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ATP Synthase: Structure, Function and Inhibition

Abstract: Oxidative phosphorylation is carried out by five complexes, which are the sites for electron transport and ATP synthesis. Among those, Complex V (also known as the F_1F_0 ATP Synthase or ATPase) is responsible for the generation of ATP through phosphorylation of ADP by using electrochemical energy generated by proton gradient across the inner membrane of mitochondria. A multi subunit structure that works like a pump functions along the proton gradient across the membranes which not only results in ATP synthesis and breakdown, but also facilitates electron transport. Since ATP is the major energy currency in all living cells, its synthesis and function have widely been studied over the last few decades uncovering several aspects of ATP synthase. This review intends to summarize the structure, function and inhibition of the ATP synthase.

Keywords: ATP, ATP Synthase, Electron Transport Chain, Proton motive force, Rotational Catalysis, ATP synthase Inhibition.

ATP: The Fuel for Life

Often referred as "molecular currency" for intracellular energy transfer, Adenosine Triphosphate (ATP) functions as a chemical fuel by powering many organic processes of life. ATP generation is the principle energy generating procedure found in all forms of life. ATP is the fuel for the operation of almost all metabolic pathways of the cell. In an ATP molecule, two high-energy phosphate bonds, called phosphoanhydride bonds, are responsible for high energy content. Hydrolysis of the third phosphate group produces adenosine diphosphate (ADP) and inorganic phosphate (Pi), along with considerable release of energy. ADP can absorb energy and regain the group to regenerate an ATP molecule to maintain constant ATP concentration.

Other than supporting almost all the cellular functions that require energy, ATP also works as a coenzyme during phosphorylation reactions. Besides, ATP has crucial role in RNA and DNA synthesis and in amino acid activation during protein synthesis. Khakh and Burnstock, established that ATP also serves as a critical signaling molecule that allows inter and intracellular communications. The ubiquitous distribution of ATP allows for signaling functions that have a uniquely broad influence on physiological functioning [1].

Estimates show that the normal body uses 40 kg of ATP on a daily basis; hence ATP production is one of the most frequent processes occurring in the body [2]. The aggregate amount of ATP in the human body is about 0.1 Mole. The energy utilized every day by an adult requires the hydrolysis of 100 to 150 Moles of ATP. This implies every ATP molecule must be reused 1000 or more multiple times in a day [3]. ATP cannot be stored and so its synthesis is closely linked to its consumption. ATP is produced under aerobic conditions through glycolysis, citric acid cycle/oxidative phosphorylation and beta-oxidation and under anaerobic condition through fermentation, photophosphorylation and replenishment reactions catalyzed by nucleoside diphosphate kinase [4].

ATP Synthase

ATP is synthesized from its precursor, ADP, by ATP synthases. These enzymes are found in the cristae and the inner membrane of mitochondria, the thylakoid membrane of chloroplasts, and the plasma membrane of bacteria [5]. Usually, there is a general understanding that ATP generation occurs in mitochondria. However, in the case of bacteria and archaea that lack mitochondria, ATP synthase is found in their plasma membrane. Additionally, ATP synthases are licensed to inhabit the chloroplast of plant cells. The structure and procedure of ATP synthesis is similar in all three locations except that light energy excites electrons enabling transmembrane movement of H^+ ions in

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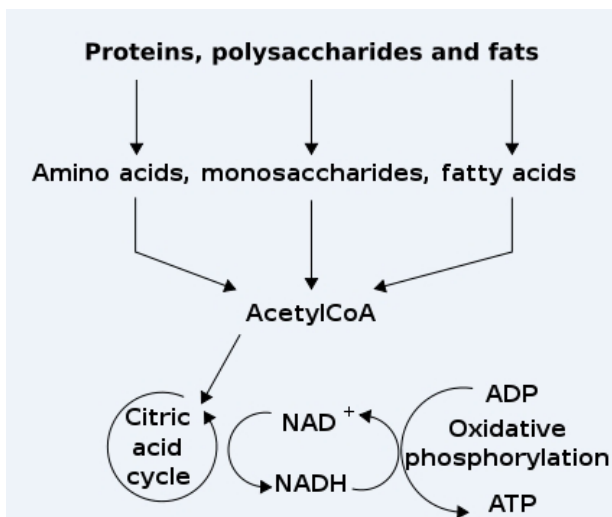
Figure 1: Production and Utilization of Acetyl CoA: The complex macromolecules present in dietary food are processed through various metabolic pathways into Acetyl CoA. Acetyl CoA in the mitochondria is then oxidized to carbon dioxide and water through the Citric Acid Cycle and Oxidative Phosphorylation. The electrochemical energy generated due to the transfer of electrons from NADH or $FADH_2$ to O_2 in a series of electron transfer reaction is utilized to synthesize ATP.

chloroplasts. The general nomenclature of ATP synthase as F_oF_1 changes to CF_oCF_1 for chloroplast ATP synthase and ECF_oECF_1 for *Escherichia coli*'s ATP synthase [6].

