The mitochondrial F1Fo ATP synthase

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1.6 The Mitochondrial F_1F_o ATP Synthase

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1 Introduction

Adenosine triphosphate (ATP) is the general energy currency of living organisms from simple prokaryotes to the more complex eukaryotes. It is continually produced at the expense of nutrients and utilized by endergonic biological processes in large amounts that usually exceed the weight of the organism. In aerobic prokaryotic and eukaryotic cells, although some ATP is produced by soluble enzymes, the largest proportion comes from oxidative phosphorylation of coupling membranes. Oxidative phosphorylation is an integrated process in which the free energy, made available by downhill electron flow in the respiratory chain as a transmembrane electrochemical gradient of protons ($\Delta\mu H^+$, or protonmotive force (PMF)), is utilized by the F_1F_o ATP synthase complex to make ATP from ADP (adenosine diphosphate) and P_i (inorganic phosphate). The F_1F_o ATP synthase, also known as complex V, is a proton pump that converts the PMF into mechanochemical energy to drive ATP synthesis. When the PMF becomes limiting, as in extremely hypoxic conditions, the ATP synthase is reversed and it hydrolyzes ATP produced essentially by glycolysis until the PMF is reestablished.

Research on the structural organization and the catalytic mechanism of the ATP synthase began in the 1960s and evolved contextually to Peter Mitchell's chemiosmotic hypothesis. In 1962, ATP synthase was directly observed, for the first time, by electron microscopy. Many spherical "bead-like structures" were visible at the surface of the inner mitochondrial membrane (Fernandez-Moran, 1962). The spheres appeared to be connected to the membrane by a thin stalk. A pioneer in this field was E. Racker who succeeded in isolating the hydrosoluble "factor" (F_1) from the mitochondrial inner membrane. F_1 could be reconstituted with the stalk moiety in the membrane in the presence of the "oligomycin sensitivity conferring protein" (OSCP), a protein required for the sensitivity of complex V to the antibiotic oligomycin (Kagawa and Racker, 1966). Oligomycin sensitivity is a feature of mitochondrial F_1F_o ATP synthase and is used to distinguish it from the other two major types of ion-motive ATPases (P-type and V-type ATPases). Since then many significant advances in understanding the structural organization and the catalytic mechanism of F_1F_o ATP synthase have been made, culminating in the X-ray crystallographic analysis of the three-dimensional structure of the F_1 catalytic moiety from bovine heart (Abrahams et al., 1994) and other sources (Shirakihara et al., 1997; Bianchet et al., 1998; Hausrath et al., 1999; Groth and Pohl, 2001). The resolution at 2.8 Å of the atomic structure of bovine heart F_1 (Abrahams et al., 1994) provided the structural basis for the development of the rotary model of the F_1F_o ATP synthase. This was further supported by the demonstration of ATP-driven rotation of the γ subunit of F_1 (Noji et al., 1997).

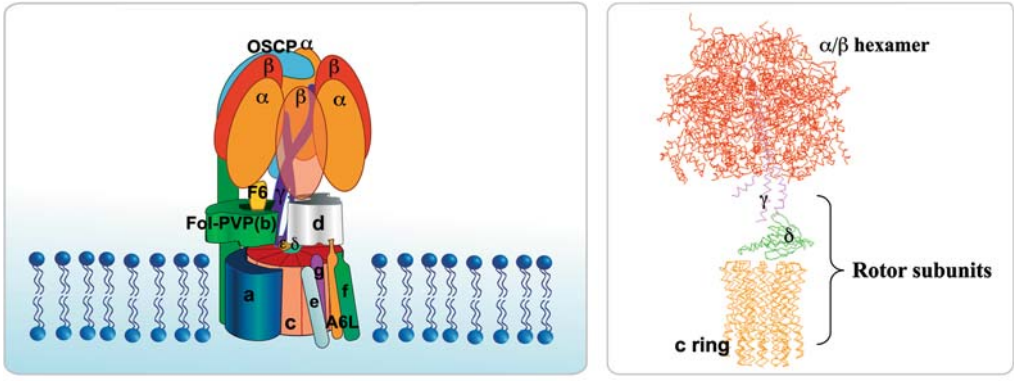
F_1F_o ATP synthase is a large oligomeric complex whose general structure is highly conserved among bacteria, chloroplasts, and mitochondria. Very recently, the presence of a F_1F_o ATP synthase complex has been demonstrated in the plasma membrane of human endothelial cells (HUVEC) where it produces extracellular ATP, which is involved in angiogenesis, and appears to be the angiostatin receptor (Moser et al., 1999, 2001; Arakaki et al., 2003; Burwick et al., 2004).

In mammalian mitochondria, the F_1F_o ATP synthase is composed of 16 subunits (only eight in bacteria), with an overall molecular weight of about 550 kDa. In mitochondria, but not in chloroplasts and bacteria, the F_1F_o complex has been shown to exist as a dimer (Arnold et al., 1998; Schagger and Pfeiffer, 2000) or even as an oligomer (Krause et al., 2005). In yeast mitochondria, the ATP synthase oligomer is thought to have a role in modulating the morphology of the mitochondrial inner membrane (Gavin et al., 2004). Furthermore, a recent report has indicated the presence of an ATP supercomplex containing F_1F_o, adenine nucleotide, and P_i carriers (Chen et al., 2004).

