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ORIGINAL PAPER

Regulation of the F_1F_0-ATP Synthase Rotary Nanomotor in its Monomeric-Bacterial and Dimeric-Mitochondrial Forms

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Abstract The F_1F_0-adenosine triphosphate (ATP) synthase rotational motor synthesizes most of the ATP required for living from adenosine diphosphate, Pi, and a proton electrochemical gradient across energy-transducing membranes of bacteria, chloroplasts, and mitochondria. However, as a reversible nanomotor, it also hydrolyzes ATP during de-energized conditions in all energy-transducing systems. Thus, different subunits and mechanisms have emerged in nature to control the intrinsic rotation of the enzyme to favor the ATP synthase activity over its opposite and commonly wasteful ATPase turnover. Recent advances in the structural analysis of the bacterial and mitochondrial ATP synthases are summarized to review the distribution and mechanism of the subunits that are part of the central rotor and regulate its gyration. In eubacteria, the ε subunit works as a ratchet to favor the rotation of the central stalk in the ATP synthase direction by extending and contracting two α-helixes of its C-terminal side and also by binding ATP with low affinity in thermophilic bacteria. On the other hand, in bovine heart mitochondria, the so-called inhibitor protein (IF_1) interferes with the intrinsic rotational mechanism of the central γ subunit and with the opening and closing of the catalytic β-subunits to inhibit its ATPase activity. Besides its inhibitory role, the IF_1 protein also promotes the dimerization of the bovine and rat mitochondrial enzymes, albeit it is not essential for dimerization of the yeast F_1F_0 mitochondrial complex. High-resolution electron microscopy of the dimeric enzyme in its bovine and yeast forms shows a conical shape that is compatible with the role of the ATP synthase dimer in the formation of tubular the cristae membrane of mitochondria after further oligomerization. Dimerization of the mitochondrial ATP synthase diminishes the rotational drag of the central rotor that would decrease the coupling efficiency between rotation of the central stalk and ATP synthesis taking place at the F_1 portion. In addition, F_1F_0 dimerization and its further oligomerization also increase the stability of the enzyme to natural or experimentally induced destabilizing conditions.

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Keywords F_1F_0 ATPase · F_1F_0 ATP synthase · IF_1 · Inhibitor protein · Epsilon · Dimeric · Rotation · Interface · Cristae · Regulation · F_1 ATPase

Abbreviations

EF_1, EF_1F_0	*Escherichia coli* F_1 and F_1F_0 complexes
EM	electron microscopy
F_1F_0	the whole ATP synthase complex with its catalytic (F_1) and proton channel (F_0) parts
F_1F_0I	the whole ATP synthase containing its physiological inhibitor protein (IF_1)
IF_1	the intrinsic inhibitor protein of the mitochondrial ATP synthase
MF_1, MF_1F_0	bovine heart mitochondrial F_1 and F_1F_0 complexes, respectively
NMR	nuclear magnetic resonance spectroscopy

1 Introduction

The mitochondrial adenosine triphosphate (ATP) synthase is a ubiquitous motor enzyme that provides most of the cellular chemical energy in the form of ATP to fuel all kinds of work in biological nature. This motor functions as a coupling factor between the condensation of adenosine diphosphate (ADP) and Pi that takes place at its catalytic F_1-ATPase portion and proton flow through the transmembranous F_0-proton channel that consumes energy from electrochemical proton gradients. According to the well-established chemiosmotic theory, this proton gradient is established by oxidative or photosynthetic electron transfer chains of the plasma membrane of bacteria, the inner mitochondrial membrane, and the thylakoid membranes of chloroplasts. Because of thermodynamic and mechanical reversibility, the F_1F_0-ATP synthase becomes a proton-pumping F_1F_0-ATPase under conditions of partial or total collapse of the proton gradient; for instance, during anoxia in bacteria where it works as a primary pump to drive secondary transporters, during ischemia in mitochondria, or under dark conditions in chloroplasts. In all these systems, different subunit structures control gyration of the central stalk by favoring rotation in the ATP synthase turnover direction. Chloroplast ATP synthase possesses a unique disulfide bridge in the γ subunit that controls rotation of the central stalk; however, only the structures of the bacterial and the bovine enzymes are reviewed here.

Important new information such as the dimerization of the ATP synthase and the role of the inhibitor protein (IF_1) in this process are also reviewed and used to propose a model of the structure of the ATP synthase dimmer that explains the inhibitory and dimerizing roles of IF_1. This model also explains how dimerization of the ATP synthase may confer a higher stability and efficiency of the dimeric enzyme to synthesize ATP. This also sheds light on how F_1F_0 dimerization promotes formation of tubular cristae membrane structures in mitochondria after further polymerization.

2 Structure and Rotational Mechanism of the ATP Synthase

The catalytic part of the enzyme is a water-soluble portion (F_1) that can be released in vitro from the membrane, retaining its capacity to hydrolyze ATP (F_1-ATPase) [1]. ATP synthesis occurs in the whole F_1F_0 when the energy derived from proton conduction through the F_0 membrane channel is combined with the nucleotide (Mg-ADP) and Pi binding energies

[2–5] to drive the release of newly synthesized ATP from each of the three alternating catalytic sites of F_1. The coupling between F_1 and F_0 is critical for efficient ATP synthesis to occur and major progress in the understanding of this coupling mechanism has been achieved. Several approaches at different laboratories showed that a central rotor actually gyrates relative to a stator that holds the catalytic subunits; this rotation induces the alternating binding, catalysis, and product release from three catalytic sites of F_1 (for reviews, see [5–9]). These studies also indicated that the γ subunit, together with ε and the ring of 9–15 c subunits of F_0, form the rotor in the central part of the enzyme. The more direct evidence demonstrating this rotational movement was the observation by fluorescence microscopy of rotation of a fluorescent actin filament attached to the γ, ε, or c subunits of immobilized F_1 and F_1F_0 complexes [10–13]. These experiments established that the rotor of the enzyme is formed by the central γ–ε–c_{9-15} domain. This core rotor–stator structure is preserved in bacterial and mitochondrial ATP synthases and is shown in Fig. 1 indicating the reversible rotational mechanism of the enzyme.

