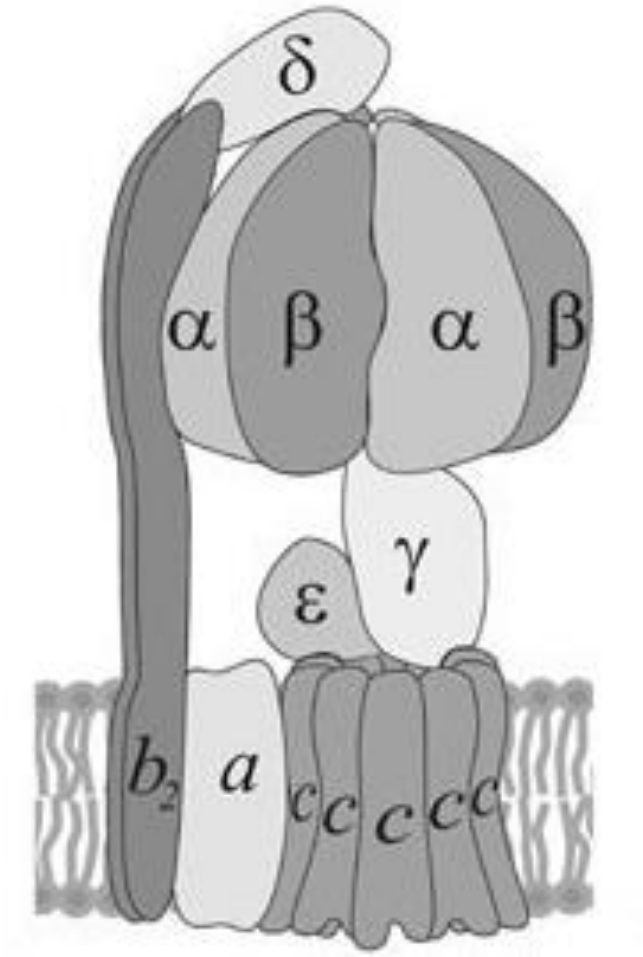
# The Motor of Life

How energy is made by the ATP synthase




*Britt Neijzen*
*s2729202*

*Begeleider: Arnold Driessen*
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## Abstract

The $F_1F_0$ ATPase molecule is a rotary enzyme that makes the energy rich ATP molecule. It consists of two parts, the $F_1$ portion and the $F_0$ portion of the enzyme. The $F_0$ portion of the enzyme is responsible for the translocation of protons over the membrane. This creates the rotation that the $F_1$ portion of the enzyme can use to create the ATP molecule from an ADP molecule and a $P_i$ molecule. The molecule can either synthesize ATP or hydrolyse it. During the synthesis, the molecule uses proton motive force to create ATP. During the hydrolysis ATP molecules are used to create proton motive force.

The $F_1$ portion of the F-ATPase molecule consists of three α subunits, three β subunits, a γ subunit, a δ subunit, and an ε subunit. The α and β subunits are very similar in their structure, but the β subunit has a catalytic binding site where the α subunit contains a non-catalytic binding site. The α and β subunits are packed together in a $\alpha_3\beta_3$ hexamer. The γ subunit and the ε subunit together form the central stalk. The δ subunit forms the peripheral stalk together with the b subunits of the $F_0$ portion of the protein. The synthesis and hydrolysis of ATP takes place in the catalytic site of the β subunits. The synthesis is driven by the conformational changes in the subunit due to the rotation of the central stalk. This rotation is driven by proton motive force. The hydrolysis of ATP is driven by the energy that is released when ATP breaks down to ADP and $P_i$. The hydrolysis drives conformational changes of the subunits and the rotation of the central stalk in the counter clockwise direction.

During the hydrolysis of the ATP molecule, the first step is the binding of ATP to the catalytic binding site of the $\alpha_3\beta_3$ hexamer. This happens at the beginning of the rotation of the central stalk. This point is also called the ATP waiting dwell. After this the ATP is hydrolysed into ADP and $P_i$. There is a lot of discussion about what happens after this. It could be that $P_i$ is released first and after that ADP. Others say that it is the ADP molecule that is released first. It has also been said that there are both released at the same time, at the end of the rotation.

During the synthesis of the ATP molecule the ADP and $P_i$ must bind to the catalytic site of the $\alpha_3\beta_3$ hexamer. The binding of ADP happens spontaneous. The binding of $P_i$ however, can only happen when there is a proton gradient present. The release of the ATP molecule is a process that needs a lot of energy. This reaction needs as much energy as it costs to form a ATP molecule.

# Contents

# Introduction

The ATP molecule is the adenosine triphosphate molecule, this molecule is an energy carrier. The energy that is released by the hydrolyses of ATP to ADP and inorganic phosphate can be used to drive a large number of cellular processes in living organisms. The synthesis of ATP is a process that requires energy. This energy is contributed by free energy that is created by oxidation of food and photosynthesis. (Junge & Nelson, 2015) (Berg, Tymoczko, & Stryer, 2012) $F_1F_0$ ATP synthase or F-ATPase is a membrane bound enzyme that is able to synthesize ATP from ADP and inorganic phosphate ($P_i$). To do this the enzyme uses an electric potential gradient that is created by photophosphorylation and electron transport. (Okuno, Iino, & Noji, 2011; Walker, 2013) The $F_1F_0$ ATPase is found in the mitochondria of mammals, chloroplasts of plants and also in bacteria and archaea. Roughly said, all prokaryotes, eukaryotes and archaea have $F_1F_0$ ATPase that can create ATP. (Junge & Nelson, 2015; Morales-Rios, Montgomery, Leslie, & Walker, 2015)

The name of the enzyme is derived from the fact that F-ATPase consist of two major groups of subunits. There is the membrane section called $F_0$. This is the part of the protein that is almost completely embedded in the membrane. It is responsible for translocation of $H^+$ across the membrane. (Schneider & Altendorf, 1987) The $F_0$ section of the enzyme consist of one *a* subunit, a ring of *c* subunits and two *b* subunits. The $F_0$ section of the enzyme is shown in figure 1. The amount of *c* subunits in the ring depends on the organism in which the ATPase protein is found. For example in bovine mitochondria the amount of *c* subunits is eight, but in yeast there are 10 *c* subunits and in spinach chloroplasts there are even 14 *c* subunits.(Junge & Nelson, 2015) Among bacteria the amount of subunits in the *c* ring varies between 10, 11, 13 and 15. (Junge & Nelson, 2015) The two *b* subunits connect $F_0$ to the $F_1$ by binding to the $\alpha_3\beta_3$ hexamer. Also, it is essential for the ability of the *a* and *c* subunits to translocate $H^+$. It has been shown that without the *b* subunit, the remaining two subunits in the $F_0$ are unable to bind $F_1$ and they will not show the translocation. (Schneider & Altendorf, 1984, 1987)