The monomeric ATP synthase complex consists of two major domains, a large globular catalytic moiety known as F_1 that consists of an assembly of five subunits with the stoichiometry of $\alpha 3\beta 3\gamma\delta\varepsilon$, protruding into the mitochondrial matrix, and a membrane-embedded domain known as F_o, involved in transmembrane proton translocation coupled to enzyme catalysis (*Figure 1.6-1a*). The F_o domain has a variable number of different subunits depending on the species. In mammals, there are 11 subunits, including the IF_1 inhibitor protein, which plays a key role in ATP hydrolase regulation. Subunits a, b, and c, represent the conserved core of F_o with the stoichiometry 1:1:10–12. In the mammalian F_oF_1 complex, all the subunits are

Figure 1.6-1

Model of the mitochondrial ATP synthase. (a) Subunit arrangement of the mitochondrial ATP synthase. The model is based on electron microscopic (EM) images, crystal structures, and on other biochemical and reconstitution studies reviewed in the text. (b) Electron density map of the F_1-c_{10} subcomplex obtained from *Saccharomyces cerevisiae* at 3.9 Å resolution (Stock et al., 1999) (PDB = 1Q01). The subunits that constitute the rotor, except subunit ε which is not resolved in the crystal structure, are shown. Molecular graphics by RasMol 2.6



a b

nuclear encoded except two, subunits a (mt gene ATP6) and A6L (mt gene ATP8), which are encoded by the mitochondrial DNA (mt DNA) (Table 1.6-1) (Papa et al., 2000).

The F_1 and F_o moieties are structurally and functionally connected by two stalks: a central stalk, which constitutes the rotor and a peripheral stalk referred to as the stator of the rotary-motor model of the ATP synthase (Collinson et al., 1996; Ogilvie et al., 1997).

Table 1.6-1

The mitochondrial F_1F_o ATP synthase subunits

Subunits	ncopies	Location	Mass (Da)	Gene
F_1				
α	3	External hexagon	55164	Nuclear
β	3	External hexagon	51595	Nuclear
γ	1	Internal cavity and stalk	30141	Nuclear
δ	1	Stalk	15065	Nuclear
ε	1	Stalk	5652	Nuclear
IF1	1	Surface	9582	Nuclear
F_o				
F_oI-PVP(b)	1	Stalk membrane	24670	Nuclear
ATP6(a)	1	Transmembrane	24815	Mitochondrial
OSCP	1	Surface F_1 and stalk	20968	Nuclear
d	1	Stalk membrane	18603	Nuclear
g	1	Transmembrane	11328	Nuclear
f	1	Transmembrane	10209	Nuclear
F_6	1	Stalk membrane	8958	Nuclear
e	1–2	Transmembrane	8189	Nuclear
c	10–12	Transmembrane	7608	Nuclear
A6L	1	Transmembrane	7964	Mitochondrial

This chapter deals with the following aspects of the F_1F_o ATP synthase in mammalian mitochondria:

1. F_1: X-ray crystallographic studies and catalysis
2. Structure and functional mechanism of the F_1F_o complex
3. The inhibitor protein: structure and physiopathological role
4. ATP synthase and human diseases

2 F_1: X-ray Crystallographic Studies and Catalysis

In 1994, Walker's group in Cambridge published the first high-resolution structure of $F_1\alpha_3\beta_3\gamma$ subcomplex at 2.8 Å resolution (Abrahams et al., 1994). This structure, prepared in the presence of the inhibitory ATP analog AMP-PNP, shows that the α and β subunits are alternatively arranged to form a spherical hexamer. The β subunits appear different in terms of nucleotide binding states; the first β binds Mg-AMP-PNP, the second Mg-ADP, and the third is empty (no bound nucleotide). These sites are termed β_{TP}, β_{DP}, and β_E and correspond to "tight," "loose," and "open" conformations, respectively (*Figure 1.6-2a*). All three α subunits bind the ATP analog AMP-PNP. Only the coiled coil C and N termini regions of the γ subunit that penetrate the α/β spherical hexamer are visible, the rest of the protein not being ordered in the crystal (*Figure 1.6-2a*). The empty conformation of β_E appears to be induced by the γ subunit, which seems to prevent the rotation of the β lower C-terminal part by pushing it toward the central axis (*Figure 1.6-2a*). The asymmetry of β subunits is the most important feature of this F1 crystal structure (Abrahams et al., 1994). Similar asymmetric β structure has also been observed in subsequent crystal structures analyzed by Walker and coworkers (Abrahams et al., 1996; van Raaij et al., 1996a; Orriss et al., 1998; Braig et al., 2000; Gibbons et al., 2000), except for a structure obtained in the presence of MgADP and an excess of aluminum fluoride (Menz et al., 2001). In this structure all the catalytic sites are filled (closed conformation) and the β subunit, corresponding to the former β_E (open conformation), appears in a "half closed" conformation. This partial closure of the β C-terminal region, apparently induced by binding and hydrolysis of ATP, is thought to result from a 20° rotation of the γ subunit, which presents a different feature with respect to all the other crystals examined. The β subunit, in a half-closed conformation, binds ADP and sulfate, the latter mimicking P_i, so that this structure represents a posthydrolysis intermediate with β_{ADP+Pi} ready to pass to the "open conformation" and release the products (Menz et al., 2001).