The rotary mechanism of the enzyme implies that, in the central stalk, the γ subunit (together with ε and c_{9-15} subunits) rotates relative to a stator where the catalytic α–β interfaces are held. Therefore, this stator must be somehow anchored to an F_0 subunit at the lipid bilayer. A peripheral second stalk was literally "invoked" and found by means of high-resolution electron microscopy studies [14, 15] and by cross-linking of α, δ, b,

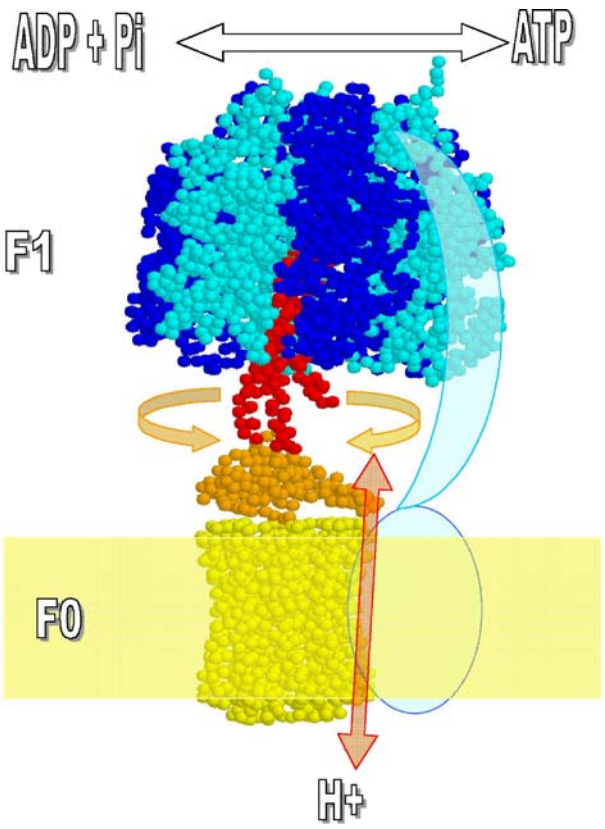

Fig. 1 Rotor–stator subunit distribution in the mitochondrial F_1F_0-ATP synthase. Only the core subunits present in bacteria and mitochondria are shown for simplicity. Rotating subunits are shown in *red* (γ), *orange* (ε), and *yellow* (ring of c subunits) whereas static subunits are in *dark blue* (β), *blue* (α), and *cyan* (subunits *a* and *b*). The *arrows* indicate the reversible rotation of γ–ε–c subunits relative to α and β catalytic subunits of F_1 that takes place during ATP synthesis ("*clockwise*" or *right direction*) and hydrolysis ("*counterclockwise*" or *left direction*). Bidirectional proton flow at the c-ring–sub *a* interface occurs associated with the gyration of the rotor as indicated by the *red arrow*. The second-stalk structure is simplified as two cyan subunits (*a* and *b*) that work as stator to anchor the catalytic $\alpha_3\beta_3$ to the membranous *a* subunit. Image is generated in RasMol 2.6 from the mitochondrial F_1F_0 structure of *S. cerevisiae* (PDB code 1Q01) and edited as shown

Table 1 Subunit composition of the *E. coli* and mitochondrial ATP synthases

	E. coli subunit	Bovine subunit	Yeast subunit
F_1	α^3	α^3	α^3
	β^3	β^3	β^3
	γ^1	γ^1	γ^1
	δ^1	OSCP^1	Sub 5^1
	ε^1	δ^1	δ^1
	–	ε^1	ε^1
	–	IF_1^1	IF_1^1
F_0	A^1	Sub. 6^1	Sub. 6^1
	B^2	b^1	*Sub* 4^1
	C^{9-15}	c^{9-15}	Sub 9^{10}
	–	d^1	Sub 7^1
	–	e^1	e^1
	–	f^1	f^1
	–	g^1	g^1
	–	$F6^1$	h^1
	–	A6L^1	Sub 8^1

There are eight and 16 different subunits in the bacterial and bovine enzymes, respectively. Subunits are accommodated according to their corresponding homologs. For example, *E. coli* δ and ε correspond to bovine OSCP and δ, respectively. Corresponding subunit stoichiometries are indicated as superscripts. The enzyme from yeast (*S. cerevisiae*) contains at least three additional subunits, namely i, j, and k.

and *a* subunits along this peripheral second stalk [7]. The anchoring part of the second stalk with the α subunit of F_1 has been solved by nuclear magnetic resonance (NMR) for the *Escherichia coli* enzyme [16], whereas most of the structure of the second stalk of the bovine mitochondrial enzyme has been resolved by X-ray crystallography [17]. Thus, the whole picture of the simplest ATP synthase of *E. coli* involves a stator formed by $(\alpha–\beta)_3$, δ, b_2, and *a* subunits and a central rotor formed by the $\gamma–\varepsilon–c_{9-15}$ domain. This core rotor–stator structure of bacterial ATP synthase becomes more complex with about twice as many different subunits present in chloroplasts and mitochondria. Besides the core subunits and structure of the EF_1F_0 motor, there are six to eight additional or "supernumerary" subunits that are well described in mitochondrial yeast and bovine ATP synthases. These subunits are d, e, f, g, F6 (h in yeast), A6L (8 in yeast), the inhibitor protein (IF_1), and mitochondrial subunit ε which does not have a bacterial counterpart (see below and Table 1). Three additional F_0 proteins are also found in the yeast enzyme (see legend of Table 1). The roles of these additional subunits are related to regulation and oligomerization of the ATP synthase as will be described below.