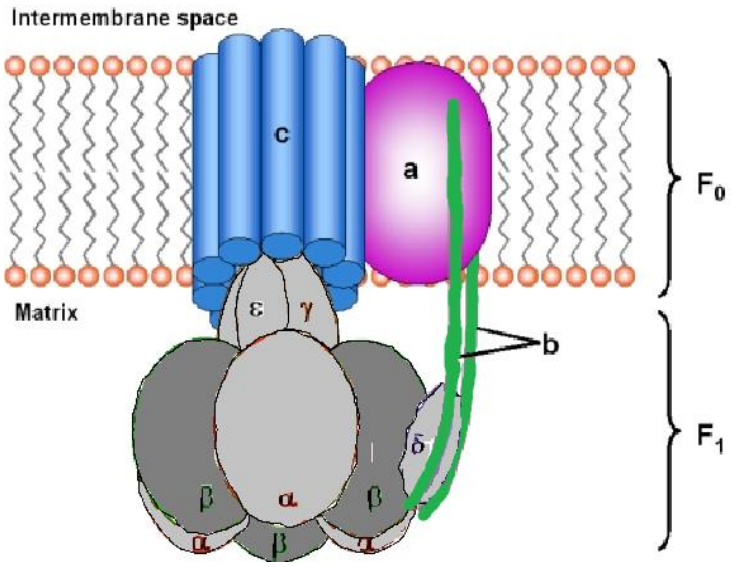


Figure 1: The $F_0$ section of the F-ATPase enzyme. The $F_0$ section consist of the *a* and *c* subunits that are mostly positioned inside the membrane, and the *b* subunit that is anchored in the membrane and connects the $F_0$ section to the $F_1$ section of the enzyme. (Blaber, 2001) (altered)

Subunit *b* contains a hydrophobic α-helix that functions as an anchor to keep the subunit attached to the membrane (see figure 2). This segment is only 20% of the subunit. The rest of the structure is extremely hydrophilic. This is the part of the subunit that resides in the cytoplasm of the cell. (Fillingame, 1992; Schneider & Altendorf, 1987) Subunit *a* is entirely hydrophobic and resides only embedded in the membrane. The subunit spans the membrane about five to seven times (see figure 2). (Schneider & Altendorf, 1987) The *a* subunit has two half-channels that interact with a *c* subunit. (Berg et al., 2012) The *c* subunit is also hydrophobic, maybe even more than subunit a. It spans the membrane twice, as is shown in figure 2. (Schneider & Altendorf, 1987)

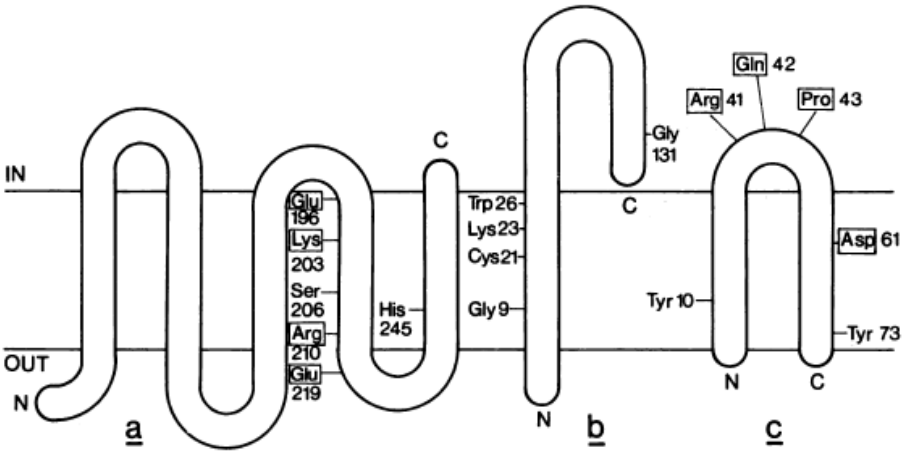


Figure 2: The orientation of the *a, b,* and *c* subunits in the membrane. The *a* subunit spans the membrane five times. The *b* subunit is embedded in the membrane by its hydrophobic anchor. One c subunit spans the membrane twice. (Schneider & Altendorf, 1987)

As is mentioned before, the F-ATPase can synthesize or hydrolyse the energy containing molecule ATP. This molecule can be synthesized using ADP and $P_i$. To do this the enzyme uses the membrane proton gradient that is created by photophosphorylation or electron transport. (Okuno et al., 2011) The enzyme functions as a rotary motor. (Mukherjee, Warshel, Benson, & Bassham, 2017)
The mechanism of the F-ATPase depends on the conditions of the surroundings. If the proton gradient over the membrane is high enough and the concentration of $H^+$ on the outside of the membrane is higher than the concentration in the cytoplasm inside the membrane, the ATPase enzyme will synthesize ATP. This is because the difference in electrochemical gradient and therefore pH. This difference drives the protons into the cell. This is called the proton motive force (pmf). (Berg et al., 2012; Junge & Nelson, 2015) If the proton gradient is to low and the concentrations inside the membrane are almost at equilibrium with the concentration outside the membrane, the enzyme will hydrolyse ATP in order to create a proton gradient. This is necessary because there are a lot of processes in the cell that are driven by pmf instead of ATP. If there is no proton gradient, these processes will not be able to function. (Junge & Nelson, 2015)

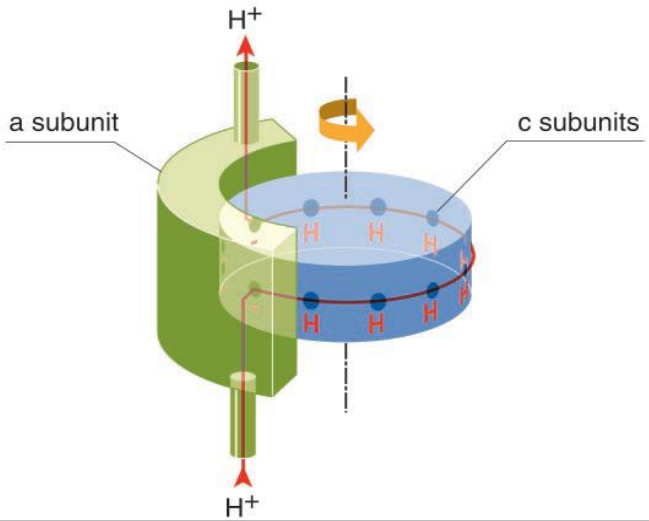


Figure 3: A model of proton transport across the membrane to create a rotational torque. The blue part represents the ring of *c* subunits. The green part represents the single *a* subunit. The red line indicates the path of the proton. In this figure the rotation is counterclockwise as protons are moving out of the cell. (Stock et al., 1999; Walker, 2013)