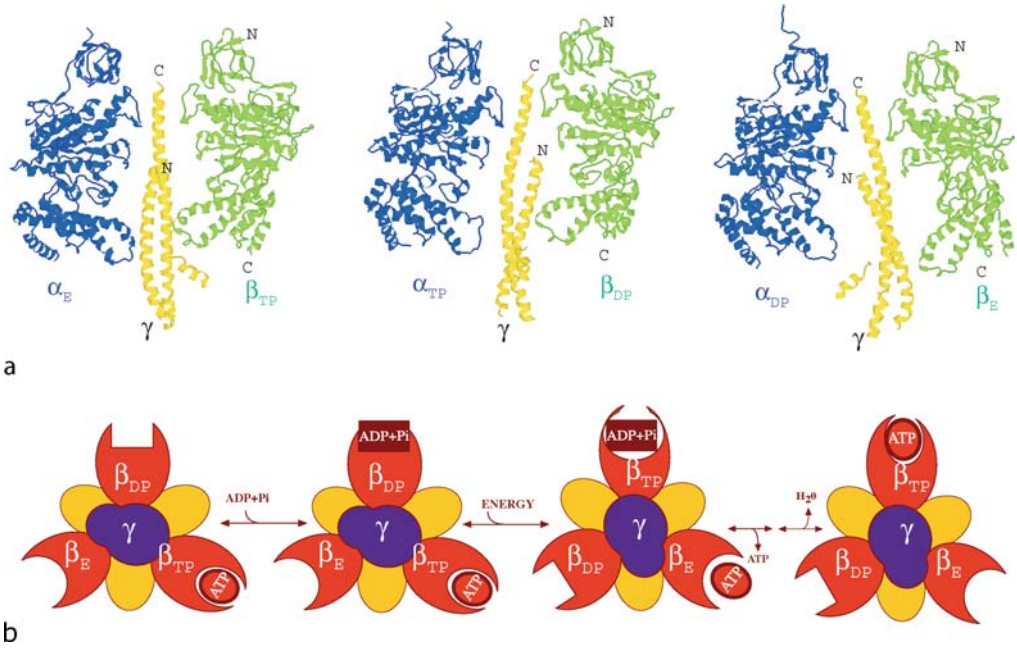
The structural data described have been of high impact and are generally considered to be illustrative of the reaction mechanism of the enzyme. They all appeared to be consistent with what Boyer's "binding change mechanism" predicts (*Figure 1.6-2b*) (Boyer, 1993, 1997). During catalysis, the three catalytic sites pass sequentially through three different nucleotide conformations, namely "tight," "loose," and "open," and cooperative interconversion of the states is associated with a rotation of the γ subunit (*Figure 1.6-2b*) (Boyer, 1993, 1997). The γ rotation, driven by transmembrane proton translocation through the complex, results in a decrease in the affinity of F_1 for ATP (and probably increases the affinity for ADP and P_i) with ATP release in the medium (*Figure 1.6-2b*). ATP formation at one catalytic site would take place after binding of ADP and P_i without energy expenditure.

Kinetic data have, in fact, shown that ATP synthase catalysis can occur involving only one catalytic site (unisite catalysis), two catalytic sites (bisite catalysis) (*Figure 1.6-2b*), or all the three catalytic sites (trisite catalysis). Unisite catalysis, measured only for ATP hydrolysis, can take place at very low ATP concentrations (ATP:F_1 ratio of 1:3). In these conditions, the rate of ATP hydrolysis is much lower (10^{-5} to 10^{-6}) than that measured during steady-state (multisite) catalysis; the release of products P_i and ADP is also very slow. Thus, when only one catalytic site is filled with the substrate, the substrate is tightly bound and the reaction proceeds slowly. The successive binding of the substrate dramatically enhances ATP hydrolysis (or synthesis) and product release. Whether two catalytic sites (bisite catalysis) or three catalytic sites (trisite catalysis) have to be filled in order to achieve a significant rate of hydrolysis is still a matter of debate (see below).

The "binding change mechanism" postulates that the catalytic sites on β subunits are in three different states during catalysis, this being due to the physical rotation of the γ subunit in the internal cavity of the α3β3 hexamer. Cross and coworkers performed experiments in which a labeled β subunit was reversibly cross-linked with the γ subunit. They observed that the addition of ATP after cleavage and before

Figure 1.6-2

(a) Different structural conformations of F_1 α, β, and γ subunits as observed in the F_1 crystal structure inhibited by AMP-PNP. Longitudinal sections of F_1 moiety, each showing partially resolved subunit γ with facing α and β subunits, are depicted. The three different conformations adopted by the three β subunits are visible: β_{TP}, which corresponds to the tight state with high affinity for substrate and catalytically active β_{DP}, which corresponds to the loose state with low affinity for substrate and catalytically inactive; and β_E, which corresponds to the open state with very low affinity for substrates and catalytically inactive. Structural data are from bovine heart mitochondrial F_1 crystal structure (Abrahams et al., 1994) (PDB ID = 1 bmf). Molecular graphics by RasMol 2.6. (b) The binding change mechanism. Bisite catalysis scheme in which only two catalytic sites in the β subunits are occupied by adenine nucleotides (ATP and ADP + P_i) during steady-state catalysis. From left to right (ATP synthesis), an ATP molecule can been seen bound at the high-affinity site (β_{TP}); the binding of ADP and P_i at the loose site (β_{DP}); and interconversion of the catalytic sites in association with rotation of the γ subunit. In the last step ATP is released from the open site (β_E). The feature of this model is that the energy-requiring steps are substrate binding and product release (see Abrahams et al., 1994)