3 The Central Stalk is Part of the ATP Synthase Rotor

Crystallographic studies have solved most of the central stalk structure in *E. coli* (EF_1) [18] and bovine mitochondrial (MF_1) F_1-ATPases [19–22]. The tertiary structure and orientation of the globular domain of the γ subunit is very similar in both species (Fig. 2), and it is in agreement with previous cross-linking data obtained with the enzyme from *E. coli*. However, the ε subunit of *E. coli* (bovine δ) was found far away from the cross-linking distance to the α or β subunits but closer to the F_0 subunit c in the crystals of the yeast [23] and bovine [20–22] enzymes. It was therefore unclear how ε could cross-link with α

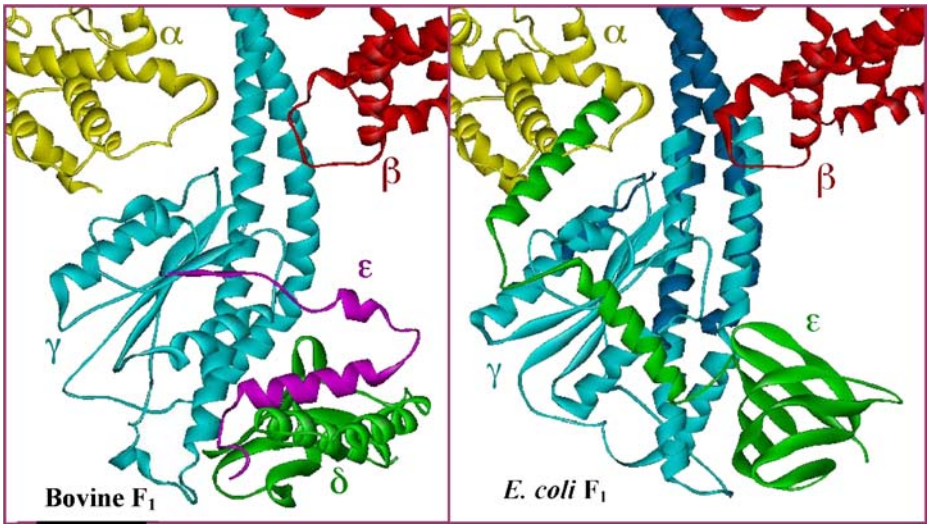

Fig. 2 Comparison of the bovine (*left*) and *E. coli* (*right*) F_1F_0-ATP synthases at the central stalk domain. Crystallographic structures of MF_1 and EF_1 central stalks are shown in the same orientation. Homologous subunits are drawn in the same color, γ (*blue*), ε subunits (*green*). For clarity, only one α subunit (*red*) and one β subunit (*yellow*) are shown. The structure shown on the *right* is a composite of the *E. coli* γ–ε structure [18] and the bovine MF_1 structure [19], constructed by aligning segments of γ present in both structures. Segments of MF_1γ subunit are shown in *darker blue*, and those of *E. coli* γ subunit are shown in *lighter blue*. This figure was modified from an original courtesy of Dr. Andrew J.W. Rodgers

or β subunits in the native enzyme as found before, until another X-Ray diffraction analysis was made with a soluble γ–ε complex from *E. coli* [18]. In this study, the structure of soluble ε was very different from the bovine δ subunit F_1. The soluble ε subunit associated with γ was found rotated in relation to the vertical axis of the central stalk and extending its two C-terminal helices toward the C-termini of α and β. This position placed the appropriate residues in cross-linking distance [18] (Fig. 2). The conclusion is therefore that ε experiences dramatic changes in conformation that are important for its role as an inhibitor of the ATPase activity of the enzyme, controlling the rate of rotation of the central stalk. Engineered cross-linking in the *E. coli* F_1F_0 complex entrapped these two conformations of the ε subunit [24]. Interestingly, when the C-terminus of ε is compacted as an antiparallel α-helix coil with its N-terminal β-sheet domain, the F_1F_0-ATPase activity is enhanced and the enzyme is coupled during ATP hydrolysis and synthesis. However, when the C-terminus of ε extends toward F_1 (as shown in Fig. 2), ATP hydrolysis is inhibited but ATP synthesis remains unaffected [24]. In agreement with this work, the structure of the γ–ε domain in the *E. coli* F_1-ATP synthase [25] was found very similar to that of the isolated subunits [18]. Furthermore, it has been found that the ratchet mechanism of ε can be regulated by ATP binding in some bacteria [9, 26]. When ATP is bound, the closed conformation is stabilized, thus favoring rotation of the central stalk in the ATPase direction; conversely, at low ATP concentrations, ε is unable to bind ATP, and therefore the extended conformation is favored, thus leaving the enzyme prone to rotate into the ATP synthase turnover. This model is supported with the recent crystal structure of the *Bacillus* PS3 subunit ε with ATP associated to the C-terminus of this subunit [27]. In summary, these studies show that ε works as an ATP sensor in bacteria that posses a novel ATP-binding motif in this subunit [26–29].