ATP synthesis is the most widespread chemical reaction inside the biological world. ATP synthase is the very last enzyme in oxidative phosphorylation pathway that makes use of electrochemical energy to power ATP synthesis [7 - 10]. ATP synthase is one of the most ubiquitous and plentiful protein on the earth, accountable for the reversible catalysis of ATP to ADP and Pi. This is also one of the most conserved proteins in Bacteria, Plants and Mammals with more than 60% of the amino-acid residues of the catalytic β-subunit resisting evolution [11]. ATP synthases are classified as F (Phosphorylation Factor), V (Vacuole), A (Archaea), P (Proton) or E (Extracellular) ATPases based on their functional differences, although they all catalyze ATP synthesis and/or hydrolysis.

The mitochondrial ATP synthase is a multi-subunit protein complex having an approximate molecular weight of 550 kDa. The human mitochondrial ATP synthase or F1/F0 ATPase or complex V (EC 3.6.3.14) is the fifth component of oxidative phosphorylation chain [12]. This enzyme is the smallest known biological nanomotor and plays a crucial role in ATP generation. In plants, energy acquired from photons is transferred through photosynthetic electron transport chain (ETC), which induces an electrochemical

gradient to build up across the membrane. ATP synthase uses energy conferred by this electrochemical gradient for phosphorylation of ADP to generate ATP [7].

Electrochemical gradient and the Chemiosmotic Theory

The chemiosmotic hypothesis proposed by Peter Mitchell states: "The differential of electrochemical activity of the hydrogen and hydroxyl ions across the membrane generated by electron transport causes the specific translocation of hydroxyl and hydrogen ions from the active centre of the so called ATPase system thus effectively dehydrating ADP+P". This pioneered the research on the coupling of the ETC and ATP synthesis. Basically, protons are pumped across the inner mitochondrial membrane as electrons pass through the electron transfer chain. This induces a proton gradient, with a decreased pH in the intermembrane space and an increased pH in the matrix of the mitochondria. The proton gradient and membrane potential are the major forces involved in ATP synthesis. Essentially, the pH gradient acts as a 'battery' which stores the electrochemical energy to be used later for ATP production.

It is well established that the electrochemical potential of protons delivered by electron transfer chains across the mitochondrial, chloroplast or bacterial membrane provides the energy for ATP synthesis [14]. Cellular respiration in the mitochondria is a widely studied process that incorporates chemiosmosis for the production of ATP.

Mitochondria, the chief organelles producing ATP, are absent in prokaryotic organisms. In the absence of mitochondria, archaea and bacteria maneuver chemiosmosis to produce ATP through photophosphorylation. This process, taking place across the inner membrane, is coherent with the ETC, proton gradient, and chemiosmosis of H^+ [15].