reinduction of cross-linking caused the γ subunit to be cross-linked to a β subunit different from the one initially labeled (Duncan et al., 1995). Junge's group employed the technique of "polarized absorption recovery after photobleaching" (PARAP) of a probe labeled to γ subunit, which allowed observation of rotation of this subunit during ATP hydrolysis (Sabbert et al., 1996). Finally, rotation of γ subunit induced by ATP hydrolysis was directly observed by optical microscopy with attachment of fluorescent actin filaments to γ or ε subunits in a $\alpha 3\beta 3\gamma\varepsilon$ subcomplex, (Noji et al., 1997; Kato-Yamada et al., 1998). Seen from the F_o side, the γ subunit rotated anticlockwise, proceeding by a 120° step upon hydrolysis of one ATP molecule. This fact indicated that the γ subunit rotates, interacting with all the three β subunits. Later, better resolution experiments showed that the 120° step could be divided into two substeps (Yasuda et al., 2001; Shimabukuro et al., 2003; Nishizaka et al., 2004). These were proposed to correspond to ATP binding and ADP release, respectively. Thus, according to these "mechanical" data, rotation of the γ subunit is driven solely by ATP binding and not by its hydrolysis. On the other hand, other models based on kinetic data support the proposal that the initial rotation substep involves not only nucleotide binding but also

both binding and hydrolysis of MgATP (Senior et al., 2002; Weber and Senior, 2003). This is one of the aspects of the ATP synthase mechanism that is still a matter of debate.

Subunit γ protrudes out of the α3β3 hexamer (Abrahams et al., 1994; Stock et al., 1999), extends throughout the stalk, and contacts the polar inner loop of membrane-embedded c subunits (Watts et al., 1995; Fillingame, 1997). Mutational analysis, cross-linking experiments, and X-ray crystallography (Aggeler et al., 1997; Stock et al., 1999) have shown that the γ subunit, together with the ε subunit in *Escherichia coli* and the δ subunit in the mitochondrial ATP synthase (Karrash and Walker, 1999), constitutes the central stalk. A crystallographic analysis at 2.4 Å resolution of bovine F_1 provided additional insight into the nature of the F_1 protruding central stalk (Gibbons et al., 2000). This X-ray structure revealed a new domain in the γ subunit, containing a Rossman fold, which together with subunits δ and ε, forms a foot-like structure in contact with the F_o subunit c (*Figure 1.6-1b*) (Stock et al., 1999). F1 δ subunit, as it appears in this structure (homolog to the bacterial ε subunit), consists of two domains—an N-terminal β sandwich and a C-terminal α-helical hairpin—while the mitochondrial ε subunit, which has no counterpart in prokaryotes or chloroplasts, has a helix–loop–helix structure. This subunit appears to stabilize the foot of the central stalk, where γ, δ, and ε subunits may interact with the c-ring and couple the transmembrane protonmotive force to catalysis in the α3β3 domain (Gibbons et al., 2000). More recently a key role of the δ subunit in the mechanical coupling of the c-ring with subunit γ has been proposed (Duvezin-Caubet et al., 2003).

Despite all this work, there is no consensus as yet on the mechanism by which the ATP synthase operates. The reasons for this controversy are both structural and kinetic. From a structural point of view, the strong asymmetry of the β subunits detected in the crystal structures described (Abrahams et al., 1994, 1996; van Raaij et al., 1996a; Orriss et al., 1998; Gibbons et al., 2000) is not observed in other X-ray studies where a more symmetric situation of β subunits is apparent (Shirakihara et al., 1997; Bianchet et al., 1998; Groth and Pohl, 2001; Menz et al., 2001; Groth, 2002). Such a difference could essentially be due to the different crystallization conditions.

Another controversial aspect is related to the number of catalytic sites that must be filled for efficient steady-state catalysis (bisite or trisite catalysis). A steady-state mechanism in which all three catalytic sites are filled (three-site catalysis) appears to gain more acceptance with respect to bisite catalysis (Boyer, 2000, 2002). The three-site catalysis, initially based on results of tryptophan fluorescence experiments (Weber and Senior, 2000), is also supported by data from single-molecule experiments (Nishizaka et al., 2004).

Two possible reaction schemes for ATP hydrolysis, both supporting trisite catalysis, have recently been proposed based on various structural data obtained and by integrating these with tryptophan fluorescence measurements (Weber and Senior, 2000) and mechanics of rotation (Noji et al., 1997; Nishizaka et al., 2004). The two mechanisms differ essentially in the assignment of the β subunit (β_{DP} or β_{TP}) where ATP hydrolysis occurs (for details see Kagawa et al., 2004).

3 Structure and Functional Mechanism of the F_1F_o Complex

Although many attempts have been made, a three-dimensional structure has not been obtained for the complete F_1F_o enzyme purified from various sources. The first model proposed, for the *E. coli* F_o, consisted of a structure in which the a and b subunits were surrounded by a ring of c subunits. With reference to the flagellar motor, it was postulated that rotation of subunits b, inside the c-ring together with F_1 γδε subunits, would drive ATP synthesis (Cox et al., 1984, 1986). Later, different experimental approaches (Schneider and Altendorf, 1987) showed that the a and b subunits are located on one side of the ring of c subunits. It was then proposed that the c-ring is made to rotate, by transmembrane proton translocation, relative to the a and b subunits. Images obtained by electron microscopy (Birkenhager et al., 1995) and atomic force microscopy (Singh et al., 1996; Takeyasu et al., 1996) confirmed the asymmetric organization of the F_o subunits, showing that two b subunits are anchored to the membrane at one side of the c-ring by the N terminus hydrophobic segment, while the C terminus protrudes toward the F1 moiety.