Besides the control of rotation described so far for the bacterial enzymes by the ε subunit, it is important to introduce a novel inhibitory 11-kDa protein that we recently found in the ATP synthase of the α-proteobacteria *Paracoccus denitrificans* (Morales-Ríos et al. 2008, submitted). The ATP synthase from *P. denitrificans* has been only described functionally as the fastest ATP synthase and the slowest ATPase found to date [30]; however, it has never been isolated until we addressed this issue. This novel inhibitory 11-kDa protein is present in F_1-ATPase and F_1F_0-ATPase preparations obtained from *P. denitrificans* membranes, and it will likely add a novel control and inhibitory mechanism to the α-proteobacteria family where the open reading frame exists. Importantly, the ε subunit of this enzyme does not inhibit the ATPase activity of the F_1-ATPase or F_1F_0-ATPase complexes in *P. denitrificans* (Morales-Ríos et al. 2008, submitted). Thus, the 11-kDa protein will add a novel control mechanism to the ATP synthases, in addition to the classical inhibitory mechanisms of bacterial, chloroplast, and mitochondrial F_1F_0 complexes. Unidirectional functioning of ATP synthase turnover has been also described for another bacterial enzyme of the thermoalkaliphilic type, *Bacillus sp*. TA2.A1 [31]. However, instead of additional regulatory proteins, unique polar interactions at the rotor–stator interface of the F_1 subunits allow almost exclusively unidirectional rotation in the ATP synthase direction for this enzyme [31]. To our knowledge, only two other bacterial proteins have been found encoded in the *atp* operon in addition to the eight core subunits of bacterial F_1F_0 ($\alpha, \beta, \gamma, \delta, \varepsilon, a, b, c$); these two proteins are encoded by the *unc*-I and *urf*-6 genes that correspond, respectively, to an assembly factor of the c-ring [32] and to majastridin, a cytosolic protein nonassociated with the *Rhodospirillum blasticus* ATP synthase [33]. In contrast, the gene encoding the 11-kDa protein of *P. denitrificans* is located upstream to both *atp* operons (one for F_0 and another for F_1 subunits) already sequenced on chromosome II of *P. denitrificans* (see Morales-Ríos et al. 2008, submitted, and the following link: http://genome.jgi-psf.org/finished_microbes/parde/parde.home.htm). Therefore, it seems that the 11-kDa regulatory protein that we found in the F_1F_0 complex of *P. denitrificans* is one of the first, if not the first, supernumerary subunit added to bacterial ATP synthases as an exogenous gene of the *atp* operon (formerly known as the *unc* operon). This 11-kDa protein therefore emerged during α-proteobacterial evolution and previous to the endosymbiotic event from which mitochondria emerged.

4 Supernumerary Subunits and Their Role in the Regulation and Dimerization of the Mitochondrial ATP Synthase

Most of the supernumerary subunits in the mitochondrial enzyme correspond to membrane proteins associated with the F_0 proton channel. These additional subunits are d, e, f, g, F6, and A6L. Subunits d and F6 are part of the second stalk, and A6L is a membrane protein of F_0 that is essential for the assembly of subunit 6, the one that forms the proton-conducting interface with the c_{9-15} ring. On the other hand, the roles of some of these subunits were recently unveiled by studies in yeast showing that subunits e and g are needed to form F_1F_0 dimers in situ [34, 35]. However, an unexpected result was the finding that genetic removal of these e and g subunits deformed the inner mitochondrial membrane and the classical cristae transformed into concentric membrane layers inside enlarged mitochondria [35]. This demonstrated that dimerization of the ATP synthase is not an artifact of detergent extraction as suspected but a natural and important biological process that improves the ATP synthase activity and the stability of the enzyme. Thus, besides dimerizing to improve

somehow the ATP synthesis reaction, the dimeric enzyme also promotes mitochondrial cristae formation, thus optimizing the overall process of oxidative phosphorylation.

Two supernumerary subunits are part of the mitochondrial F_1, namely ε and the so-called inhibitor protein (IF_1). Bovine ε is different with its bacterial homonym; it is a 5.7-kDa protein, whereas that of *E. coli* is 15 kDa in size (Table 1 and Fig. 2). Bovine ε stabilizes the structure of the central stalk by interacting with the globular part of γ (Figs. 2, 3, and 4). Closely interacting with ε, bovine subunit δ has a similar structure to that of its homologous *E. coli* ε. However, neither bovine δ or ε subunits inhibit the ATPase activity of bovine F_1F_0; together, they form a compact and noninhibitory structure at the central stalk, in contrast to the flexible structure of *E. coli* ε (Fig. 2) [20]. The bovine and *E. coli* δ subunits also correspond to different proteins. *E. coli* δ does not form part of the central stalk as the bovine δ does (Table 1, Fig. 2). *E. coli* δ is the connection between the "tip" of the F_1 subunit α and the "top" of the peripheral second stalk (reviewed in [7]). It is homologous to bovine