When ATP is synthesised, the proton concentration outside of the membrane is higher than the concentration in the cytoplasm. Therefore, the protons are driven to move to the cytoplasm. The translocation of the protons is regulated by the *a* and *c* subunits of the $F_0$ membrane section as is shown in figure 3. The proton will go through the half-channel in subunit *a*, and is thereby directed to the *c* subunit. After the *c* subunit completes its rotation, the proton can leave the membrane through the other half-channel of the *a* subunit. Due to the movement of the protons and the fact that they go from the place with a high concentration to a place with a low concentration, the *c* ring will start rotating. (Berg et al., 2012) The translocation op the protons generates a rotary torque. The ring of *c* subunits starts rotating in the clockwise direction as seen from the $F_0$ portion of the enzyme (see figure 3). (Okuno et al., 2011) The *c* ring is connected to the γ subunit of the $F_1$ complex. When the *c* subunit is rotating, it will make the γ subunit rotate as well. The γ subunit then makes the α and β subunits go through a set of conformations. (Boyer, 1997) These subunits bind ADP and $P_i$ and due to the changing of the conformations, the ADP and $P_i$ will form ATP. (Okuno et al., 2011)

When the proton gradient is not high enough to force the protons to translocate, ATP will be hydrolysed to ADP and $P_i$. This means that the *c* ring and the γ subunit are turning in the counter clockwise direction as seen from the $F_0$ portion of the enzyme. Now the protons are actively transported from inside the cytoplasm to outside the membrane. This creates a new proton gradient. (Okuno et al., 2011)
Researches into the structure and the mechanism of the F-ATPase are done in a great variety of organisms. All species have slightly different F-ATPase molecules. There are some subunits (the subunits described above) that are the same in all the organisms. A lot of research has been done in E. coli. Therefore this enzyme is characterized most extensively. (Cretin, Baggetto, Denoroy, & Godinot, 1991) Also, the structure of the E. coli $F_0$ is the simplest that is known thus far. For studies into mammal F-ATPase, cow tissue is mostly used. A lot of research is done using the mitochondria from a bovine heart. Other mammals that are studied often are rats and pigs. The enzyme in the pig and cow mitochondria are indistinguishable, but the mitochondria of rats however do show slight differences. (Cretin et al., 1991).

Synthesis of ATP is mostly studied in the eubacteria Paracoccus Denitrificans. These organisms are related to the protomitochondria, the ancestors of the now engulfed mitochondria in eukaryotic cells. The F-ATPase of these eubacteria can only preform ATP synthesis, not ATP hydrolysis. Therefore it is easier to study the synthesis in these organisms. (Morales-Rios et al., 2015) The F-ATPase molecule can also be studied in the chloroplasts of plants. The F-ATPase enzymes are located in the Thylakoid membranes of the chloroplasts. The structure of the F-ATPase molecules is similar to mitochondrial F-ATPase molecules. (Junge & Nelson, 2015) In this thesis the nomenclature of the bacterial ATPase will be used, unless it is specifically said otherwise.

Though the general working of the F-ATPase enzyme is quite well known, there is still a lot of research into the precise structures of the subunits and their interactions. Also, there are still a lot of questions about the precise working mechanism of the synthesis of ATP. This includes the way in which the ADP, $P_i$ and ATP are bound and released by the molecule. Important for the understanding of these questions is to know what the precise conformations are in the different states of the molecule. To answer these questions the focus will be on the $F_1$ section of the F-ATPase molecule. This is because in this section the synthesis and hydrolysis of ATP takes place.

# Chapter 1: The Structures of the $F_1$ Subunits

**The structure of the α and β subunits**

The structures of the α and β subunits are very similar to each other. They both consist of three domains. First there is the N-terminal domain. This domain is composed of six β-strands. (Morales-Ríos et al., 2015a) The so called 'crown' of the $F_1$ portion of the ATPase enzyme exists because the N-terminal domains of the alternating α and β subunits are connected by hydrogen bonds. This makes the structure of the 'crown' very stable. (Morales-Ríos et al., 2015a) The N-terminal regions of the α subunits protrude from the 'crown' of the $\alpha_3\beta_3$ hexamer. The α-helices of the $\alpha_E$ and $\alpha_{TP}$ subunits interact with the α-helices of the δ subunit. (Morales-Rios et al., 2015) Here are also connections to the *b* subunits. (Walker, 2013) The structures are shown in figure 4.

After the N-terminal domain there are the central nucleotide-binding domains. These domains are slightly different in the two subunits. In the α subunit there are ten β-strands and eight α-helices. In the β subunit there are seven β-strands and five α-helices. The nucleotide-binding domains contain a P-loop region. (Morales-Ríos et al., 2015a) This P-loop thanks its name to the fact that the loop binds to the phosphate molecule of the ATP or ADP molecules that are bound in the nucleotide-binding domains. (Bason, Montgomery, Leslie, & Walker, 2015) The P-loop of the β subunit contains the Walker motif. This is the following motif: GxxxxGKT/S. In the motif, the x can refer to any residue. (Walker, Saraste, & Gay, 1982) The nucleotide binding domain of the β subunit contains an arginine sidechain that is contributed by the α subunit next to it. This sidechain coordinates the phosphate molecule in the $\beta_{TP}$ conformation. If there is no ATP present, the sidechain will turn away from this position. (Abrahams, Leslie, Lutter, & Walker, 1994)

The last domain is the C-terminal domain. This domain consist in the α subunit of six and in the β subunit of seven α-helices that are folded into a bundle. (Morales-Ríos et al., 2015a) At the end of the C-terminal domain of the β subunit there is a helix-turn-helix motive, also called the hinge. The convex side of the γ shaft pushes against this hinge and thereby forces the β subunit into the $\beta_E$ conformation at the end of a rotational step. (Junge & Nelson, 2015) In the different conformations of the β subunit, the subunit can be in an 'open' or closed conformation. During the $\beta_E$ conformation,