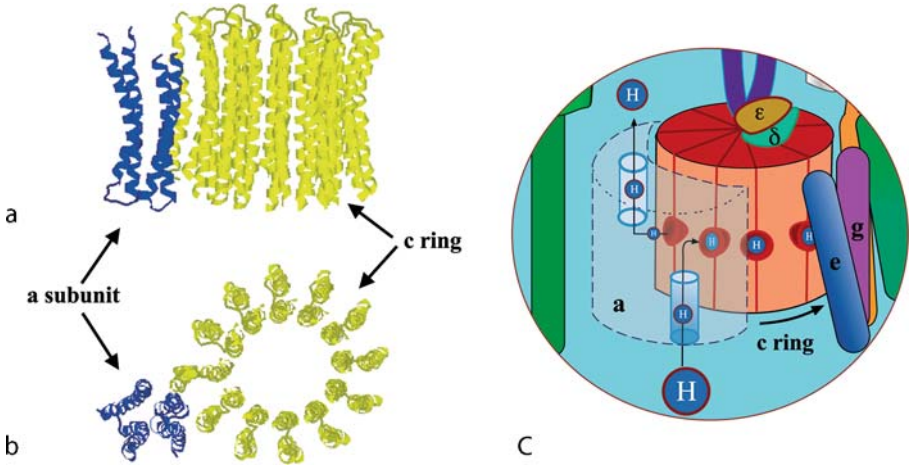
In the late 1990s, contrary to the general view (Gogol et al., 1987; Capaldi et al., 1994; Walker and Collinson, 1994), according to which the stalk subunits were assembled in a single central structure

connecting F_1 and F_o moieties, cross-linking results (Ogilvie et al., 1997; Wilkens et al., 1997) and better resolved electron microscopy images (Bottcher et al., 1998; Wilkens and Capaldi, 1998; Karrash and Walker, 1999) showed that, in addition to a central stalk, there exists a second lateral stalk connecting the periphery of F_1 and F_o (*Figure 1.6-1a*). This observation came concomitantly with the first single-molecule experiment showing directly subunit γ rotation in the F_1 moiety (Noji et al., 1997). The lateral moiety was thus conceived to function as a static element (stator) of the ATP synthase rotary motor, holding the α_3 β_3 hexamer relative to the central rotor. It was proposed that the latter involves the membrane-embedded subunit c-ring (*Figure 1.6-1b*), in addition to γ δ, and ε F_1 subunits (γ and ε in prokaryotes).

Subunit c has been studied extensively. Cross-linking experiments and NMR structural analysis of the *E. coli* protein have shown that this highly hydrophobic subunit folds in the membrane as a hairpin with two membrane-spanning α-helices connected by a polar loop region that comes in contact with the F_1 sector on the matrix side (Girvin et al., 1998; Dmitriev et al., 1999) (*Figure 1.6-3a–c*). There are several

Figure 1.6-3

Structure of the c subunit ring and model of the rotational movement of the two half-channels in the F_o rotary motor. (a) Side view of the NMR model for the ac_{12} subcomplex, which includes four of the five transmembrane helices of subunit a (Rastogi and Girvin, 1999) (PDB = 1c17). The c subunits are arranged in a ring with subunit a on one side. (b) Top view of the ac_{12} subcomplex. (c) The interaction between the a subunit and the c ring is shown. Proton flow (toward the F1 region) through the two half-channels in the a subunit is allowed by the c oligomer stepwise rotation. In each step a different c subunit comes into contact with the a subunit, thus allowing proton translocation across the membrane. Molecular graphic by RasMol 2.6



copies of the c subunit organized in an oligomer with a ring-like shape (*Figure 1.6-3a, b*). Each copy has the N-terminal transmembrane helix on the inside of the c-ring and the C-terminal helix on the outside. Evidence obtained shows that the number of c-subunit copies range from 10 to 14 in bacteria, chloroplasts, and mitochondria (Stock et al., 1999; Seelert et al., 2000; Jiang et al., 2001; Vonck et al., 2002). In bacteria, the c-subunit stoichiometry may vary depending upon metabolic conditions (Schemidt et al., 1998; Tomashek and Brusilow, 2000).

In the middle of the C-terminal helix of subunit c, there is a highly conserved residue (D61 in *E coli*; E65 in mitochondria) essential for proton translocation through F_o (Miller et al., 1990). A low-resolution electron density map of the *Saccharomyces cerevisiae* F_1ATPase connected with the membrane-embedded c subunits has been obtained (Stock et al., 1999) (*Figure 1.6-1b*). This structure shows an oligomer of 10 c subunits and confirms the structural data obtained from the *E. coli* enzyme. It also shows that the

central stalk, and in particular subunits γ and δ of F_1, makes extensive contacts by "sitting" asymmetrically on the polar loop regions of six c subunits (*Figure 1.6-1b*).

Subunit a of F_o is located at one side of the c-subunit ring (Fillingame et al., 2000). It is encoded by mtDNA and consists of five membrane-spanning helices (Long et al., 1998; Wada et al., 1999; Dmitriev et al., 2004). Arg210 in the fourth transmembrane helix of subunit a interacts with D61 in subunit c. These two residues, together with other neighboring polar residues, are directly involved in proton translocation through the complex (Valiyaveetil and Fillingame, 1997; Jiang and Fillingame, 1998; Cain, 2000; Fillingame et al., 2002a, b). Different models have been proposed to explain how protons are transferred through F_o and how this passage causes the rotation of subunits. In the "two half channel" model (*Figure 1.6-3c*), subunit a, located at one side of the subunit c-ring, contributes two half channels providing a gate for protons toward or from subunit c carboxylate (D61 in *E. coli*; E65 in mitochondria) (Vik and Antonio, 1994). The sequential protonation and deprotonation of subunit c carboxylate would be coupled to a stepwise movement of the c-subunit ring. Another elegant model, derived from NMR studies of subunit c at two different pHs (Rastogi and Girvin, 1999), proposed that deprotonation of D61 (*E. coli* residue) causes structural changes leading to a 140° rotation of subunit c C-terminal helix, with respect to the N-terminal helix, followed by the movement of the c-ring. Recently, it has been proposed that in addition to subunit c, the fourth transmembrane helix of subunit a also rotates to alternately expose the two half-channels to D61 of subunit c during the proton transport cycle. Thus, subunits a and c would both rotate in a concerted mode, and this motion would generate the mechanical force necessary to drive the rotation of all the c-oligomers (Fillingame et al., 2003).