Electrons from NADH, FADH and other oxidizable substrates pass through the complexes of the ETC arranged asymmetrically in the inner membrane of mitochondria. Electron flow is accompanied by the transfer of protons (H^+) across the membrane, producing both chemical gradient (ΔpH) and electric gradient ($\Delta\Psi$). The electrochemical energy built through the difference in proton concentration and separation of charge across inner mitochondrial membrane translates to the proton motive force (PMF). The PMF drives the synthesis of ATP as proton flow back into the matrix through the proton specific channels (F_o) component of the ATP synthase.
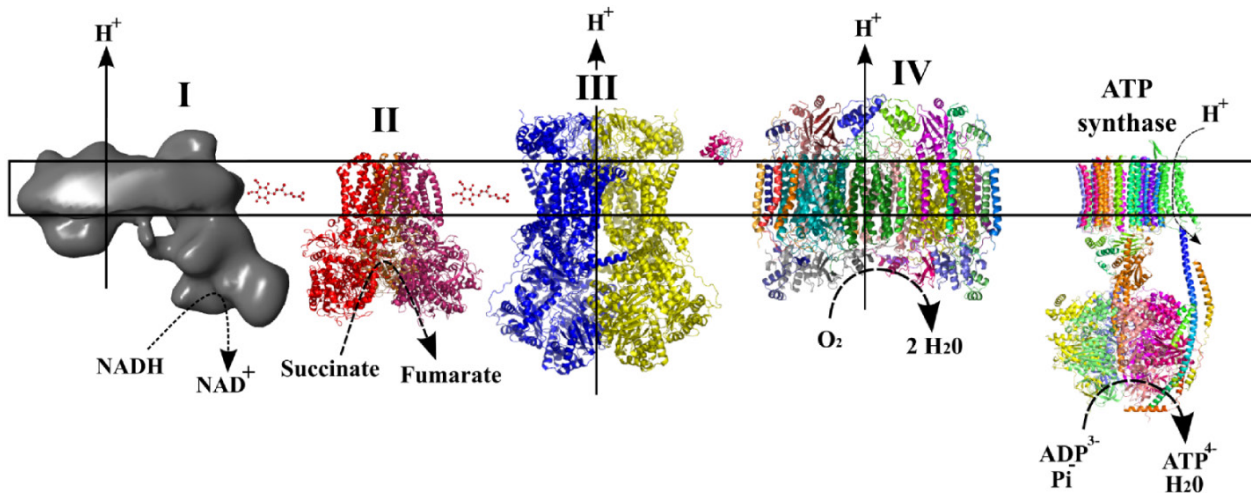
Figure 2: The Mitochondrial Electron transport chain and ATP synthase.The Electron transport chain composed of four different multi-subunit complexes transfer electrons (e-) in a sequential manner ultimately reducing O_2 to H_2O. Electron transfer is coupled to a vectorial proton translocation outdoor into the matrix via three of the four complexes (I, III and IV). Protons gather and create an electrochemical gradient throughout the inner mitochondrial membrane. This osmotic potential is used to power ATP synthesis when protons re-enter the mitochondrial matrix through ATP synthase [13].

This also satisfies a main criterion stated by Mitchell for the chemiosmotic coupling to occur: the inner mitochondrial membrane must be impermeable to protons. Thus, protons are compelled to re-enter matrix through F_0 while F_1 catalyzes the synthesis of ATP [16].

Feniouk, described ATP synthesis/hydrolysis by the reaction:

$$ATP^{4-} + H_2O <=> ADP^{3-} + P_i^{2-} + H^+$$
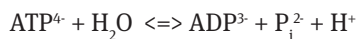
ATP synthase is powered by the transmembrane electrochemical proton potential difference ($\Delta \hat{\mu} H^+$) measured in Joules per mole (J mol^{-1}) and is defined as:

$$\Delta \hat{\mu} H^+, = -F \Delta \Psi + 2.3 RT (pH_P - pH_N),$$

where, P and N denote the positively and the negatively charged sides of the coupling membrane; F is Faraday constant (96 485 C mol^{-1}); R is the molar gas constant (8.314 J mol^{-1}K^{-1}), T is the temperature in Kelvin, and $\Delta \Psi$ is the transmembrane electrical potential difference in volts. Protons, being charged particles, are driven by $\Delta \hat{\mu} H^+$, from the positively charged side to the negatively charged side. The value of $\Delta \hat{\mu} H^+$ gives energy required or released when 1 Mole of proton move across the membrane. During hydrolysis, the enzyme operates as ATP-driven proton pump generating $\Delta \hat{\mu} H^+$. The equation for reaction catalyzed is:

$$ADP^{3-} + P_i^{2-} + nH^+_P <=> ATP^{4-} + H_2O + (n-1)H^+_N$$
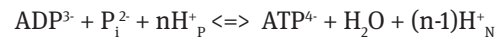
$\Delta \hat{\mu} H^+$ is more conveniently replaced by proton-motive force (*pmf*) measured in millivolts and defined as:

$$\boldsymbol{pmf} = - \Delta \hat{\mu} H^+ / F$$
$$= \Delta \Psi (- 2.3 RT)/F (pH_P - pH_N)$$

At room temperature (25°C),
$$\boldsymbol{pmf} = \Delta \Psi (- 59) (pH_P - pH_N)$$

The *pmf* value, for most biological membranes involved in ATP synthesis, lies between 120 and 200 mV ($\Delta \hat{\mu} H^+$ between 11.6 and 19. 3 kJ mol^{-1}). This energy is capitalized by ATP synthase to catalyze the formation of ATP from ADP and inorganic Phosphate [17].

