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Crucial aminoacids in the F_0 sector of the F_1F_0 -ATP synthase address H^+ across the inner mitochondrial membrane: molecular implications in mitochondrial dysfunctions

Fabiana Trombetti, Alessandra Pagliarani, Vittoria Ventrella, Cristina Algieri, Salvatore Nesci

Department of Veterinary Medical Sciences – University of Bologna

Via Tolara di Sopra 50 – 40064 Ozzano Emilia (BO) Italy

Corresponding author: alessandra.pagliarani@unibo.it

Abstract

The eukaryotic F_1F_0 -ATP synthase/hydrolase activity is coupled to H^+ translocation through the inner mitochondrial membrane. According to a recent model, two asymmetric H^+ half-channels in the a subunit translate a transmembrane vertical H^+ flux into the rotor rotation required for ATP synthesis/hydrolysis. Along the H^+ pathway, conserved aminoacid residues, mainly glutamate, address H^+ both in the downhill and uphill transmembrane movements to synthesize or hydrolyze ATP, respectively. Point mutations responsible for these aminoacid changes affect H^+ transfer through the membrane and, as a cascade, result in mitochondrial dysfunctions and related pathologies. The involvement of specific aminoacid residues in driving H^+ along their transmembrane pathway within a subunit, sustained by literature and calculated data, leads to depict a model consistent with some mitochondrial disorders.

Keywords: crucial aminoacids; a subunit; F_1F_0 -ATP synthase; H^+ pathway; mitochondrial dysfunctions

Abbreviations: Δp , protonmotive force; IMM, inner mitochondrial membrane; MILS, Maternally Inherited Leigh Syndrome; MLASA, Myopathy, Lactic Acidosis and Sideroblastic Anemia; NARP, Neuropathy, Ataxia and Retinitis Pigmentosa; PTP, permeability transition pore.

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Introduction

Severe human diseases are linked to mitochondrial dysfunctions and particularly to mismatches of the enzyme machinery of the mitochondrial F₁F₀-ATP synthase/ATPase (EC 3.6.3.14). This multi-subunit enzyme, localized in the inner mitochondrial membrane (IMM), possesses an unique energy transmission mechanism which synthesizes or hydrolyzes ATP according to the mitochondrial bioenergetics requirements (Junge and Nelson 2015). The enzyme complex consists of two main domains: the hydrophilic F₁ domain, which protrudes in the mitochondrial matrix and can synthesize or hydrolyze ATP (Adachi et al. 2007), and the membrane-embedded F₀ domain, which translocates H⁺ (Nesci et al. 2016). ATP synthesis is driven by the electrochemical energy produced by the respiratory chain in the form of Mitchell's proton motive force (Δp). *Vice versa* ATP hydrolysis is due to the reverse working mode of the same enzyme complex, which exploits ATP breakdown to pump H⁺ from the mitochondrial matrix to the intramembrane space, thus energizing the inner mitochondrial membrane (IMM) by re-building the transmembrane potential (Junge and Nelson 2015). The bi-functional activity of ATP synthesis/hydrolysis coupled to H⁺ translocation is a mechanism unique in biology. The F₀ motor (Junge et al. 1997), guarantees the H⁺ translocation across the IMM in both directions, an universal feature shared by all the F₁F₀ complexes (Kühlbrandt and Davies 2016).

Catalysis (ATP synthesis or hydrolysis) and proton direction across the IMM are mutually linked by an amazing torsional mechanism. While H⁺ translocation driven by Δp generates the counterclockwise (viewed from the matrix) rotor rotation and produces ATP, ATP hydrolysis provides the energy required for the uphill H⁺ flux of the reverse working mode coupled to the clockwise (viewed from the matrix) rotation (Nesci et al. 2015) (Fig. 1). The core of the F₀ rotor is the *c*-ring, a sort of cylinder embedded in the IMM and built by the circular arrangement of *c*-subunits, whose number (*n*) is species-dependent. During the *c*-ring rotation in either direction, the H⁺ binding sites, identified in specific acid aminoacids in each *c*-subunit (the *c*Glu-59 of *c_n*-ring), are reversibly protonated and deprotonated. This chemical mechanism, repeated many times, allows H⁺ translocation across the F₀ domain. Protons pass through two asymmetric half-channels of *a* subunit, also part of F₀, each of which opens on one side of the IMM (Klusich et al. 2017). The H⁺ movement across the IMM exploits charged and polar residues of the *a* subunit (Allegretti et al. 2015), which connects the *c*-ring to the matrix-side half-channel. On the other side, the luminal half-channel, which opens in the intermembrane space, consists of aminoacid residues in the *f*, *b* and H5/H6 hairpins of *a* subunit (Srivastava et al. 2018) (Fig. 2).