Direct evidence of ATP driven subunit c rotation from different experimental approaches has confirmed that the c-subunit oligmer functions as the main rotor element of the F_1F_o ATP synthase motor (Sambongi et al., 1999; Panke et al., 2000; Tsunoda et al., 2001; Nishio et al., 2002). Single-molecule experiments have shown that, upon ATP hydrolysis, the oligomer rotates counterclockwise when viewed from the membrane side (Sambongi et al., 1999; Panke et al., 2000; Nishio et al., 2002). Proton-driven rotation of the F_1F_o complex has also been observed and the direction is opposite to that generated by ATP hydrolysis (Diez et al., 2004). The rotational properties of the intact F_1F_o complex have been shown to be the same as those of the F_1 subcomplex, indicating negligible drag due to the F_o moiety (Ueno et al., 2005). All these data show that the c-subunit oligomer is part of the rotor element of the ATP synthase motor; it can rotate either clockwise or anticlockwise depending on the direction of the proton flow. This rotation is in both cases strictly related to the rotation of the central stalk, allowing the transfer of mechanical energy from or to the catalytic sites.

The lateral stalk, also known as stator, is the other critical part of the ATP synthase molecular machinery. In the *E. coli* enzyme, as well as in other bacteria and chloroplasts, the lateral stalk is made up of the membrane-embedded a subunit and two neighboring copies of b subunits, which arises from the membrane, extends till the αβ hexamer, and comes into contact with one of the α/β interface region (Weber et al., 2004b) and with the δ subunit (Ogilvie et al., 1997). The δ subunit sits with its C-terminal domain on the C-terminal domain of b subunits while its N-terminal domain is connected to the top of the F_1 moiety where it contacts the N-terminal domain of the α subunit (Ogilvie et al., 1997; McLachlin et al., 1998; Wilkens and Capaldi, 1998; Weber et al., 2004a). It has recently been shown that a correct interaction between α and δ subunits is essential for the membrane assembly of ATP synthase (Weber et al., 2004a).

The lateral stalk in the mitochondrial F_1F_o ATP synthase appears to be more complex than in prokaryotes. The lateral stalk of the bovine enzyme has been studied extensively with different approaches such as limited proteolysis of subunits, cross-linking analysis, in vitro assembly of stalk complexes, and immunodecoration by subunit-specific antibodies. It is made up of single copies of subunits a, OSCP, d, e, F6, FoI-PVP(b), A6L, f, and g (*Figure 1.6-1a*) (Zanotti et al., 1988, 1994; Collinson et al., 1994, 1996; Papa et al., 1999, 2000). By employing subunit-specific antibodies in mitoplasts and in inner membrane inside-out vesicles, it has been shown that FoI-PVP(b), d, F6, and OSCP subunits are exposed at the matrix, but not at the cytosolic side of the inner mitochondrial membrane, while subunits c and a are shielded to their antibodies on both sides (Hekman et al., 1991). Subunit A6L, which is encoded by mtDNA, has a membrane-embedded N terminus while the C terminus protrudes into the matrix side. Subunits f and g both have the N terminus at the matrix side of the membrane, while the e subunit appears to be essentially

exposed at the cytosolic side (Belogrudov et al., 1996). Subunit e has been shown to exist as a dimer in yeast (Arnold et al., 1998; Shagger and Pfeiffer, 2000) and in rat liver mitochondrial ATP synthase (Arakaki et al., 2001). It has also been shown that this subunit plays a central role in the dimerization process, in the stabilization of dimer-specific F_o subunits, subunits g and k, in the modulation of the mitochondrial morphology of yeast ATP synthase (Arnold et al., 1998; Everard-Gigot et al., 2005), as well as in the Ca^{2+}-dependent regulation of H^+ ATP synthase activity (Arakaki et al., 2001).

FoI-PVP(b) subunit contributes to the functional coupling of F_1 and F_o and is located externally, at one side of the ring of c subunits (*Figure 1.6-1a*), like the *E. coli* subunit b (Zanotti et al., 1988). As in *E. coli*, the FoI-PVP(b) N-terminal region folds in the inner mitochondrial membrane, while the hydrophilic part (residues 79–214) protrudes toward the α3β3 hexamer. Oligomycin sensitivity of ATP hydrolysis appears to be strictly dependent on the correct assembly of the hydrophilic portion of FoI-PVP(b), OSCP, and F6 subunits (Guerrieri et al., 1991).