Structural Assembly of F_1F_0 ATP Synthase

E.coli ATPase/synthase comprises of 8 different subunits. There are only slight variations in its structure in the chloroplast and in the mitochondria. The chloroplast ATPase has two isoforms and in the mitochondria it has 7-9 additional subunits. Besides these differences, ATPases are structurally and functionally similar. The ATP synthase, also called Complex V, has two major subunits designated
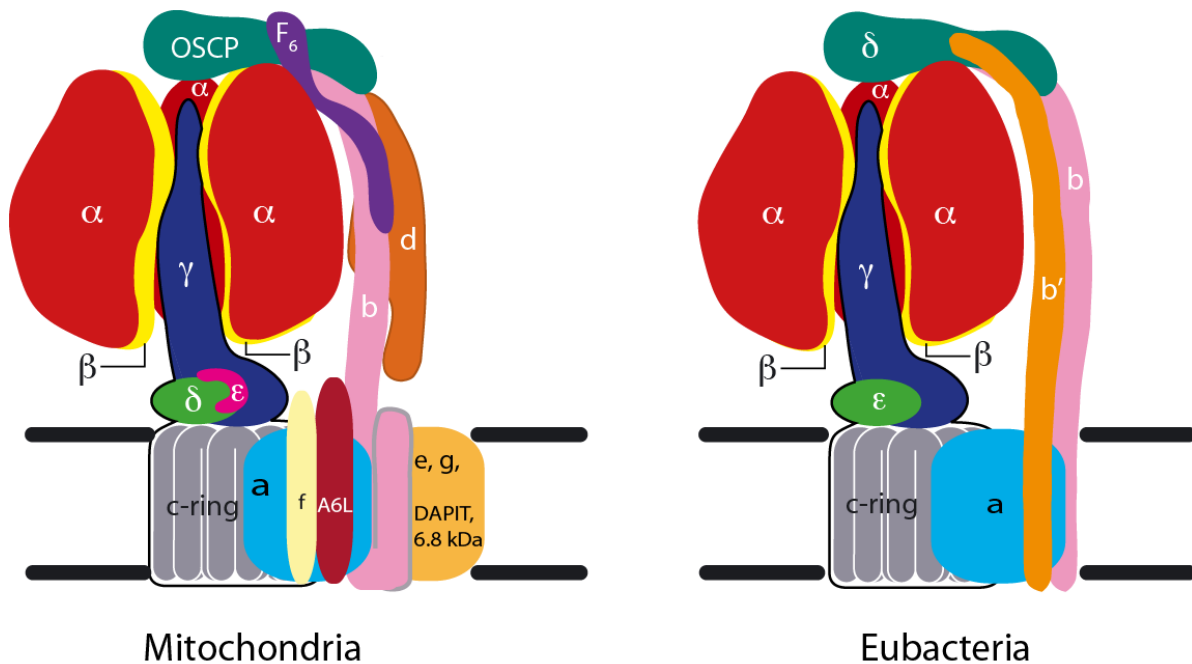
Mitochondria   Eubacteria

Figure 3: Schematic subunit composition of ATP synthase. The bacterial and chloroplast ATP synthases have comparable subunit composition, with $\alpha_3\beta_3\gamma\delta\epsilon$ constituting F_1 and F_0 comprising of ab_2c_{10-15}. Subunits γ and ϵ shape a primary stalk that non-covalently interacts with the membrane bound, hydrophobic c-ring. The proteolipidic oligomer c rotates in opposition to the peripheral stator that is made from ab_2 and δ. The stator restricts rotation of the $\alpha_3\beta_3$ hexamer, which provides six nucleotide binding sites (each located at $\alpha\beta$-subunit interface) for reversible ATP synthesis. Ions are translocated across channels at the a−c ring interface, generating a torque on the central stalk $\gamma\epsilon$. The γ-subunit which forms α-helical coiled-coil structure is embedded in the F_1-hexamer. The torque of γ causes alternating conformational adjustments in the β-subunits, leading to differences in nucleotide binding affinities (open, tight and free), which eventually bring about ATP synthesis or hydrolysis. Mitochondrial ATP synthase possess the bacterial core set of subunits, but contain additional subunits in the F_0 domain (subunits DAPIT and 6.8 kDa Proteolipid) and in the stator (subunits d and F6). OSCP (Oligomycin sensitivity-conferring protein) is equivalent to δ subunit of bacterial enzyme.

F_0 and F_1. The F_0 part, bound to inner mitochondrial membrane is involved in proton translocation, whereas the F_1 part found in the mitochondrial matrix is the water soluble catalytic domain. F_1 is the first factor recognized and isolated from bovine heart mitochondria and is involved in oxidative phosphorylation. It was named so from the term 'Fraction 1'. F_0 was named so as it is a factor that conferred oligomycin sensitivity to soluble F_1 [18].

