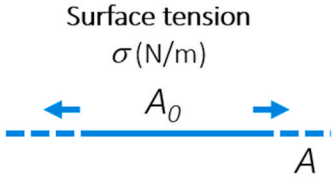
Lipid tubes, also known as tethers, are cylindrical tubules of 10–100 nm radius, made up of a lipid bilayer growing from a larger patch of

Box 1



The spontaneous curvature (c_0) is defined as the curvature of a membrane patch has if it was unconstrained. Deviations to the spontaneous curvature (actual curvature, c), then the membrane patch is under stress as it is prevented from reaching the configuration of minimum curvature energy (equation below). The difference between the actual and spontaneous curvature is key information to understand how the membrane system will evolve under particular conditions.

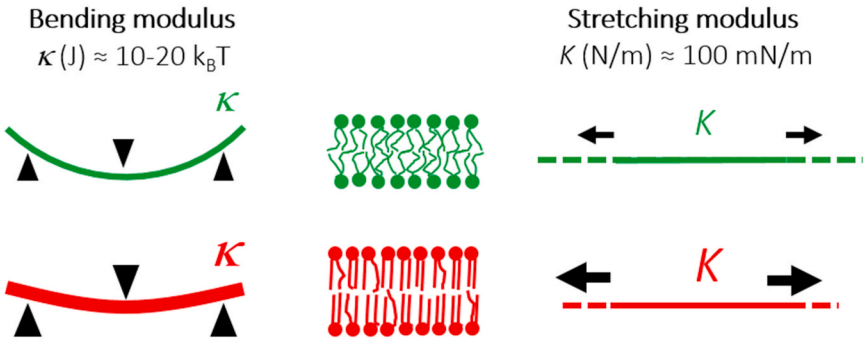
$$\Delta E_{bending} = \frac{\kappa}{2}\int dA\,(c - c_0)^2$$



The surface tension gives the surface energy required to stretch a patch of membrane with an initial area A_0. Deviations to the initial area (actual area, A), then the membrane patch is under stress as it is prevented from reaching the configuration of minimum stretching energy (equation below). The difference between the actual and initial area is key information to understand how the membrane system will evolve under particular conditions. For deflated vesicles surface tension is considered to be close to zero (σ<<10^{-6} N/m)

$$\sigma = \frac{K}{2}\int dA\left(\frac{A - A_0}{A_0}\right)^2$$

***Elastic moduli** are the proportional constants between the energy of the system and the deformation. It measures the resistance of a material to being deformed*



Elastic moduli depend on the lipid composition. For exemple and generally speaking, unsaturated lipids are more deformable than saturated lipids

membrane acting as a lipid reservoir and headed by a membrane tip. Tethers occur in cells and play an important role in fundamental processes such as cell migration [84], intracellular trafficking [85] and signalling [86]. Clearly, we emphasize here the tether-like structures forming *cristae* (see Fig. 1). Artificially pulled from giant vesicles, lipid tubes have been used to study the mechanical properties of lipid membranes and understand how the bilayer composition and protein activity dictate membrane behaviour. Evans and Yeung were the first to provide the tubulation force in the absence of spontaneous curvature [87]. The equilibrium pulling force is given by the expression

$$f_{tub} = 2\,\pi\,\sqrt{2\kappa\sigma} \tag{2}$$

where σ is the surface tension and κ is the bending modulus. One might expect that σ and κ tend to reduce or increase the radius of the tube respectively (see Box 1). Certainly, the radius of the tube is given by $R_t = \sqrt{\kappa/2\sigma}$. Also, a direct consequence from Eq. (2), is that tensionless and softer membranes are more easily tethered. This agrees with the picture that deflated vesicles characterized by vanishing surface tension, can act as membrane reservoirs. Finally, note that the surface tension is increased during tether formation unless it remains constant through a "tension buffering" mechanism [88]. Experimentally, the surface tension can be maintained by micropipette aspiration on giant vesicles for example. In the absence of a constant surface tension, the bending energy cost associated to the formation of very narrow tethers prevents reaching very high surface tensions.