Cross-linking studies in submitochondrial particles have shown that, in the absence of a transmembrane electrochemical proton gradient, FoI-PVP(b) C-terminal segment is in close contact with the γ subunit. The cross-linking between FoI-PVP(b) and γ subunits is completely prevented by the presence of a transmembrane electrochemical proton gradient, which, probably causing conformational changes, places the two subunits in a position that prevents their cross-linking (Gaballo et al., 1998). It must be recalled that mutational deletions in the *E. coli* b subunit indicate that this protein has a flexible structure (Sorgen et al., 1998, 1999). Furthermore, the elasticity of subunit b has been postulated to allow reorientations of the stator for rotation in opposite directions (clockwise and anticlockwise) during either ATP synthesis or ATP hydrolysis (Grabar and Cain, 2003).

Limited cleavage experiments have shown that OSCP, like subunit δ of the *E. coli* enzyme, sits on the C-terminal region of subunit FoI-PVP(b) and extends toward the top of F_1, where it interacts with the N terminus of the α subunit (*Figure 1.6-1a*) (Xu et al., 2000; Rubinstein and Walker, 2002, 2003).

4 The Inhibitor Protein: Structure and Physiopathological Role

In mitochondria, the F_1F_o complex functions essentially as an ATP synthase. In conditions of anoxia or ischemia, the enzyme can alternatively hydrolyze ATP, pumping protons in the reverse direction with respect to that in ATP synthesis. In vivo, regulation of the ATP synthase involves ADP, pH, membrane potential, and a natural protein inhibitor, IF_1 (Pullman and Monroy, 1963; Harris and Das, 1991; Lebowitz and Pedersen, 1993). IF_1 was first isolated from bovine heart mitochondria in 1963 (Pullman and Monroy, 1963). It is absent in bacteria and chloroplasts where an analogous counterpart is thought to be represented by the ε subunit of the F_1 moiety (Pullman and Monroy, 1963). The inhibitor protein acts by binding reversibly to the F_1F_o ATP synthase and selectively inhibiting ATP hydrolysis, but has no effect on ATP synthesis (Panchenko and Vinogradov, 1985; Harris and Das, 1991). The respiratory protonmotive force ($\Delta\mu H^+$) across the mitochondrial inner membrane displaces IF_1 from its binding site in the ATP synthase complex (Harris and Das, 1991). The ΔpH component of the $\Delta\mu H^+$ and in particular the pH of the matrix side, where IF_1 binds to the complex, is the critical factor that modulates binding and inhibitory activity. IF_1 exerts a low inhibitory capacity at alkaline pH, becoming very active at a pH around 6.5.

IF_1 is a small basic protein of 10 kDa. Its primary sequence is significantly conserved among species, presenting a 75% identity between bovines and humans. The binding and inhibitory activity of IF_1 appears to be associated with its central segment (van Raaij et al., 1996b). By using synthetic peptides it has been found that the segment from Leu 42 to Lys 58 is as active as the intact bovine IF_1 (Papa et al., 1996), displaying the same kinetic, temperature, and pH dependence as the native IF_1 (Zanotti et al., 2000). The pH dependence is an essential feature of IF_1 and appears to be related to the presence of His49 in the central segment. Three other histidine residues are also present in the primary sequence of the protein (positions 48, 55, 56), which are likely to increase the stability of the interaction between the inhibitor and the ATP synthase complex.

In yeast mitochondria, the binding site of IF_1 to the F_1F_o complex has been shown to involve both α and β subunits (Mimura et al., 1993), probably including the DELSEED sequence of the β subunit, which, on

the other hand, contacts the γ subunit during catalysis (Abrahams et al., 1994). A bovine IF_1 three-dimensional structure has been obtained at 2.2 Å resolution (Cabezon et al., 2001). It shows that the purified bovine inhibitor protein (IF_1) exists in vitro at pH 6.7 as a dimer made up of two monomers that associate through an antiparallel α-helical coiled coil in the C-terminal region, leaving the N termini protruding in opposite directions (Cabezon et al., 2001).

More recently, the structure of crystal bovine IF_1–F_1 complex has also been solved (Cabezon et al., 2003). It shows that an IF_1-active dimer associates with two F_1 regions supposed to belong to an ATP synthase dimer. In particular, each of the two N termini of the IF_1 dimer, located between two F_1 moieties, interacts with the 4–40 and 4–47 residues respectively, and also with the α/β interface of one of the two F_1 regions in the ATP synthase dimer. Contribution of IF_1 to the ATP synthase dimerization is still a matter of debate. Evidence obtained shows that the ATP synthase dimerization occurs in the membrane independently of the binding of the inhibitor protein IF_1 (Tomasetig et al., 2002). Recently, Zanotti et al. have shown that the binding of IF_1 with the α/β subunits is pH dependent and involves, in particular, the Leu42–Lys58 inhibitory segment. In addition, evidence has been obtained indicating that the C-terminal region of IF_1 interacts with the OSCP subunit of the F_o moiety in a pH-independent process (Zanotti et al., 2004). This pH-independent binding of IF_1 to OSCP in the lateral stalk could be relevant at alkaline pH of the matrix when the inhibitory segment is detached from the αβ subunits, keeping the inhibitor protein (IF_1) attached to the complex.

The role of IF_1 in pathophysiology has been essentially investigated in the context of ischemia and tumor cell growth. In the absence of oxygen, the electrochemical gradient vanishes and glycolysis becomes the only cellular source of ATP. Under these conditions, ATP can be rapidly hydrolyzed by F_1F_o ATP synthase, causing rapid cellular energy depletion. The inhibitory action of IF_1, by preventing dissipative hydrolysis of glycolytic ATP, can contribute to prevent ischemic cell injuries. The protective role of IF_1 can be especially relevant for cardiac muscle tissue and other tissues with a high oxidative metabolism (Rouslin, 1991; Rouslin and Broge, 1996).