The structure of enzyme ATP synthase mimics an assembly of two motors with a shared common rotor shaft and stabilized by a peripheral stator stalk. The F1 part of ATP synthase is made up of 8 subunits, 3α, 3β, γ, δ and ε, where the γ, δ and ε subunits add up to the central stalk (or the rotor shaft) and an alternate arrangement of 3α and 3β form a hexameric ring with a central cavity. The γ subunit inserted in the central cavity protrudes out to meet ε which binds on its side and together they bind the F_0.

Bacterial F_0 has the simplest subunit structure consisting a_1, b_2 and c_{10-14} subunits. Eukaryotic F_0 has several subunits including d, F_6 and the oligomycin sensitivity-conferring protein (OSCP). Subunits b, d, F_6 and OSCP form the peripheral stalk, which connect both F_1 and F_0 and keep the stators (F_1-$\alpha\beta_3$ and F_0a) from spinning along with the rotor (γδε and F_0c). Other additional subunits such as subunit e, f, g, and A6L extending over the membrane cohort with F_0 [5, 10, 20].

Rotational catalysis and ATP generation

Paul Boyer proposed a simple catalytic scheme, commonly known as the binding change mechanism, which predicted that F-ATPase implements a rotational mechanism in the
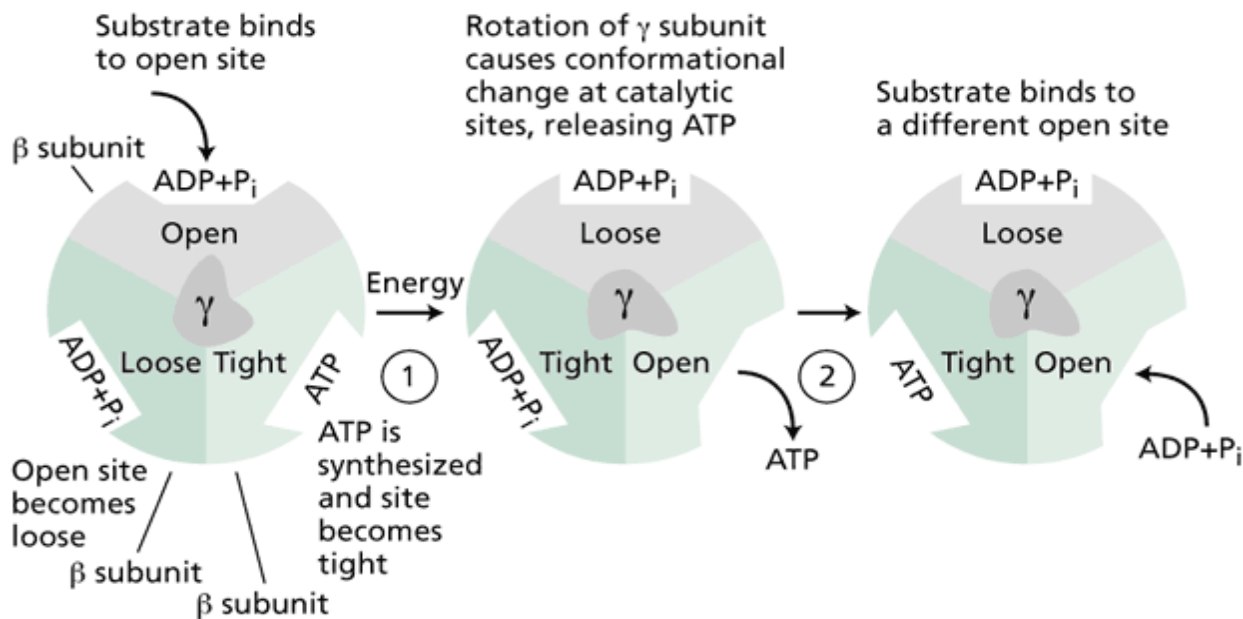
Figure 4: The binding-change mechanism as seen from the top of the F_1 complex. There are three catalytic sites in three different conformations: loose, open, and tight. (For clarity, only the three β subunits are shown.) Substrate (ADP + P_i) initially binds to the open site and is converted to ATP at the tight site. In step 1, rotation of the γ subunit causes a conformational change, resulting in a change in the formation of the sites. As a result, ATP is released from the enzyme. In step 2, substrate again binds to the open site, and another ATP is synthesized at the tight site [25].

catalysis of ATP [21]. The movement of subunits within the ATP synthase complex plays essential roles in both transport and catalytic mechanisms. Each catalytic site would achieve and change three conformations during a complete 360° turnover and a cycle would be completed at a different catalytic site with a rotation of 120°. When a nucleotide binds to ATPase, it undergoes a conformational change in order to be tightly bound to ATP. Another subsequent change in conformation brings about the release of ATP. These conformational changes are accomplished by rotating the inner core of the enzyme. The core itself is powered by the proton motive force conferred by protons crossing the mitochondrial membrane.