Motivated by the experimental observations of spontaneous tubulation, i.e. in the absence of external forces, Lipowski has demonstrated that an asymmetrical adhesion of molecules on the different leaflets of lipid bilayers leads to the generation of an additional spontaneous curvature [89]. Depending on the nature of the adsorbed molecules the generated spontaneous curvature can range from 0.01 to 10 μm^{-1}. The membrane bends away from the leaflet with smaller coverage of adsorbed molecules. Interestingly, four remarkable predictions are obtained using energetics and stability arguments: a) tubes are preferred to vesicle budding upon spontaneous curvature generation on planar supported bilayers and slightly inflated large vesicles as long as the elastic properties are homogeneous along the membrane patch; b) the formation of outward and inward tubes simultaneously in the same vesicle is not allowed and only all inward or outward tubes are

produced; c) additionally, all the connected tubes to the spherical membrane have the same radius; d) inward tubes are stable in large vesicles, as the osmotic pressure difference ΔP across the membrane (larger inside the vesicle) balances the surface tension generated upon tubulation. Relevant for mitochondrial shaping; similar predictions can be straightforward translated for multilamellar liposomes. The external bilayer of the multilayer stack behaves as a supported bilayer in adhesion with the inner layer experiencing a mutual attractive interaction.

3.2. Active ATP synthases optimize the mechanical behaviour of lipid membranes to be shaped

We have already pointed out in the previous section that lipid vesicles at equilibrium can exhibit very complex structures, sometimes very similar to *cristae*. A set of mechanical parameters can explain shape changes without any energy input and dissipation, including the growth of highly curved tubes in the absence of external forces. However, *cristae* structures are most likely to have an out-of-equilibrium origin since the cell is continuously disspating energy. In conjunction with other identified protein key players [5–9] here we describe how active ATP synthases can lead to mechanically adapted membranes. This involves soft membranes with a high compliance to bend and adopt high curvatures.

Analysing the shape fluctuations or normal undulations of quasi-spherical vesicles containing the ATP synthase, a method called flicker spectroscopy [90], the bending modulus and the surface tension of membranes were obtained in the absence (passive case, κ_{pass} and σ_{pass}) and in the presence of protein activity (active case, κ_{act} and σ_{act}) [91]. Upon rotation, ATP synthase promoted large nonequilibrium deformations at particular sites of the membrane and the surface tension ($\sigma_{act}/\sigma_{pass} \approx 0.1$) and bending modulus ($\kappa_{act}/\kappa_{pass} \approx 0.25$) were reduced in consequence. Although Eq. (2) requires the addition of other second order terms for multicomponent systems, one might expect that the membrane softening and the surface tension lowering promoted by ATP synthase activity might result in weaker forces ($f_{act}/f_{pass} \approx 0.1$) to produce membrane tubulation in such mechanically active membranes. The mechanical energy savings due to ATP synthase activity might contribute to improve the elastic behaviour of the mitochondrial inner membrane from the point of view of *cristae* generation.

From symmetry, it remains clear that a pure rotation does not couple to normal displacements of the membrane unless a change in spontaneous curvature emerges from protein activity. The emergence of a spontaneous curvature is firstly supported by the proton pump activity of ATP synthase through the membrane-spanning component. The localized pH gradients would be the driving force for a preferred spontaneous curvature [89]. The effect might be enhanced by the change in lipid packing induced by pH in cardiolipin-containing bilayers [92]. *In vitro* experiments have shown that *cristae*-like invaginations can be created by an externally controlled pH gradient in giant vesicles comprising cardiolipins [93]. The out of equilibrium membrane deformations can be also maintained by a diffusive flux of protons [94]. A second mechanism that gives structural support to a positive spontaneous curvature and thus to a localized membrane softening is provided by the coupling between the protein shape and the membrane curvature under active conditions [95]. Consequently, the apparent bending modulus shifts to lower values. The enhanced nonequilibrium fluctuations observed in Ref. [91] were compatible with an accumulation of active proteins at highly curved patches. However, the stability of the membrane might be compromised if the curvature produced by protein activity attracts an excess of proteins, i.e. the clustering of many proteins into curved regions. For stable conditions, the curvature-protein coupling mechanism might be essential for *cristae* maintenance upon ATP synthase dimerization [10–12].