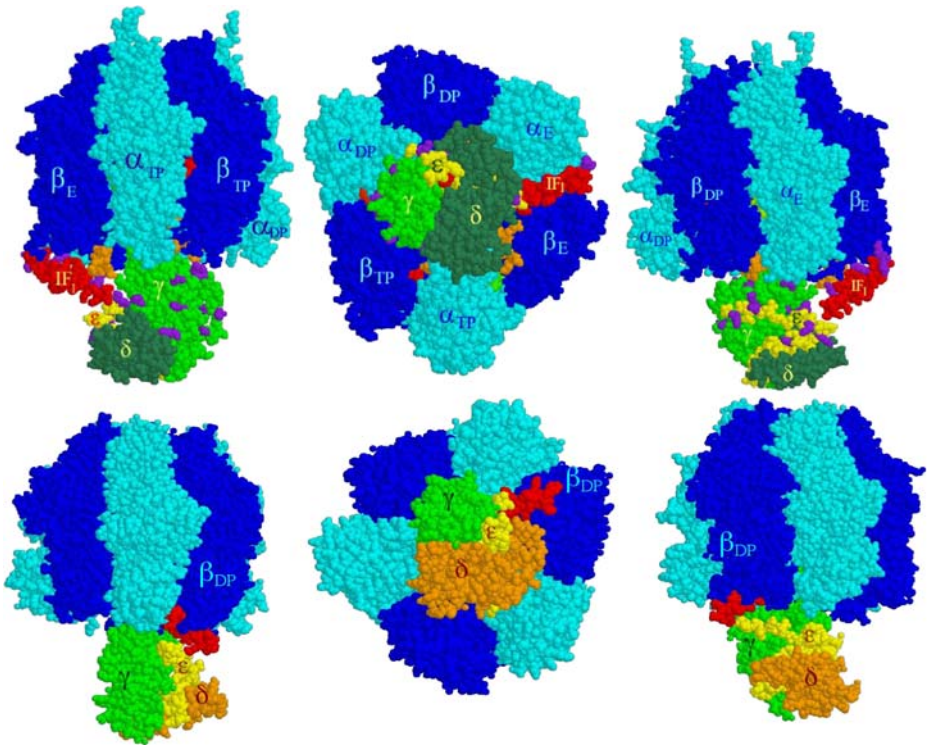

Fig. 3 Model and crystal structures of the F_1–IF_1 complex from bovine heart mitochondria. *Top three panels*, our model: we positioned the IF_1 N-terminal domain at an entrance-binding site (α_E–β_E interface) at about 12-Å cross-linking distance from γ and ε subunits as we found [44]. From the side (*left* and *right*) and "bottom" (*center*) views, it was clearly shown and proposed for the first time that IF_1 is close enough to the rotor of the enzyme to block gyration of the central stalk as part of its inhibitory mechanism [44]. *Bottom panels*, the crystal structure from the F_1–IF_1 crystal with a nondimerizing fragment of IF_1 [21]: the same IF_1 N-terminal side was resolved and observed actually bound to the γ subunit at an α_{DP}–β_{DP} interface [21, 22]. The *top structure* depicts the entrance site of IF_1, whereas the *bottom structure* shows the final inhibited structure where IF_1 is locked into the same α_E–β_E interface that became α_{DP}–β_{DP} after two counterclockwise 120° gyration steps (shift from *top* to *bottom panels*). IF_1 therefore inhibits rotation of the central stalk and the opening and closing conformational changes of a single catalytic interface

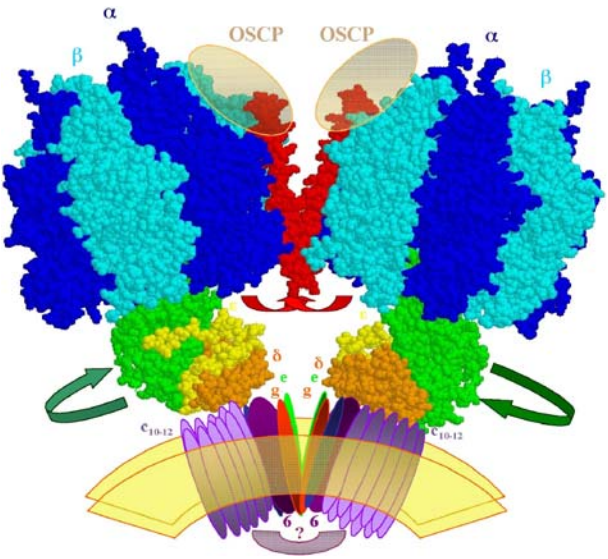

Fig. 4 Model of the dimeric-mitochondrial ATP synthase: possible localization of the IF_1 protein and its movements to allow rotation of the central stalk during ATP synthesis. The model depicts the overall shape of the dimeric ATP synthase molecule that we observed for the bovine mitochondrial enzyme [54]. The dimeric interface involves F_0 subunits (*e* and *g*) and two protein bridges, one at the F_0–F_0 side of unknown composition (*question mark*) and another at the F_1–F_1 interface where the second stalks (not shown for clarity) and the IF_1 protein (*red*) are likely to be located. The C-terminal side of the IF_1 molecule is assumed to cross the dimer interface and to stabilize the dimer by interacting with subunits OSCP [65] and possibly subunits of the second stalk. The N-terminal inhibitory domain that in the absence of the proton gradient blocks rotation of the central stalk by entering at an α–β–γ interface (Fig. 2) is removed from this position and exposed into the media after establishment of a transmembrane proton gradient, thus allowing rotation of the central stalk during ATP synthesis. The F_1 structures were constructed from the bovine F_1-DCCD coordinates available (PDF code 1E79)

oligomycin sensitivity conferring protein (OSCP) which also interacts with subunits of the second stalk (see Fig. 4, and [17, 36–38]). As mentioned above, the connection between *E. coli* δ and α subunits has been resolved by NMR [16].