Masamitsu *et. al* reviewed that 3αs and 3βs are positioned alternatingly in a circle, each having different function as well as conformation [22]. Although the α subunit has bound ATP, it neither releases the ATP nor participates in the reaction. Each β subunit has three catalytic sites that differ in nucleotide binding states [23]. The first is occupied by Mg·AMP-PNP1 (an analog of ATP), the second is occupied by Mg·ADP, and the third is empty (no bound nucleotide); these sites are termed βT, βD, and βE, respectively. The F_1 system has inherent asymmetry within the β subunit conformations, which depend on orientation of the γ subunit. When the γ subunit rotates,

it induces conformational changes in each β subunit conducive to the surface of γ subunit in contact. The rotation of the shaft (γ subunit) is effectuated by the flow of protons into the matrix through F_0, which connectedly provides the energy for the release of ATP [24].

Conformational transitions that are significant in rotational catalysis are directed by the passage of protons through the F_0 assembly of ATP synthase. The flow of protons through the F_0 pore brings about the rotation of the cylinder of c subunits and the attached γ subunit around the axis of γ. The γ subunit runs along the central canal of the $(\alpha\beta)_3$ assembly, which is held stationary relative to the membrane surface by the b_2 and δ subunits and meets the c ring via δ and ε subunits. With every rotation of 120°, γ comes into contact with a different β subunit, and the contact forces that β subunit into the βE conformation. The three subunits switch conformations in such a way that when one holds the βE conformation, one of its flanking subunit assumes the βD form, and the other takes βT form. As a consequence of a complete rotation of the γ subunit, each β subunit courses through all three possible conformations and three ATP's are synthesized and delivered by the enzyme.

A sturdy prediction of the binding-change model is that the γ subunit must rotate in one direction when FoF_1

is synthesizing ATP and in the opposite direction whilst hydrolyzing ATP, was confirmed by the experiments of Masasuke Yoshida and Kazuhiko Kinosita, Jr.. In biological conditions, when the concentration of H^+ ions for F_0 motor is greater compared to F_1 motor, protons enter the matrix through the F_0 pore and the F_0 motor rotates anticlockwise to turn around F_1, thereby driving ATP synthesis. On the other hand, when the proton concentration is higher in the mitochondrial matrix, the F_1 motor reverses the F_0 motor bringing about the hydrolysis of ATP to power translocation of protons to the other side of membrane.

A team of Japanese scientists have succeeded in attaching magnetic beads to the stalks of F_1-ATPase isolated *in vitro*, which rotated in presence of a rotating magnetic field. F_1-ATPase synthesized ATP from ADP and Pi when rotated in a clockwise direction at a rate of about 5 molecules per second. Additionally, ATP was hydrolyzed when the stalks were rotated in the counterclockwise direction or when they were not rotated at all [26].

ATP Synthase Diseases

Defects or mutations in this enzyme are known to cause many diseases in humans. The first defect in ATP synthase was reported by Houstek *et. al.*in the case of a child with severe lactic acidosis, cardiomyopathy and hepatomegaly, who died 2 days after birth. Their study indicated that this enzyme was under expressed by 70-80% and there was no observed mutation or expression deficit in the enzyme. It was postulated that mutations in some factors explicitly involved in the assembly of ATP synthase could have caused the defect [27]. Kucharczyk *et. al.*in their review have discussed mutations in both the nuclear and mitochondrial DNA. A mutation in one or many of the subunits in ATPase synthase can cause these diseases [28].