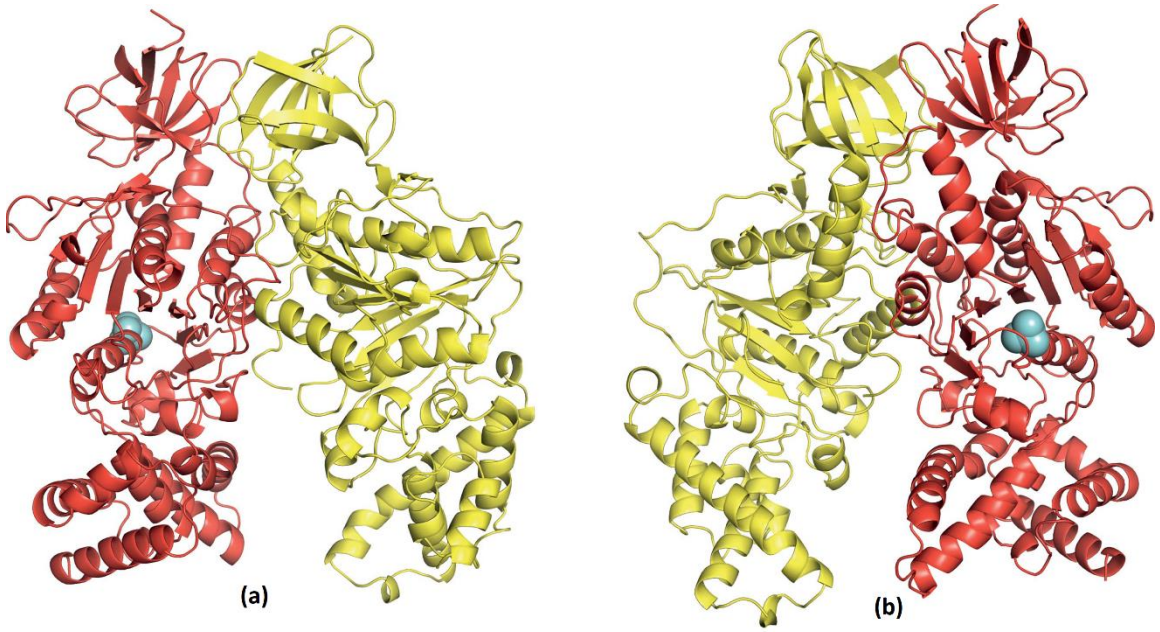


Figure 4: The structures of an α (red) and β (yellow) subunit of the F-ATPase of the Paracoccus Denitrificans with a bound phosphate. (a) Front view, looking inwards toward the rotary stalk. (b) view from the inside of the complex, looking towards the outside. (Morales-Ríos et al., 2015a)

the subunit is open. This means that the N-terminal region and the C-terminal region are further apart. In the closed conformation during the $\beta_{TP}$ and $\beta_{DP}$ states, the C-terminal region is swung closer to the N-terminal region. (Shirakihara et al., 2015)

**The structure of the γ subunit**

The rotation of the γ subunit in the $\alpha_3\beta_3$ hexamer causes changes in the way it interacts with the α and β subunits close to where the stalk emerges from the $\alpha_3\beta_3$ hexamer. However, it doesn't change the interactions between the γ subunit and the 'crown' region of the $\alpha_3\beta_3$ hexamer. (Bason et al., 2015) The γ subunit is folded into two α-helices in the N-terminal and the C-terminal regions. Between these two regions there is an intervening Rossmann fold. This means that there are five β-strands alternating with α-helices. The α-helices at the N-terminal and C-terminal ends of the subunit form an anti-parallel coiled-coil. This is the coiled-coil that lies in the $\alpha_3\beta_3$ hexamer (see figure 5). (Ferguson, Cook, Montgomery, Leslie, & Walker, 2016)

**The structure and conformations of the ε subunit**

The ε subunit can take on two different conformations. It can be in the 'up' or in the 'down' conformation. It is in the down conformation when there is an ATP molecule bound to the subunit. The ATP molecule and an accompanying magnesium ion are bound by the two α-helices of the C-terminal region of the ε subunit. (Ferguson et al., 2016) The N-terminal domain consists of nine β-strands that are arranged in a β-sandwich. The C-terminal domain lies in a hairpin alongside the N-terminal domain. (Yagi et al., 2007) The β-sandwich connects the ε subunit to the γ Subunit and to the *c* ring in the membrane (see figure 5). The conformations are created by the α-helices of the C-terminal domain. (Ferguson et al., 2016) The N-terminal domain of the subunit remains unchanged in the 'up' and the 'down' position. The changes between the conformations occur in the C-terminal domain and in the loop that connects the C-terminal domain to the N-terminal domain. When the subunit moves from the 'down' to the 'up' state, the C-terminal domain forms a ε-hook, where it folds itself in the direction of the N-terminal domain. In this conformation the ε subunit can interact with the Rossmann fold in the γ subunit. The ε subunit will move to the 'up' position when there is not enough ATP left in the surroundings. (Shirakihara et al., 2015)

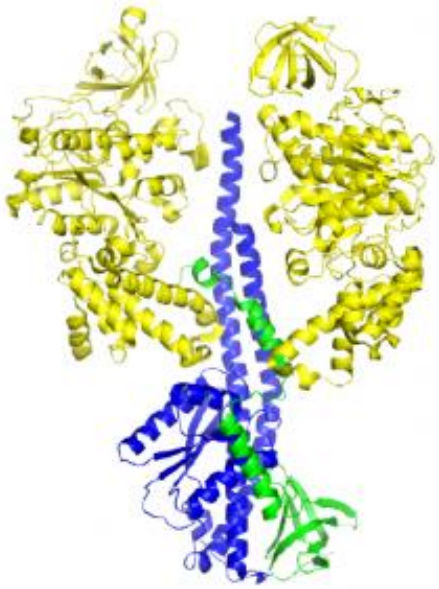

Figure 5: the structure of F-ATPase in E. Coli. The β, γ and ε subunits are shown as yellow, blue and green respectively. For clarity the three α subunits and one of the β subunits have been removed. (Walker, n.d.)(Walker et al., 2015)

**The structure and interactions of the δ subunit**

The δ subunit consist of a N-terminal domain and a C-terminal domain. Between these two domains, there is a flexible region that is called the elbow. Except for this part, the peripheral stalk is very rigid. (Walker, 2013) The δ subunit is connected to the C-terminal domain of the *b* subunit by its own C-terminal domain. (Vinothkumar et al., 2016) The N-terminal domain of the δ subunit interacts with the N-terminal domain of the α subunits in the $\alpha_3\beta_3$ hexamer. This is the main interaction between the peripheral stalk and the $F_1$ portion of the enzyme. The N-terminal region of the subunit is folded into a bundle of six α-helices. These α-helices interact with the α-helices that protrude from the N-terminal region of the α subunits. This interaction between the α and δ subunits is specific and also very stable. (Wilkens, Borchardt, Weber, & Senior, 2005) The δ subunit binds to one of the α subunits in the hexamer, not all three of them. (Walker, 2013)