5 The Inhibitor Protein (IF_1) and its Inhibitory and Dimerizing Roles on the Mitochondrial ATP Synthase

A key regulatory subunit absent in bacterial or chloroplast F_1F_0 is the mitochondrial inhibitor protein (IF_1). Since its first isolation in 1963 by Pullman and Monroy [39], this protein was shown to inhibit the ATPase activity of the catalytic F_1 part. This protein is therefore crucial to preventing the hydrolysis of newly synthesized ATP in conditions of low membrane potential in mitochondria. Upon membrane energization, IF_1 is believed to be relocated from its inhibitory site into an unknown position within [40, 41] or outside the F_1F_0 complex [42, 43], therefore allowing ATP synthesis to occur. In de-energized or uncoupled conditions, such as ischemia, the bovine IF_1 is productively associated with the enzyme, inhibiting the ATPase turnover of the F_1I or F_1F_0I complexes. However, this protein allows the rotational ATP synthesis turnover during energization of mitochondrial membranes. Therefore, IF_1 is an important physiological regulator of the functioning of the ATP synthase.

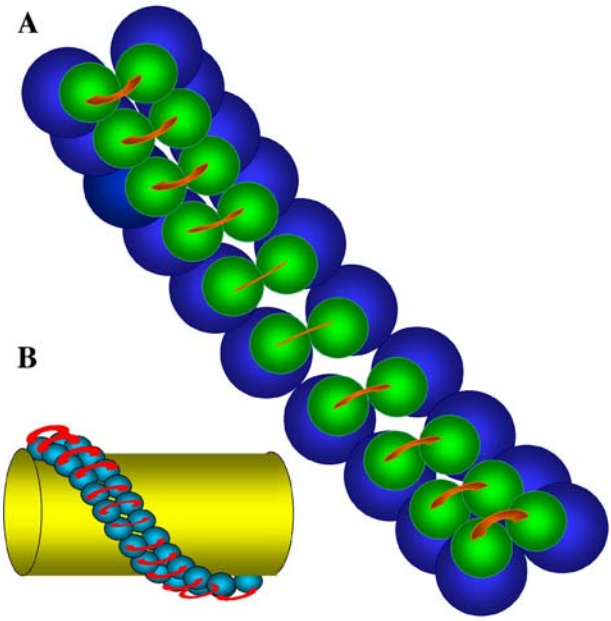

Fig. 5 Possible arrangement of the ATP synthase helical polymer that wraps and gives shape to the mitochondrial tubular cristae. **a** Inner view of the polymer from the interior of the cristae (membrane is transparent); the green spheres represent the F_0 channels connected by the protein bridge we observed [54]; the blue spheres are the F_1 heads. **b** The ATP synthase polymer is depicted from the outer surface of a single cristae (*yellow*). The F_1 particles are in *blue* and connected with a protein bridge that could be composed by the IF_1 and second-stalk subunits. The original model is from Allen et al. [75]

Although the location of the endogenous IF_1 in the whole F_1F_0I-ATP synthase remains unknown, on the basis of the available structural studies, we proposed a model of the binding site for the IF_1 that would explain the inhibitory role of IF_1 [44]. It is well known that the inhibitory domain of IF_1 lies on the N-terminal side of the molecule [45–47]; thus, according to our cross-linking data showing for the first time a relatively short distance (12 Å) between IF_1 and the γ and ε subunits, we placed the N-terminal inhibitory side in a cross-linking position of about 12 Å from the γ and ε subunits [44]. Figure 3 (top panels) shows this position of the N-terminal side on a cleft formed by the β_E catalytic subunit and the γ–ε part of the central rotor; lysine residues that are at 12-Å cross-linking distance are shown in purple. This β_E–γ–ε cleft was the wider binding site available for entrance of the IF_1 N-terminal side into the rotor–stator interface. It is clearly demonstrated from different perspectives that the binding of the N-terminal side of IF_1 in this position interferes not only with the conformational changes of the β subunits, as proposed before [19, 48], but also with the intrinsic rotation of the central stalk. Thus, we supported and proposed a novel mechanism of action for this protein that was later confirmed by the elegant crystal structure of the reconstituted dimeric F_1–IF_1 complex [22]. In the latter complex, the central coiled α-helixes of the γ subunit that extend along the central pseudosymmetry axis of the F_1-ATPase particle were found not only in proximity but in actual close contact with the its N-terminal side of IF_1 [21, 22] (see Fig. 3, bottom panels). The IF_1 was found locked into a β_{DP}–γ cleft rather than in a β_E–γ–ε interface, as we originally proposed [44]. This shows that what we found by cross-linking and model building was the entrance site of IF_1 into the F_1-ATPase particle and that two further angular movements of the γ subunit of 120° lock the IF_1 into the β_{DP} form of the catalytic subunit that previously received the IF_1 in its open β_E conformation (see Fig. 3 and [21]). Thus, the mechanism of action of IF_1 as inhibitor involves blocking the rotation of the central stalk and inhibiting the opening–closing conformational changes of the catalytic β subunit that leads to substrate binding, catalysis, and product release from F_1.