3.3. Curvature sorting of ATP synthases

Although isolated mitochondrial ATP synthase is fully active as a monomer, the ubiquitous occurrence of ATP synthase dimers in eukaryotic cells suggests that they may have a critical role in oxidative phosphorylation and the subsequent bending of the mitochondrial membrane, which is necessary for *cristae* formation [47,96]. The binding through the F_o domains of the ATPase is essential for the dimer formation (see Section 2.2). The ability of conic-like ATP synthase dimers to partition into highly curved membranes has been also proposed to induce membrane curved shapes characterized by a wedge angle within the range of 55° and 120° [10–12]. Dimers, in turn, can assemble into rows, which account for the formation of highly curved ridges in mitochondrial *cristae* [13–16]. By means of molecular simulations, it has been suggested that the dimers of ATP synthases organize into rows spontaneously, driven by a long-range attractive force that arises from the relief of the overall elastic strain of the membrane. In other words, the bending energetic costs of gathering dimers into rows is smaller than a homogeneous distribution of dimers within the membrane [97].

Compared to their mitochondrial counterparts, bacterial ATP synthases lack some extra subunits responsible for specific interactions for dimerization and prokaryotic ATP synthase is thought to occur exclusively as a monomer. This and the existence of a turgor pressure inside the bacterial cytoplasm might prevent the formation of *cristae*-like structures in prokaryotic cells. However, the activity of ATP synthase from *E. coli* in giant vesicles (see Section 3.2) promoted localized nonequilibrium membrane fluctuations where the active proteins might be clustered [91]. Does it mean that protein activity alone, in the absence of dimeric proteins, can generate spontaneous curvatures and thus contribute to the generation curved regions? In other words, the dimeric protein might sense the curvature of pre-existing *cristae* and the active monomeric ATP synthase act as a spontaneous curvature generator. However, there is no experimental demonstration for an active contribution to protein sorting into lipid tethers and/or the curvature sensing already sense preformed curved regions. This might allow to allocate a definitive role for ATP synthase activity to protein clustering into curved regions and hence *cristae* biogenesis and maintenance. Note that the cross-linking of ATP synthase complexes *in vivo* eliminates the mitochondrial cristae, so the structural arrangement of protein dimerization might not be enough for curvature generation [98].

Tube formation is now conventionally used for studying membrane curvature sorting and sensing of proteins [99]. We propose here to measure the protein sorting of monomeric ATP synthases into lipid tethers under active and passive conditions to demonstrate if the softening effect of the protein activity on the membrane bending modulus might trigger the protein sorting. We anticipate that for a large protein ($Area_{F_o} \approx 300\ nm^2$) in a softened membrane ($\kappa_{act}/\kappa_{pass} \approx 0.25$), the sorting curvature $c_{sorting} \approx 100 nm^{-1}$ is rather small and compatible with the *cristae* size [100,101].

4. *Cristae* and mitochondrial dynamics

4.1. Cristae biogenesis and mitochondrial dynamics

The current view of the inner mitochondrial membrane defines two different membrane regions. The inner boundary membrane (IBM) and the *cristae*. Both are linked together through the *cristae* junctions (CJ), which are believed to act as diffusion barriers across the two compartments to prevent protein mixing between them (Fig. 3). However, the inner mitochondrial membrane, as well as the whole organelle, is not a static structure but undergoes multiple fission and fusion events upon mitochondrial dynamics [102]. A process that is mainly orchestrated by dynamin-like proteins, Opa1 among them [103], which is responsible for mitochondrial inner membrane fusion. The formation and maintenance of *cristae* is therefore coupled to the continuous exchange of