# Chapter 2: Binding and Release of Substrates

**ATP binding**

An ATP molecule is bound by the β subunit after the ATP waiting dwell (figure 6). (Nam, Pu, & Karplus, 2014) This is the dwell that takes place at the 0° pause of the rotation of the γ subunit. (Masaike, Koyama-Horibe, Oiwa, Yoshida, & Nishizaka, 2008) The binding site of the catalytic domains has a high affinity for binding the MgATP substrate when the proton gradient is set for hydrolysis. In this case the release of the MgADP and $P_i$ is stimulated by the binding of the MgATP molecule to additional sites. (Penefsky & Cross, 1991) At one point in time, the three catalytic binding sites have different affinities for the MgATP substrate. There is one with the highest affinity, one with an intermediate affinity and one with a low affinity for binding the substrate. (Weber & Senior, 1997) The binding of ATP to a high affinity catalytic binding site generates enough energy to accommodate the binding energy. This indicates that the binding of ATP to the catalytic site is an energy coupling step. (Al-Shawi, Parsonage, & Senior, 1990)

**The release of ADP and $P_i$**

Nam et al. found that $P_i$ is released after a full rotation of the γ stalk substep (120°). This means that it is released when the β subunit has gone back to the $\beta_E$ conformation. In this conformation $P_i$ is very weakly bound to the β subunit and therefore it can be easily released. The release of $P_i$ results in a fluctuation in the P-loop region of the β subunit. The release of $P_i$ is a spontaneous reaction; however, it is hindered by the presence of ADP. Therefore the $P_i$ will probably be released after the ADP has left the subunit and the hindrance is gone. (Nam et al., 2014) The hindrance is created because the ADP molecule blocks the pathway to the P-loop. This prevents the $P_i$ from escaping the catalytic binding site. (Okazaki & Hummer, 2013) On the other hand it has been found that at the end of the hydrolysis process, the ADP molecule is the only molecule left in the $\beta_E$ subunit. The $Mg^{2+}$ and the $P_i$ were already gone in this structure. This suggest that the ADP molecule is the last to leave the catalytic site. (Bason et al., 2015) The release of ADP takes place after the final substep, when the conformation of the subunit is back to $\beta_E$ (figure 6). (Masaike et al., 2008)

**Binding of ADP and $P_i$**

There is a high energy barrier to the binding of $P_i$. This suggests that this is an energy coupling step in the synthesis of ATP, together with the release of the formed ATP molecule. (Al-Shawi et al., 1990) The binding of ADP only generates 30% of the binding energy that is needed. This indicates that the conformations of the binding site are different for the binding of ATP and ADP. The remaining 70% of the binding energy is contributed by the binding of $P_i$. The affinity of the catalytic site to bind $P_i$ is greatly enhanced by the presence of a proton gradient. (Al-Shawi et al., 1990) As the affinity of the catalytic site for the binding of $P_i$ rises, a phosphate binding pocket is formed. (Weber & Senior, 2000) As is mentioned before, the binding of ADP is unaffected by the proton gradient. (Weber & Senior, 1997)

It is important that there is a block on the binding of ATP during synthesis, otherwise the ATP molecule that was just synthesized would immediately rebind to the catalytic site and would be hydrolyzed, even though the extra proton gradient was not necessary. To prevent the rebinding of the ATP molecule, the $P_i$ creates a blockade. When the $P_i$ is energetically bound to the catalytic site, the ATP molecule can no longer rebind. (Weber & Senior, 1997)

The ATP molecule binds to the catalytic binding site accompanied by a $Mg^{2+}$ ion. Therefore, the ATP that is hydrolyzed does not only give the products ADP and $P_i$, but also the $Mg^{2+}$ ion. This ion is released together with the $P_i$. (Walker, 2013)

**ATP release**

The dissociation of ATP from the $\alpha_3\beta_3$ hexamer needs as much energy as would be released by ATP hydrolysis. (Souid & Penefsky, 1995) The energy that is needed for this dissociation of ATP from the catalytic site, is provided by the free energy that is generated by the proton-motive force. (Penefsky, 1985) The energy needed for the dissociation of ATP from the catalytic site can also be contributed by a NADH molecule that is made during the oxidative respiration. This NADH molecule has been shown to dissociate ATP in an energy dependent fashion. The NADH molecule could also be replaced by a succinate, as long as the substrate can be oxidized. By oxidizing these substrates, enough energy can be released to lower the binding affinity of the catalytic site for ATP. When the affinity is lower, the ATP molecule can be released. (Penefsky, 1985)

**Binding of $Mg^{2+}$**

The $Mg^{2+}$ that accompanies the ATP or ADP molecule as it binds to the catalytic site and undergoes the hydrolysis or synthesis, is necessary for the cooperative binding of the substrate and the catalytic binding site. It also makes sure that the binding site has a high affinity for the substrate. Without the $Mg^{2+}$ all the catalytic sites would have the same, low affinity for ADP and ATP. (Weber & Senior, 1997) In the catalytic binding site the $Mg^{2+}$ is coordinated by three water molecules and a residue of the β-subunits P-loop. When the β subunit moves to the $\beta_E$ conformation, the $Mg^{2+}$ loses its coordination with the water molecules. This is a state that is energetically unfavorable for the $Mg^{2+}$ and therefore it will be released from the β subunit. (Rees, Montgomery, Leslie, & Walker, 2012)