Besides showing the close-up view of the IF_1–γ interaction, the isolation and resolution of the F_1–IF_1 crystal structure also showed that reconstitution of recombinant IF_1 induces dimerization of the soluble F_1-ATPase particles in the expected 1:1 IF_1–F_1 stoichiometry [22, 49]. Earlier blue native polyacrylamide gel electrophoresis analyses of mitochondria showed a dimeric ATP synthase species that appears after mitochondrial solubilization with several detergents [50] and that some F_0 subunits such as e and g are essential for ATP synthase dimerization [34, 35]. Thus, the question emerged of whether the IF_1 participates in the dimerization of the whole F_1F_0-ATP synthase in mitochondria, besides dimerizing the soluble F_1-ATPase in vitro [49]. Initially, several groups found that genetic or physical removal of the yeast or bovine IF_1, respectively, did not prevent F_1F_0 dimerization; thus, it was concluded that IF_1 does not participate in the homodimerization of the whole F_1F_0 [51, 52]. However, because the yeast IF_1 protein lacks most of the C-terminal dimerizing domain and it is much less prone to dimerize [53], it was conceivable that the role of IF_1 in dimerization of the ATP synthase might be excluded from the yeast enzyme but present in the bovine and rat mitochondrial enzymes. Besides, the results where IF_1 removal did not change the dimer to monomer ratio of the bovine ATP synthase were obtained in the presence of triton X-110 where the F_1F_0-ATPase is inactive [51]. Therefore, we reassessed the role of IF_1 as a dimerizing factor of the bovine and rat mitochondrial enzymes in digitonin-extraction conditions where the dimeric and monomeric forms of the F_1F_0 complex are functional [54]. Besides, instead of looking to the decrease in the dimeric ATP synthase after IF_1 removal, we looked for the recovery or promotion of the dimeric species after reconstitution of increasing amounts of IF_1 into submitochondrial particles. With this approach, we demonstrated that removal of IF_1 dissociated the whole ATP synthase into monomers of high ATPase activity, and the reconstitution of IF_1 into SMP brought a partial recovery of the dimer content of the SMP extract accompanied by an overall inhibition of the ATPase activity [55]. Interestingly, the larger ATP synthase oligomers were also partially recovered by IF_1 reconstitution [55], suggesting that IF_1 participates in the formation of aggregation states of the ATP synthase larger than the ATP synthase dimer. Thus, it seems that yeast IF_1 is not essential for F_1F_0 dimerization in *Saccharomyces cerevisiae* simply because it is much less prone to dimerize since it lacks most of the C-terminal coiled-coil dimerizing domain [53]. The latter seems therefore essential for bovine and rat IF_1 to promote and/or stabilize the dimer and higher oligomer structures of the mitochondrial ATP synthase. This is consistent with previous findings where it has been shown that IF_1 confers structural stability to the F_1F_0I and F_1I complexes during high-pressure denaturation that leads to dissociation of oligomeric species [56]. In line with the dimerizing role of IF_1, it has been recently shown that, among other metabolic effects, the overexpression of IF_1 increases the amount of mitochondrial cristae, and its downregulation decreases the number of cristae in mitochondria of cultured cells [57]. Taken together, these studies confirm that, besides the inhibitory role of IF_1, it is also an important factor that stabilizes dimerization and further oligomerization of the mitochondrial ATP synthase, thus promoting formation of mitochondrial cristae as detailed below.

6 Structure of the Dimeric-Mitochondrial ATP Synthase: Improving Rotational Catalysis, Adding Stability, and Giving Shape to Mitochondrial Cristae

In Fig. 4, the possible quaternary structure of the dimeric bovine F_0F_1I complex is depicted according to the structural data available from crystallographic [20], genetic [58–60],

subunit association [36, 37], cross-linking [38, 44, 61–65], and protease accessibility [38, 66, 67] evidence. How does this model accommodate the inhibitory and dimerizing functions of IF_1 in the F_1F_0 dimer? We assumed a crossed IF_1 structure at the dimer interface, given that we also resolved by high-resolution electron microscopy the dimeric F_1F_0 and found a conical homodimeric molecule containing a protein bridge at the F_1–F_1 interface [54]. In this model, the IF_1 N-terminal side is located at the rotor–stator interface in inhibitory position, whereas the C-terminal side of IF_1 crosses the dimer interface and interacts with the opposite monomer probably through the OSCP subunit at the top of the side stalk as found by cross-linking evidence [65]. This model explains both the inhibitory and dimerizing roles of IF_1; however, both functions of IF_1 would require some further distortion from the fully extended helix observed in the isolated IF_1 to a bent or random coil conformation. This distortion is necessary to introduce the N-terminal side of IF_1 into the β_{DP}–γ interface as shown by the crystal structures [21, 22]. In the F_1F_0 dimer model, we used a crystal IF_1 conformer that is bent in the middle of the IF_1 protein, and this fits better at this interface than the extended IF_1 dimer conformers [68]. Similar crossed IF_1 dimeric structures have been observed in the IF_1 crystal [68]; this arrangement would be different from the observed antiparallel coiled-coil dimer of isolated IF_1 [68]. It was necessary to invoke this crossed structure because the distance between the N-terminal inhibitory domains in the IF_1–IF_1 extended dimer is about 60 Å [68], whereas the F_1–F_1 distance observed in the soluble F_1–IF_1 dimer [22] or in the $(F_1F_0I)_2$ dimer is $\leq$10 Å [54]. This implies that the IF_1 dimer must bend or cross somehow to be accommodated at the F_1–F_1 interface of the ATP synthase dimer that had an angle of about 40° which gives its conical shape.