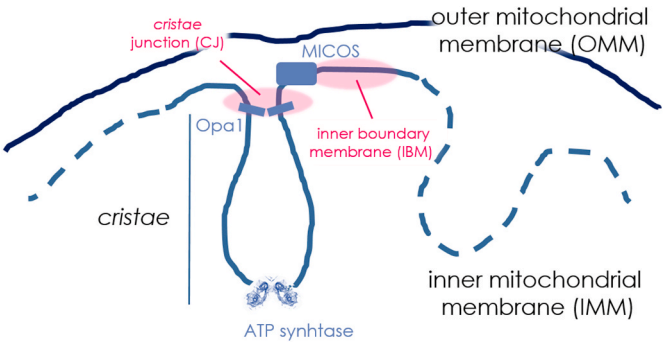


Fig. 3. Schematic representation of a mitochondrion with the inner and outer mitochondrial membranes (IMM and OMM) and *crista*. The main regulators of *cristae* morphology are included; MICOS and Opa1 at the inner boundary membranes (IBM) and *cristae* junctions (CJ) respectively and ATP synthase dimers at *cristae* rims.

membranes occurring during mitochondrial dynamics. Although there is not a fully understanding on how proteins time-regulate the biogenesis and maintenance of all membrane regions, the widely accepted structural picture of *cristae* and CJ allocates an essential role for Opa1, MICOS and ATP synthase [104,105]. Opa1 and MICOS are found at CJ and it is thought they control the number and stability of *cristae* [9,106]. As for ATP synthase, its localization at the rim of *cristae* as dimers and ribbons seems to be also important for the *cristae* modulation function of Opa1 [107]. The *cristae* are thus seen as specialized compartments within mitochondria to enhance OXPHOS efficiency.

The multiscale dynamics (*cristae* and mitochondria) also suggests the absence of a single mechanism for the formation and maintenance of *cristae* [2]. A first and intuitive pathway consists of the invagination of *cristae* from the IBM. As shown in Section 3, the emergence of a spontaneous curvature due to an asymmetric accumulation of molecules in the matrix side of the membrane is energetically favourable for membrane bending, tubulation and stabilization. A similar mechanism to the invagination model involves the action of CJ complexes as lipid and protein flowing gates, thus promoting the spontaneous curvature required for membrane invagination. Recent experimental results obtained with yeast [108] suggest that this mechanism is the leading pathway to the formation of tubular *cristae*. Moreover, the intra-mitochondrial lipid transport plays an important role in *cristae* formation [18]. A second mechanism comprises a fusion-dependent pathway, in which the outer membrane fusion and docking of inner membranes result into the evolution of an asymmetric fusion intermediate of inner membranes leading to the formation of *cristae*. Here, one could argue about the requirement of some mechanism to prevent the completion of membrane fusion. The formation of lamellar *cristae* following the fusion-dependent mechanism and the stabilizing role of MICOS is reported also in Ref. [108]. Although the presence of dimeric ATP synthases located at the *cristae* was necessary in both pathways, monomeric ATP synthases were also found in the docked inner membranes and IBM [108]. The two different population of ATP synthases, which seem to be spatially regulated, might have a different functional role. For that, the biogenesis of both ATP synthase and *cristae* should be orchestrated.