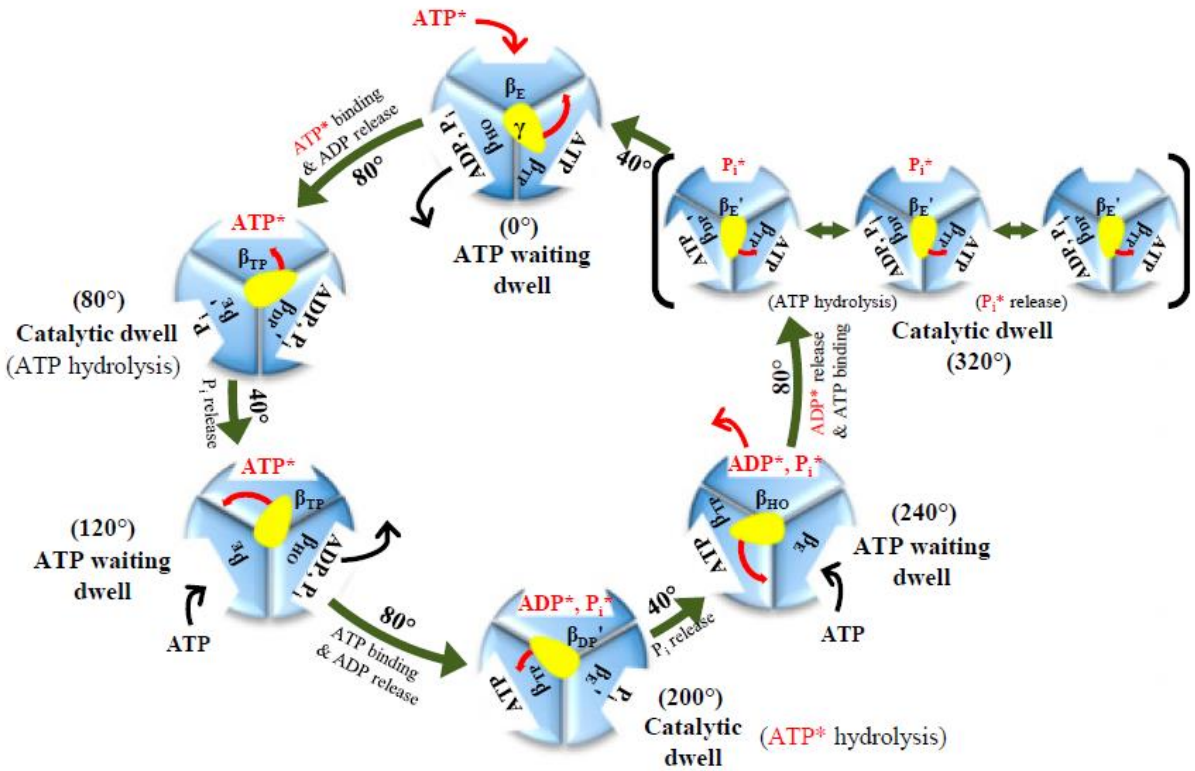


Figure 6: A schematic interpretation of the stepped rotation of the γ subunit and the binding and release of ATP, ADP and $P_i$ as interpreted by Nam et al. The ATP is bound at the ATP waiting dwell, then there is a rotation of 80° to the catalytic dwell. Here the ATP hydrolysis takes place. Then the 40° rotation takes the enzyme back to the ATP waiting dwell. During the rotational step, the $P_i$ is released and in the dwell the ADP is released. (Nam et al., 2014)

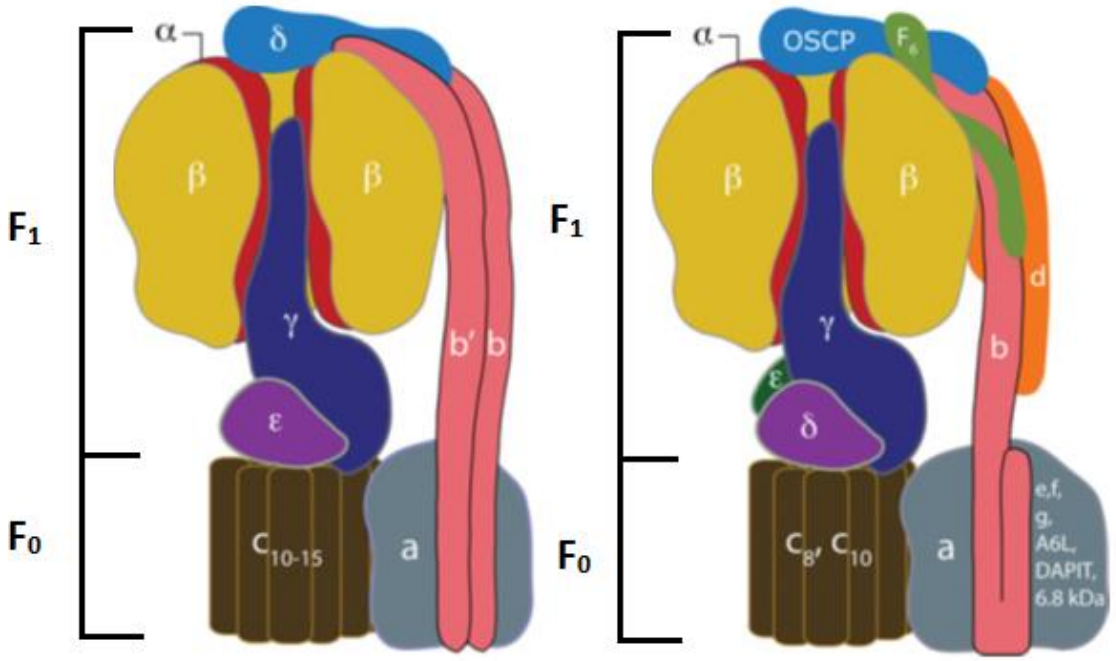


Figure 7: The composition of the F-ATPase enzyme in *E. Coli* (left) and bovine mitochondria (right). The $F_1$ part of the enzyme is the part that resides in the cytosol, the $F_0$ part mostly resides in the membrane. (Walker, 2013) (altered)

**The subunits of the $F_1$ portion of the F-ATPase**

The γ and ε subunits together form the central stalk of the F-ATPase molecule. Together with the *b* subunits, the γ subunit connects the $F_1$ to the $F_0$ portion of the enzyme. The γ subunit consists of a long helical coiled coil. This coil extends into the middle of a $\alpha_3\beta_3$ hexamer (figure 7). (Berg et al., 2012) This hexamer contains three α subunits and three β subunits that alternate. The α and β subunits are very similar to each other. They share about one fifth of the same amino acid residues. (Boyer, 1997) The α subunit can bind ATP or ADP in a non-catalytic binding site. The molecules that are bound in these sites will be replaced during catalysis that takes place at the catalyzing binding sites. These are located in the β subunits. In these catalytic binding sites the hydrolysis and synthesis of ATP can take place. (Boyer, 1997) In the F-ATPase enzyme there are parts of the enzyme that can be referred to as the rotor and parts that can be referred to as the stator. The α and β subunits are part of the stator. This means that these subunits are stationary and they don't rotate. The central stalk does rotate and therefore is part of the rotor. (Vinothkumar et al., 2016)