Mutation in α subunit has been associated with neuropathy, ataxia, retinitis pigmentosa syndrome, the familial bilateral striatal necrosis and one form of Leigh syndrome (neuromuscular disorder with a 50% survival rate to 3 year old children) [29, 30]. These diseases also result decrement in intermediary metabolism and functioning of the kidneys in removing acid from the body due to increased production of free oxygen radicals. A low expression of the β subunit and the cytosolic accumulation of the α subunit are known to cause Alzheimer's disease. Dysfunction of F_1 specific nuclear encoded assembly factors causes selective ATPase deficiency [31]. Similar inborn defects in the mitochondrial F-ATP synthase,

termed ATP synthase deficiency, have been noted where newborns die within few months or a year.
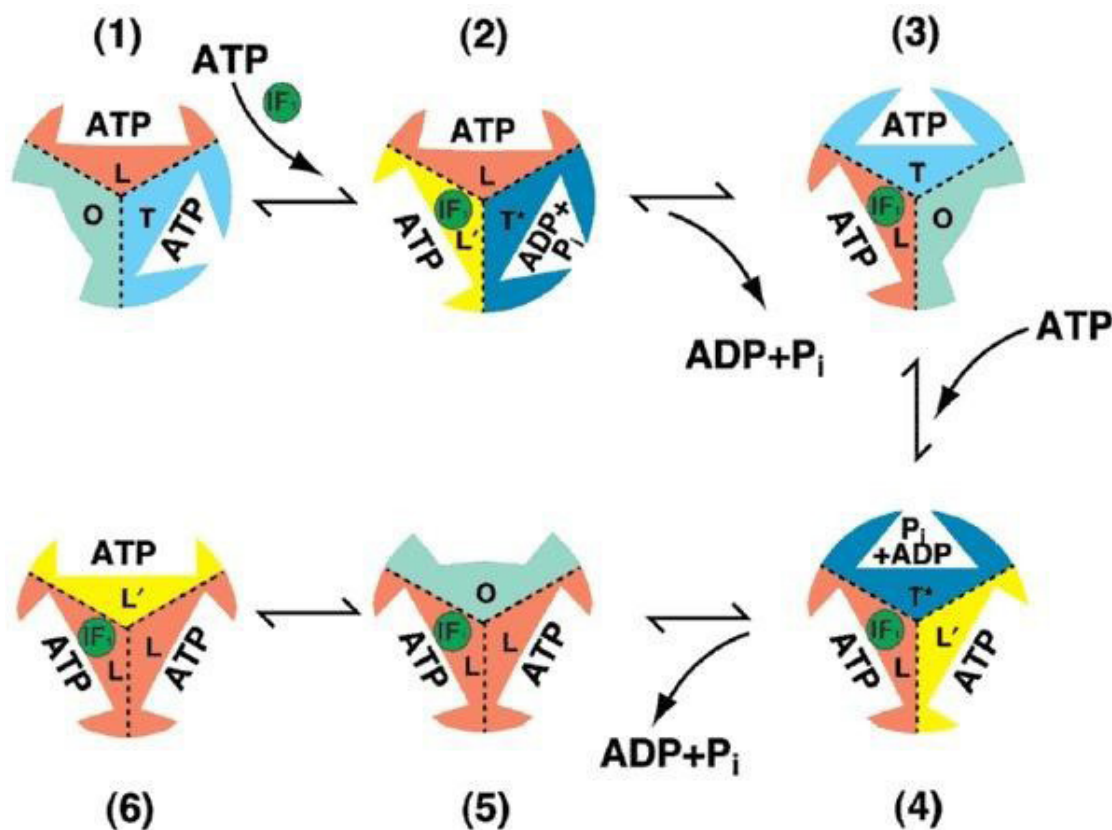
ATP Synthase Inhibition

Current research on ATP synthase as a potential molecular target for the treatment for some human diseases have produced positive consequences. Recently, ATPase has emerged as appealing molecular target for the development of new treatment options for several diseases. ATP synthase is regarded as one of the oldest and most conserved enzymes in the molecular world and it has a complex structure with the possibility of inhibition by a number of inhibitors. In addition, structure elucidation has opened new horizons for development of novel ATP synthase-directed agents with plausible therapeutic effects. More than 250 natural and synthetic inhibitors have been classified to date, with reports of their known or proposed inhibitory sites and modes of action [30].

We look to explore a few important inhibitors of ATP synthase in this paper. A drug, diarylquinoline (also known as TMC207) developed against tuberculosis is known to block the synthesis of ATP by targeting subunit *c* of ATP synthase of tuberculosis bacteria. Another such diarylquinone, Bedaquiline, is used for the treatment of multidrug resistant tuberculosis.

Among other ATP synthase inhibitors, Bz-423 is pro-apoptotic and 1,4-benzodiazepine binds the oligomycin sensitivity conferring protein (OSCP) component resulting in the generation of superoxide and subsequent apoptosis [32 - 34]. Melittin, a cationic, amphiphilic polypeptide is yet another ATP synthase inhibitor with documented inhibition of catalytic activities in mitochondrial and chloroplast ATP synthases [35].