4.2. ATP synthase biogenesis and mitochondrial dynamics

The genes coding for the eukaryotic ATP synthase are expressed in both nuclear and organellar genomes. The products of nuclear genes must be imported into the mitochondria to assemble into a functional complex. All genes of the F_1-subunits and most genes of the F_o, except for the F_o subunits *a* and *8*, are encoded from the nucleus, whereas the F_o subunits *a* and *8* are encoded from the mitochondrial chromosome [109]. During the assembly process, a F_1-dependent control of F_o-subunit translation ensures a balance between mitochondrially encoded F_o-subunits and the imported F_1-subunits [110]. This controlled assembly is chaperoned by multiple accessory factors [111] and the Oxa1 protein translocase [112]. The step by step process involves different intermediates. Once the *c*-ring is assembled, the soluble F_1 module is thought to bind independently of other F_o-subunits to the *c*-oligomer via the stalk subunits to form a larger ATP synthase intermediate [113]. Different subcomplex intermediates as the *a*-8-stator intermediate [113] and the stator subunits (*b-d-F6/h-OSCP*) [114] are preassembled and merge in a final step with the subcomplex *c-a* to the mature F_1F_o complex to reduce a futile proton efflux and the dissipation of the mitochondrial membrane potential. As the monomeric ATP synthase is assembled within *cristae* [115], the presence of monomeric ATP synthase found at the IBM (see Section 4.1) may result from protein diffusion from *cristae* to the IBM or from the protein exchange/scrambling during mitochondrial dynamics [116].

The fully assembled monomeric mitochondrial ATP synthases dimerize then via the interaction of the first transmembrane helix of two adjacent *b*-subunits and the participation of subunits *e* and *g* [11,117]. This stepwise assembly process is aided by the two small subunits *i* and *k*, which facilitate incorporation of subunits *e* and *g* into the monomer and promote stabilization of the dimer, respectively [118]. Again, it is not known if dimers are assembled previously to their clustering into the tip of *cristae* or if the accumulation of monomeric proteins into curved regions favour their stabilization through the specific interaction provided by the different F_o subunits. Protein diffusion might be a determinant factor for regulating protein location within different membrane regions of *cristae*.

Dimers, in turn, can assemble into rows, which account for the formation of highly curved ridges in mitochondrial *cristae* [13–16]. However, the activity of the ATP synthase oligomers [119] in mitochondria might be correlated and regulated by the morphology and internal architecture of mitochondria [120,121]. In particular, the operating mode (hydrolysis/synthesis) has been shown to be dependent on the oligomeric state of the protein and its location in the inner membrane [122]. Thus, the increased ATP synthesis activity under glucose starvation through deoxyglucose incubation leads to the ATP synthase to be characterized by a smaller average diffusion coefficient following an anomalous diffusion and accompanied with larger trajectories out of *cristae*. A deeper analysis using specific inhibitor for ATP hydrolysis/synthesis suggested a reduced dimeric/oligomeric-to-monomeric protein ratio, with the monomeric subpopulation corresponding to the more mobile proteins, operating the ATP hydrolysis function, and preferentially localized in the IMB. In contrast, proteins in the ATP synthesis mode would be characterized with a lower diffusion and mainly located in *cristae*. The changes on the specific spatio-temporal arrangement of ATP synthase were corelated with a reduction in *cristae* width [107,122,123]. However, the protein levels of OPA1 [107, 122] were not affected under starvation, which suggests that the fast alteration of the protein dimers/monomer ratio should be responsible for the change in *cristae* morphology [107,122,124–126]. In contrast, the slow mitochondrial dynamics might have a minor role on the ATP synthase biogenesis and distribution.

4.3. Collective effects of rotors in viscous membranes

ATP synthases are embedded within the mitochondrial membrane. Cell membranes behave as two-dimensional fluids [127] and thus, the transport properties of the ATP synthase within the membrane are determined by the membrane viscosity following the Saffman-Delbrück model [128]. The ATP synthase diffusion coefficient, D_0, resulting from the Brownian motion within the membrane sets a time scale that is not compatible with the time scales required for the reversible and quick reaction of ATP synthases to metabolic stress described in 4.2. Moreover, the presence of high protein concentrations within the membrane, such as those found in the mitochondrial membranes [129],

significantly affect the dynamics of the proteins, effectively reducing their diffusion coefficient [130]. Since the ATP synthase transport properties depend on the membrane viscosity, dynamical changes of this viscosity will allow to regulate the transport properties of ATP synthases. On one side, metabolic stress can induce changes in the membrane composition leading to a change in the membrane viscosity and the diffusion properties of the ATP synthases. On the other side, the ATP synthase rotational activity might also play a crucial role on the transport properties.