The δ subunit is located on top of the crown of the $F_1$ domain (see figure 7). The subunit interacts with the *b* subunits from $F_0$ and the α subunit in $F_1$.(Morales-Ríos, Montgomery, Leslie, García-Trejo, & Walker, 2015a) Together with the two *b* subunits, δ forms the peripheral stalk. This peripheral stalk is part of the stator. It makes sure that the $F_1$ and $F_0$ portion of the enzyme stay in the correct spatial arrangement so the syntheses or hydrolyses of ATP can occur. (Wilkens et al., 2005) The peripheral stalk connects the $\alpha_3\beta_3$ hexamer to the subunit *a* in the $F_0$ portion of the enzyme. Subunit *a*, the peripheral stalk and the $\alpha_3\beta_3$ hexamer together form the complete stator of the F-ATPase. (Walker, 2013)

The γ subunit is anchored to the $F_0$ portion of the F-ATPase by the ε subunit. Together with the end of the γ subunit, the ε subunit forms the 'foot' of the central stalk (figure 7). (Walker, 2013) The ε subunit also seems to play a role in choice of the enzyme to hydrolyze or synthesize ATP. The subunit

can take two different conformations. The interaction of this conformation with the $\alpha_3\beta_3$ hexamer can induce or inhibit the hydrolyses of ATP. (Ferguson et al., 2016)

**Synthesis**

As is explained earlier, the proton motive force makes the protons translocate from outside the membrane to the cytoplasm of the cell. This translocation is done by the *a* subunit and the *c* ring of the $F_0$. The translocation of protons results in the rotation of the ring of *c* subunits. (Berg et al., 2012) The *c* ring and the γ subunit are closely connected. About two thirds of the *c* ring subunits are in contact with the central stalk. Because of the tight connection, when the *c* ring starts rotating, the stalk will follow. (Stock, Leslie, & Walker, 1999) During the synthesis of ATP, the rotor will be turning in the clockwise direction as viewed from the $F_0$ portion of the enzyme. (Vinothkumar et al., 2016) As the central stalk is rotating, it interacts with the $\alpha_3\beta_3$ hexamer. (Berg et al., 2012) The α and β subunits in the hexamer can bind ADP and $P_i$ or an ATP molecule. In case of synthesis the subunits bind ADP and $P_i$. (Junge & Nelson, 2015) If ADP and $p_i$ are not bound to a free binding site of the β subunit, then proton translocation can't take place. (Boyer, 1997)

When the central stalk is rotating, the α and β subunits will take in different conformations. This happens because the γ subunit is asymmetrical. (Czub & Grubmüller, 2014) These conformations form a cycle: substrate binding, ATP formation and the release of the product (ATP). (Vinothkumar et al., 2016) There are three different conformations that the α and β subunits can take. These conformations are called Empty (E), diphosphate containing (DP) and triphosphate containing (TP). (Morales-Ríos et al., 2015b) The α subunit is not actively changing its conformation, but is forced to change because of the different conformations of the β subunit. (Oster & Wang, 1999) When the γ subunit rotates in the clockwise direction, it pulls the β subunit inward and the tip of the subunit is bended slightly to the right. This increases the affinity of the *B* subunit to bind ADP and $P_i$. (kinosita Jr., Adachi, & Itoh, 2004) For the binding of $P_i$, the proton gradient is essential. It is the proton gradient that creates the $P_i$ binding site in the α and β subunits. ADP does not need the proton gradient or the rotation of the γ subunit to bind to the α and β subunits. (Senior, Nadanaciva, & Weber, 2001) The binding of ADP to the α and β subunits happens spontaneous. In a buffer that contained Mg-ADP it was found that ADP would bind to the β subunit even without the rotational torque of the γ subunit. (Ferguson et al., 2016) This means that the conformational change in the subunit β from the $\beta_E$ conformation to the $\beta_{DP}$ conformation happens without an extra input of energy. This correlates with the fact that without the ADP and $P_i$ bound to the catalytic site, the pmf cannot be used by the ATPase. (Boyer, 1997) The ADP can however only bind to a subunit that already has $P_i$ tightly bound. (Okazaki & Hummer, 2013) When the γ subunit rotates further, it bends the tip of the β subunit to the left. This induces the formation of the ATP molecule. This ATP molecule is still tightly bound to the subunit. The γ subunit rotates further and brings the β subunit in its open conformation. Now the ATP molecule is able to leave the β subunit. (kinosita Jr. et al., 2004) The release of the formed ATP molecule is a process that needs energy. This energy comes from the rotating γ subunit. (Senior et al., 2001)

**Hydrolysis**

Every full rotation of the γ subunit, this means a rotation of 360°, takes the β subunits through all three the conformations. This means that in every full rotation all the β subunits will have been in the empty, ADP containing and the ATP containing conformations. In this one rotation three ATP molecules will be hydrolyzed (see figure 8). The rotation of the γ subunit is in the counterclockwise direction as viewed from the $F_0$ domain. (Walker, 2013) The rotation of the γ subunit happens in steps of 120°. Each of these steps drives one catalytic cycle. Each step of 120° is powered by the binding of an ATP molecule. (Masaike et al., 2008) The $\beta_E$ subunit can spontaneously go from the open conformation to a closed conformation. This is independent of the rotation of the γ subunit and also independent of nucleotide binding. (Czub & Grubmüller, 2014) The rotational step of 120° is not continuous, but it has several substeps and waiting points. (Mukherjee et al., 2017) These substeps

are 80° and 40° rotations (Masaike et al., 2008) or 90° and 30° rotations (Yasuda, Noji, Yoshida, Kinosita Jr., & Itoh, 2001). In this thesis, we will assume the 80° and 40° substeps.

Between these substeps there are two pauses. The ATP-binding pause and the catalytic pause respectively. (Czub & Grubmüller, 2014) At the ATP-binding pause or ATP-binding dwell at the 0° rotation, the ATP molecule is bound to the β subunit (figure 8). This is when the subunit changes its conformation form $\beta_E$ to the closed form $\beta_{TP}$. The stalk than rotates further to the catalytic dwell. To reach this dwell, the stalk will perform the 80° step. (Nam et al., 2014) During the catalytic dwell, three reactions are said to take place. The reaction that certainly takes place is the cleavage of bound ATP into bound ATP and $P_i$. Then there are studies that argue that there is a second reaction, the release of ADP. They also say there is a third reaction that takes place which is the release of $P_i$. This step is said to be the rate determining step. This is because it seems that the 40° substep cannot take place before both the ADP and $P_i$ are released. (Junge, Sielaff, & Engelbrecht, 2009) It is not certain that ADP is released first, in some studies it was found that it is in fact $P_i$ that is released first. This is most likely according to the kinetic and structural studies. (Rees et al., 2012) However, other studies found that the ADP molecule prevents the $P_i$ from leaving the catalytic site. They argue that ADP has to leave the site first before the $P_i$ can be released. (Okazaki & Hummer, 2013) Other researches argue that the ADP and $P_i$ are not released during the catalytic dwell, but rather at the end of the rotation, when the β subunit goes back to its open $\beta_E$ conformation. (Masaike et al., 2008) To perform the 40° rotational step, transformation of all the α and β subunits are necessary. After this step follows to the waiting dwell at the 120° rotation. (Nam et al., 2014) Here the subunits wait to bind a new ATP molecule. (Mukherjee et al., 2017)