Also, IF_1 has been found to be overexpressed in murine and human neoplastic cells, whose rapid growth is essentially supported by increased glycolytic production of ATP (Luciakova and Kuzela, 1984; Chernyak et al., 1994; Capuano et al., 1997). These observations have very recently been confirmed by cross-linking studies and kinetic data from rat hepatoma cells. These studies showed that neoplastic cells overexpress the IF_1 inhibitor protein that binds with high efficiency to the ATP synthase complex, thus preventing energy dissipation (Bravo et al., 2004).

The IF_1 ATPase inhibitor appears to be missing in Luft's disease, a mitochondrial myopathy. Oxidative phosphorylation in mitochondria isolated from skeletal muscle of patients suffering from Luft's disease is loosely coupled with abnormally low P/O ratios (Yamada and Huzel, 1992).

5 ATP Synthase and Human Diseases

Among the mitochondrial pathologies, those associated with a defect in the F_1F_o ATP synthase complex are very severe and primarily involve the pediatric population (Houstek et al., 2004). The symptoms of these disorders include muscle weakness or exercise intolerance, heart failure or rhythm disturbances, dementia, movement disorders, stroke-like episodes, deafness, blindness, limited mobility of the eyes, vomiting, and seizures. The prognosis for these disorders ranges in severity from progressive weakness to death.

In mammals, all the ATP synthase subunits, except two mitochondrial-encoded subunits (ATP6 and A6L), are nuclear encoded. The presence of proteins encoded by two separate genomes in different cellular compartments makes the biogenesis and assembly of eukaryotic complex V a complex process. Furthermore, additional nuclear factors are needed for the assembly of the enzyme. The biogenesis of complex V is a poorly understood multistep process with several assembly intermediates. Five assembly factors have been identified in yeast mitochondria. Two of them, Atp10p and Atp22p, mediate F_o assembly (Ackerman and Tzagoloff, 1990a; Helfenbein et al., 2003) while the other three, Atp11p, Atp12p, and Fmc1p, have been found to be involved in F_1 assembly. Only two human orthologs of Atp11p and Atp12p have been identified (Ackerman and Tzagoloff, 1990b; Lefebvre-Legendre et al., 2001).

Genetic defects in mitochondrial ATP synthase appear to involve either mtDNA mutations in the ATP6 gene, encoding the F_o a subunit, or nuclear mutation in genes that encode specific assembly proteins or biosynthetic factors. No pathogenic mutations involving nuclear-encoded structural subunits of ATP synthase have yet been reported.

The neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) syndrome and the maternally inherited Leigh's syndrome (MILS) are two maternally inherited primary ATPase defects associated with mutations in the mitochondrial ATP6 gene (subunit a). The ATP synthase H^+ channel of these patients displays an impairment that is often, but not always, related to decreased ATP production. No mutation has so far been described for the mitochondrial ATP 8 gene (Houstek et al., 2004).

Some other ATP synthase defects of nuclear origin have been identified by complementation analysis (Houstek et al., 1999; De Meirleir et al., 2004; Mayr et al., 2004). Usually, these patients have a selective decrease of the complex V content, caused by a diminished biogenesis of the ATPase complex. Complete loss of the ATP synthase enzyme is probably incompatible with life. However, partial loss of the complex has been associated with human diseases (Houstek et al., 2004). ATP synthase is also involved in some brain degenerative processes. Some of these display an accumulation of complex V subunits in cellular compartments other than mitochondria, while others show a decreased ATP synthase activity (Das, 2003).

Recently, evidence has been obtained for the implication of the α subunit of the ATP synthase in neurofibrillary degeneration of Alzheimer's disease. The α subunit accumulates in the cytosol of degenerating neurons in Alzheimer's disease and could have a role in the neurodegenerative process (Sergeant et al., 2003).

Some forms of neuronal ceroid lipofuscinosis (Batten disease in man), a neurodegenerative lysosomal storage disease, are characterized by structurally altered mitochondria and by an altered cellular handling of the F_o subunit c (Palmer et al., 1992). This protein appears to accumulate in lysosomes and constitutes more than 50% of the fluorescent storage bodies associated with most forms of this pathology. The relationship between subunit c accumulation and neuronal degeneration is still unknown. The occurrence of subunit c homologs on the plasma membrane of neurons, where it assembles to form high-conductance ion pores, has led to the hypothesis that subunit c accumulation in Batten disease can result in altered cation permeability due to an increased number of subunit c pores in the plasma membrane (McGeoch and Palmer, 1999). Reduced ATP levels have been observed in fibroblasts from patients with early infantile, infantile, and juvenile neuronal ceroid lipofuscinosis (CLN1, CLN2, and CLN3, respectively) (Das and Kohlschutter, 1996; Das et al., 1999).

A significant inhibition of the ATP synthase complex activity has been observed in cultures of mixed cortex cells from rat brain incubated with 3-hydroxyglutarate (Ullrich et al., 1999). 3-hydroxyglutarate contributes to neurodegeneration (Flott-Rahmel et al., 1997) and accumulates, together with glutaconic acid, in the brain and cerebrospinal fluid of patients with glutaconic aciduria, an organic aciduria due to inborn deficiency of glutaryl-CoA dehydrogenase (Hoffmann and Zschocke, 1999). The neurodegenerative process appears to be related to disturbed energy supply, which causes a "slow onset excitotoxicity" comprising membrane depolarization and successive alteration in homeostasis of calcium and other ions due to the opening of N-methyl-D-aspartate receptor ion channels (Beal, 1992, 1995).

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