On the other hand, it is also noted that, besides the bovine dimeric ATP synthase [54], other similar dimeric structures have been subsequently observed by electron microscopy in *S. cerevisiae* and *Polytomella sp* mitochondria. The latter species has a unique second-stalk composition and is therefore nonrepresentative of other mitochondrial ATP synthases [69]; however, in both cases, the dimeric structure adopted two angles of about 40° and 70° [69]. Dudkina and colleagues [70] named their open (70°) structure as the "true dimer", and our compact (40°) structure as a "pseudo-dimer"; furthermore, they also suggest that, in line with other reports, only their open "true" dimer actually participates in cristae formation [71, 72]. However, their dimer structures have several drawbacks: (1) their image averages are collected not by hand but automatically by image analysis software; in consequence, a large proportion of their dimer particles lack one or both of the F_1-portions, showing that their preparation is largely unstable compared to our preparation, which contains mostly complete F_1F_0 structures. (2) The larger detergent concentration used to isolate the enriched open dimers [69, 70] decreases the dimer yield and stability, and, importantly, it also decreases the functional coupling between F_1 and F_0; in contrast, our dimer enriched at lower detergent concentrations preserves essentially full oligomycin sensitivity, i.e., F_1F_0 functional coupling (Minauro-Sanmiguel and García-Trejo, unpublished results). This parameter has not been reported in the preparations enriched with the open (70°) and unstable dimer; it would not be surprising to find there a decreased F_0 inhibition. (3) There is emerging evidence from others [73] and from our recent studies with the yeast F_1F_0 dimer (not shown) indicating that both structures (open and closed) coexist with a wide distribution of dimers showing different angles after detergent extraction, but there is no clear evidence indicating which protein or factor is controlling the opening or closing of the dimer angle. Although IF_1 is not essential for IF_1 dimerization in yeast [52], the possibility remains that the shift from an extended to a compact conformation of the IF_1 dimer could

participate in determining the angle of dimeric F_1F_0. Therefore, we conclude that there is no reason to name arbitrarily the open or closed conformations as "pseudo" or "true" dimers; instead, we propose to refer to them just as "open" (≅70°) and "closed" (≅40°) dimers, with the understanding that the dimer population actually spreads through all angles between these values. Regardless of the observed angle values after detergent extraction, two major dimeric species correlate well with two distinct dimeric interfaces at the F_0 side that have been found in yeast F_1F_0 [74]; these two interfaces would build a helical polymer of dimers that wraps and gives shape to the tubular cristae of mitochondria [75], as it is currently proposed (Fig. 5).

In summary, the dimeric structure of the F_1F_0 ATP synthase is stabilized by the so-called inhibitor protein (IF_1) in the mitochondria of complex organisms such as rat or cow. Literally, on the other hand, the conserved N-terminal side of IF_1 inhibits the F_1F_0-ATPase activity by entering through the open catalytic α_E–β_E interface in a cleft formed by β–γ–ε subunits. With the IF_1 bound at this interface, the F_1-ATPase carries out two 120° gyrations of the central stalk and the N-terminal side of IF_1 locks at the β_{DP}–α_{DP}–γ interface, completely blocking rotation of the central stalk and the opening and closing of the catalytic sites. A further question that emerges is, how this deep inhibitory interaction of IF_1 with the rotor–stator interface of F_1 is reversed in the presence of the mitochondrial electrochemical proton gradient to allow ATP synthesis turnover? We are currently addressing this question by limited proteolysis experiments; interestingly, we observed that the N-terminal side of IF_1 becomes exposed to the media upon membrane energization, whereas the C-terminal side of IF_1 becomes shielded to proteolysis, indicating that it hides behind another F_1F_0 subunit (García-Trejo et al., unpublished). We propose here how this might happen in the dimeric F_1F_0 structure of bovine heart mitochondria. Upon membrane energization, the C-terminal side of IF_1 might become occluded between OSCP or second-stalk subunits at the dimer interface, whereas the N-terminal inhibitory domain is released from the α_{DP}–β_{DP}–γ cleft where it is bound, thus restoring rotation of the central stalk and the opening–closing conformational changes of the β subunits that are essential for F_1 catalysis. In this model, second-stalk subunits are not depicted for clarity, but they should contribute significantly to the dimer interface, as shown for the yeast H subunit (bovine subunit F6, see [75]). Once formed, the dimer structure seems more stable and in better shape to resist the rotational drag of the continuous gyration of the central stalk than its dimeric form (Fig. 4). In other words, the monomeric enzyme could lose coupling energy by rotating as a rigid body following the angular drag of the rotor; this would hardly occur in a dimerized or oligomerized ATP synthase. Indeed, it has been proposed that the rotational drag of each monomer promotes closer F_0–F_0 interactions in the dimer as observed by atomic force microscopy in the dimeric enzyme [71]. It can also be questioned whether dimerization actually increases the coupling efficiency of the enzyme, given that the monomeric bacterial enzyme is already highly efficient as a coupling factor; indeed, the most efficient and practically unidirectional ATP synthases described so far are those of *P. denitrificans* [30] and of a thermoalkaliphilic bacterium [31]. However, it is also recalled that, in α-proteobacteria and even in eubacteria such as *E. coli*, it has been described that the rotary turnover of the F_1 portion undergoes slippage from the proton conduction through F_0 under conditions of low ADP and Pi concentrations [76, 77]. This slipping has not been observed for the mitochondrial enzyme, probably because the rotor and stator interfaces of each monomer interact more efficiently in the dimeric or oligomeric forms of the enzyme. In this line, we are currently collecting evidence to respond to the question of whether the dimeric enzyme possesses a higher stability and better efficiency as ATP synthase in comparison with its monomeric species;

preliminary results indicate that it is actually the case. Together with its role in formation of the mitochondrial cristae, these studies and models shed light on the mechanisms by which the F_1F_0-ATP synthase becomes not only the most efficient nanomotor in nature by its regulation in bacteria and by its dimerization in mitochondria, but also becomes a dimeric building block of a hypothetical helical polymer that wraps and gives shape to the mitochondrial cristae (Fig. 5).

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