The 80° rotation is driven by the binding of an ATP molecule and possibly the release of ADP. The 40° substep is driven by the release of $P_i$. (Junge & Nelson, 2015) The conformational changes in the $\alpha_3\beta_3$ hexamer during the hydrolyses of ATP provide the free energy that is needed to rotate the γ subunit. (Mukherjee et al., 2017) The rotation of the γ subunit is partially driven by the release of $P_i$. This is because in the hydrolysis direction the $\alpha_3\beta_3$ are less tight than in the synthesis direction and the $P_i$ can be released more easily than in the synthesis direction were it is bound very tight. (Okazaki & Hummer, 2013) The release of ADP might also be a trigger for the rotation of the shaft. On the other hand, the presence of $P_i$ in the $\beta_E$ subunit blocks the rotation of the γ subunit. (Nam et al., 2014)
Because the conformational changes are driven by the binding of ATP and the release of ADP and $P_i$, the rotation of the γ subunit is driven in the counterclockwise direction. (Mukherjee et al., 2017) When the rotation is applied to the *c* ring and the *a* subunit, this complex will start pumping protons from the cytoplasm to the outside of the membrane. (Junge & Nelson, 2015) This means that the protons are pumped in against the electrochemical potential, a process that would not happen spontaneously. (Okuno et al., 2011)

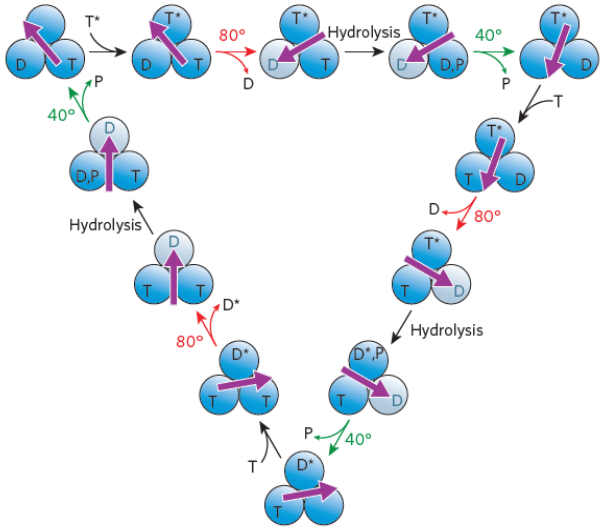


Figure 8: A schematic interpretation of the process of hydrolysis according to Junge et al. After the ATP waiting dwell, ATP is bound. During the 80° substep ADP is released. In the catalytic dwell the hydrolysis takes place. After that the $P_i$ is released during the 40° rotational step that takes the enzyme back to the ATP waiting dwell. (Junge et al., 2009)

## Discussion

After years of research on the F-ATPase there is much known about the enzyme. There are however a few subjects on which there is still a lot of discussion. For example, there is a lot of discussion and contradictory information about ADP an $P_i$ release. Many researches show that at least the $P_i$ is released during the catalytic dwell. This is supported by researches that said that the release of $P_i$ drives the 40° substep of the γ subunit. On the other hand, there have been researches that indicate that the $P_i$ is blocked by the presence of the ADP molecule in the catalytic binding site. As long as the ADP molecule is present, the $P_i$ can't escape the β subunit. (Okazaki & Hummer, 2013) Therefore it should not be possible that the $P_i$ leaves the subunit during the catalytic dwell if ADP is released after a full rotational step of 120° has been completed. There are studies that say that the ADP molecule is also released in the catalytic dwell, but I find this hard to believe, since the subunit is in the closed conformation at that point and therefore it would take a lot of energy to release the ADP molecule. Lastly there are studies that say that the ADP and $P_i$ are both released after a full rotation of 120° has been completed. (Nam et al., 2014) Even in recent studies they have no certainty on the order in which the release of the ADP an $P_i$ happens. They still propose two mechanisms; the $P_i$ is released before ADP or after it. (Sugawa et al., 2016) In short, this is a subject that requires more specific research. Hopefully that will finally give a conclusive answer to the question how the release of ADP and $P_i$ works.

Another question that remains is the exact working mechanism of the synthesis of ATP by the F-ATPase. Most of the researches have been done about hydrolysis, this is because this process is easier to study. The process of synthesis would require very specific circumstances that are hard to create in the lab. A development was the finding of the Paracoccus Denitrificans. These eubacteria have a F-ATPase that can only perform the synthesis reaction and not hydrolysis. This organism is therefore a very good subject for studies that look at the synthesis. (Morales-Rios, Montgomery, Leslie, García-Trejo, et al., 2015; Morales-Rios, Montgomery, Leslie, & Walker, 2015) For now there is a lot of discussion whether the synthesis is simply the hydrolysis in reverse or it follows another reaction path.

Much is known about the structure of the enzyme. Also, much is known about the interactions between the subunits. We have seen that the subunits in the $F_1$ portion of the F-ATPase enzyme are the α, β, γ, ε and δ subunits. These play roles in the hydrolysis or synthesis of ATP (α, β, γ and ε) or in the stabilizing and connecting of the enzyme (α and δ). The γ and ε subunits together form the central stalk and are in close contact with each other. The ε can inhibit the γ from performing synthesis.

As said before there is still a lot of uncertainty surrounding the synthesis of ATP by the F-ATPase. The hydrolysis on the other hand is quite clear. This happens in steps of 120° rotation of the γ subunit. Every full rotation of the γ subunit (360°) three ATP molecules can be hydrolyzed. The precise way of binding and release of ADP, ATP and $P_i$ are not yet certain. This will need more research to clear up.

The enzyme can both hydrolyze and synthesize ATP. Which of these two mechanisms is at work is decided by the proton gradient over the cell membrane. When the proton gradient is low, the enzyme will hydrolyze ATP. When the proton gradient is high and the amount of ATP molecules low, the enzyme will synthesize ATP. This is regulated by the ε subunit.

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