The enzymatic activity of ATP synthases is generated by the torque exerted by the proton's membrane translocation through the subunit *a* in the ATP synthesis operation mode or by the hydrolysis of ATP in the F_1 domain [1]. In both operating modes, the subunit *c* rotates with a rotational velocity, ω, against the viscous drag exerted by the membrane. The protein movement generates a rotational flow within the two-dimensional fluid, where the azimuthal velocity in spherical coordinates decays as the inverse of the squared distance from the rotating protein in the limit of small Reynolds number, as $\sim \frac{1}{r^2}$ [131]. In the presence of multiple rotating proteins, the flow field generated by the ATP synthases interact, leading to the emergence of collective effects that can significantly modify the transport properties of the proteins, as well as the membrane structure and dynamical properties.

The transport properties of a set of rotors confined in a two-dimensional fluid have been studied [131]. Depending on the rotational velocity of the rotating protein, ω, nontrivial collective behaviours can be observed. In the absence of rotation, $\omega = 0$, ATP synthases freely diffuse within the membrane with a diffusion coefficient D_0. At slow spin velocities, the hydrodynamic interactions between the rotating proteins result into an increase of the protein diffusion ($D^{'} > D_0$) due to enhanced velocity fluctuations of active proteins within the generated rotational flows. At high rotational velocities beyond a critical rotational velocity ω_c, ($\omega > \omega_c$), proteins might be kinetically arrested ($D'' \approx 0$) and self-organize into a crystalline order (Fig. 4) [131]. Above the critical rotational velocity ω_c, the secondary flows generated by the protein's rotational activity result into a repulsive interaction between the rotating ATP synthases. This repulsive force between rotating proteins is the Magnus force, which corresponds to the first order inertial correction to the Stokes solution [132]. The stability of the crystalline network is guaranteed if the thermal energy is smaller compared to the input energy. To better evaluate this, we can employ the Péclet number. The Péclet number tells how fast the flow around the object is as compared to its thermal diffusion. In our case the Péclet number can be estimated as:

$$Pe = \frac{\omega R^2}{D_0} \tag{3}$$

where R is the radius of the protein. Taking typical values of $R = 5$ nm [34] and $\omega_{max} \approx 10^2 Hz$ [27], we obtain $Pe \approx 1$ and the thermal energy simply prevents the formation of ordered phases at physiological temperature [133]. In other words, the critical frequencies for protein ordering predicted for ATP synthase (of $\omega_c \approx 10^4 - 10^8\, Hz$) are unachievable [131] but proteins might be in a melted stated characterized with $D^{'} > D_0$. The collective effects on protein diffusion can be easily demonstrated using simple fluorescence approaches such as FRAP on supported lipid bilayers or giant vesicles [134]. Other collective effects such as phase separation or synchronization [19–24] might be also explored and be relevant for ATP synthase function.

5. Concluding remarks

Fuelled with an electrochemical gradient, the beautiful rotating process of ATP synthases triggers the conformational changes on the catalytic site of the protein for ATP synthesis. The process is reversible and an excess of ATP results on a reverse rotation of the protein and a proton translocation across the membrane. Besides the main functionality of protein rotation, we have discussed in this review the importance

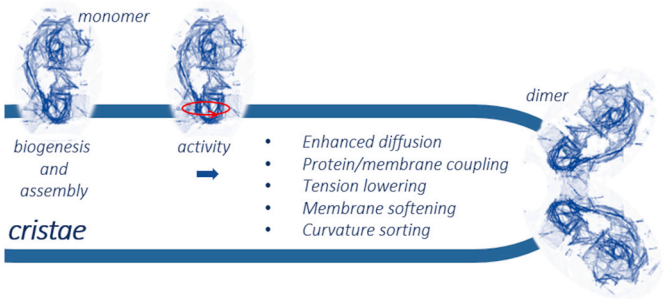


Fig. 5. Monomeric ATP synthases are synthesized and assembled within *cristae* [124]. Protein activity triggers membrane softening and tension lowering [91]. The mechanical adaptation might facilitate membrane tubulation. In addition, membrane softening might be also responsible for an enhanced protein-to-curvature coupling thus results into curvature sorting. The transport properties of ATP synthase can be regulated in a framework containing a set of rotating ATP synthases.