IF1 and oligomycin are two other important classes of ATPase inhibitors. The binding of IF1, an endogenous inhibitor protein, fundamentally locks the ATP bound to the catalytic site of ATPase and restricts ATP hydrolysis. Oligomycin, an antibiotic, blocks protein channel F_0 subunit and this inhibition eventually inhibits the electron transport chain. This further prevents protons from passing back into mitochondria, eventually ceasing the operations of the proton pump, as the gradients become too high for them to operate.

Several polyphenolic phytochemicals, such as quercetin and resveratrol, have been known to affect the activity ATPase. Resveratrol and Genistein are profound non-competitive inhibitors of F0F1-ATPase. Quercetin, a flavonoid, inhibits F-ATPase and other ATPases, such as

This scheme is based on the binding change mechanism of ATP hydrolysis [36].

Figure 5: The inhibition of the ATP hydrolytic activity of ATP synthase by IF1. IF1 is a naturally occurring 9.6 kDa basic protein that comprises of 84 amino acids and is known to inhibit the hydrolytic activity of mitochondrial ATP synthase [37]. It acts by binding to ATP synthase at the F_1 domain in the COOH-terminal region of the β-subunit in an area which is in contact with the central γ subunit. It disrupts the contact between the β and γ subunits, thereby inhibiting the function of ATPase [38].

Na^+/K^+-ATPase, Ca^{2+}-ATPase. At decreased concentrations, it inhibits both soluble and insoluble mitochondrial ATPase. However, it does not impact oxidative phosphorylation occurring in other mitochondrial entities [39 - 41].

Several other plant products also serve as ATPase inhibitors. Polyphenols and flavones has been found effective in the inhibition of bovine and porcine heart F_0F_1-ATPase [41, 42]. Efrapeptins are peptides which are produced by fungi of the genus *Tolypocladium* that have antifungal, insecticidal and mitochondrial ATPase inhibitory activities [43]. They target F_1 particles, especially in the α, β and γ subunits, thereby inhibiting both ATP hydrolysis and ATP synthesis in mitochondria, chloroplasts, and photosynthetic bacteria. The mode of inhibition is competitive with ADP and phosphate [30]. Another inhibitor piceatannol, a stilbenoid, has been found to inhibit the F-type ATPase preferably by targeting the F_1 subunit [39].

Another inhibitor of ATPase is bicarbonate. Bicarbonate anion acts as activator of ATP hydrolysis and Lodeyro *et. al.*found that bicarbonate prompts ATP hydrolysis while inhibiting steady-state ATP synthesis profoundly by increasing the affinity of ATP for the catalytic site. This inhibition of ATP synthase activity was competitive with respect to ADP at low fixed phosphate concentration, mixed at high phosphate concentration and non-competitive towards Pi at any fixed ADP concentration [44].

Other inhibitors of ATPase are tenoxin, lecucinostatin, fluro-aluminate, dicyclohexyl-carbodimide and azide. Tentoxin, a phyotoxin, specifically inhibits the activity of chloroplast ATP synthases by binding at the cleft between the α and β subunits close to N-terminal beta-barrel crown of F_1[45]. Leucinostatins bind to the F_0 part of ATP synthases and inhibit oxidative phosphorylation in mitochondria and photophosphorylation in chloroplasts [46]. Fluoro-aluminate based inhibitors bind together with ADP in catalytic sites and freeze the enzyme in a confirmation

that presumably reflects an intermediate step of ATP hydrolysis/synthesis [47]. Dicyclohexylcarbodiimide (DCCD) reacts with the carboxyl group of the conserved acidic amino acid residue of subunit c at higher pH levels. However, at a lower pH (<7), DCCD modifies several carboxyl groups in F_1 and inactivates it. So this compound can be considered as an inhibitor of both F_O and F_1. However, inhibition of F_O is highly specific, well-defined, and requires a much lower concentration of the inhibitor [48]. Azide in mitochondrial F_1 selectively inhibits ATPase activity by binding with MgADP (interacting with its beta-phosphate) in a catalytic site, and presumably prevents ADP release from this site, leaving its ATP synthesis activity unaffected [49].

The list of inhibitors that directly and indirectly inhibit the activity of ATP synthase includes, magnesium, bismuth subcitrate and omeprazole, ethidium bromide, adenylyl imidodiphosphate, arsenate, angiostatin and enterostatin, ossamycin, dequalinium and methionine, almitrine, apoptolidin, aurovertin and citreoviridin, rhodamines, venturicidin, estrogens, catechins, kaempferol, genistein, biochanin A, daidzein and continues to grow [50 - 62].

Conflict of interest: Authors state no conflict of interest

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