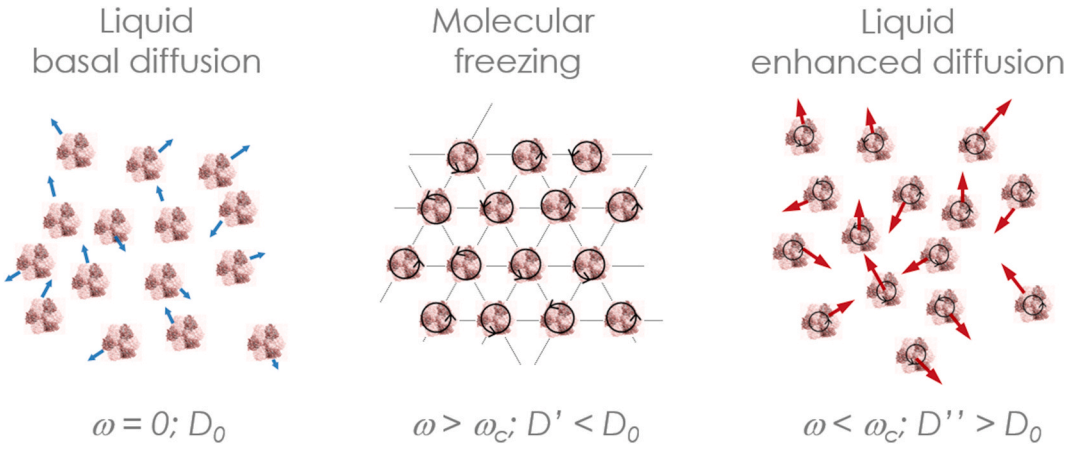


Fig. 4. Phase behaviour of rotating ATP synthases in lipid membranes. In the absence of rotation, $\omega = 0$, ATP synthases freely diffuse within the membrane with a diffusion coefficient D_0. At high rotational velocities ($\omega > \omega_c$) proteins are kinetically arrested ($D^{'} \approx 0$). At low spin velocities, protein diffusion is enhanced ($D'' > D_0$). The spin velocity can be tuned with the ATP concentration and the critical angular velocity can be modified by changing the viscoelastic properties of the embedding lipid membrane and the protein packing.

of the rotatory movement of ATP synthases on membrane remodelling, which might be fundamental for mitochondrial shaping (Fig. 5). Through specific protein-protein interactions, the arrangement of dimeric ATP synthases at the rim of the *cristae* stabilizes mitochondrial ultrastructure, morphology, and function. Notably, rotating ATP synthase monomers promote large non-equilibrium deformations in lipid bilayers inducing membrane softening [91], in agreement with theoretical predictions [95]. In translation to the biological system the rotation of ATP synthase might be convenient to reduce the mechanical energy necessary to physically remodel the mitochondrial membranes. Moreover, the membrane softening under active conditions might result in the protein clustering on curved membranes. We suggest quantifying the curvature sorting of rotating ATP synthase monomers using tether pulling experiments. A putative active sorting might contribute to understand the physical mechanism facilitating the generation and maintenance of curved regions as well as the protein distribution, which is very dynamic under bioenergetic stress [122]. The fast rearrangement of the monomeric and dimeric subpopulations suggests a control of their diffusive properties. An enhanced diffusion is predicted for a set of rotating proteins homogeneously distributed within a viscous membrane [131]. It is therefore tempting to assume a direct link between the rotatory movement of ATP synthases and the mitochondrial ultrastructure. This opens the way to a new generation of experiments which will teach us the importance of collective effects in the ATP synthase behaviour, up to now ignored.

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