To make the machinery work, proton flux must be channeled without hesitation in one direction. A conserved Arg in *a* subunit (*a*Arg-159 in mammals or *a*Arg-176 in yeasts), bears a positive charge and acts as electrostatic barrier, being localized between the two half-channels (Mitome et al. 2010). This positive Arg prevents any H⁺ leakage by direct ion translocation from one half-channel to another during the torque generation and addresses the H⁺ flow toward either direction (counterclockwise or clockwise), according to the rotor rotation (Klusich et al. 2017; Hahn et al. 2018). Interestingly, the *a* subunit is encoded by a mitochondrial gene (ATP6). mtDNA mutations are much more frequent than nuclear DNA ones (Dautant et al. 2018). Indeed, mitochondria produce free radicals, which likely enhance mutations, and lack DNA ligase and other repair enzymes (Schon 2015). Most disease-associated mutations in the ATP synthase are in the ATP6 gene (Xu et al. 2015). Any mutation in the *a* subunit may disrupt the H⁺ pathway, thus playing the role of molecular defect responsible for mitochondrial disorders (Dautant et al. 2018).

1 Accordingly, the involvement of specific aminoacid residues which, by subsequent H⁺
2 uptake/release steps, channel H⁺ along their transmembrane pathway within *a* subunit, could
3 represent the molecular link between the enzyme complex and mitochondrial disorders (Dautant et
4 al. 2018). In turn, different alterations in the H⁺ pathway result in pathological symptoms that
5 differently affect organs and tissues, giving rise to mitochondrial diseases which exhibit peculiar
6 features. The present work, which gathers the available data on H⁺ transmembrane pathway and
7 defines the distance between specific aminoacid residues of F_O, the intramembrane portion of the
8 F₁F_O-ATPase, may contribute to cast light on the link between aminoacid substitutions due to point
9 mutations in the ATP6 gene and pathological symptoms. An improved knowledge of the chemical
10 mechanisms underpinning H⁺ translocation, may be of outstanding interest in the perspective of
11 counteracting mitochondrial diseases.
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17 **Methodology**

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19 The most recent literature data were combined to the results from distance calculations and position
20 of crucial aminoacid residues, obtained by Chem3D program of ChemOffice 2017 software from the
21 deposited structures in PDB, in order to clarify a model in which the H⁺ flux through the IMM is
22 driven by crucial aminoacids, whose substitution affects the whole F₁F_O-ATPase function.
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27 **The H⁺ route**

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29 Emerging evidence shows that H⁺ pathway in either direction across the IMM is ensured by the
30 precise localization of selected aminoacids, which act as stone posts and define the fate of the
31 incoming protons (Klusck et al. 2017). In other words, the aminoacid residues are localized along the
32 H⁺ route according to a molecular strategy which, step-by-step, uptakes and releases H⁺. By this
33 chemical mechanism, based on electrostatic attractions and repulsions, the *a* subunit builds the H⁺
34 pathway. Two asymmetric and discontinuous half-channels establish the direction of H⁺ flux and of
35 the rotor rotation during ATP synthesis/hydrolysis (Nesci et al. 2015). The depicted model, based on
36 the calculated distances between selected aminoacids involved in H⁺ channeling and literature data,
37 shows how the substitution of only one aminoacid alters or even prevents the correct H⁺ movement
38 across the IMM by modifying the electrostatic interactions which constitute the chemical bases of H⁺
39 translocation across the IMM. Structural and chemical aspects should be considered.
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47 *Spatial arrangement*

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49 Some structural details of the protein arrangement in the intramembrane portion of the enzyme
50 complex are relevant in building the H⁺ pathway. Even if half-channels arranged across the membrane
51 (orthogonal to the IMM) were expected on the basis of H⁺ movement, quite surprisingly, no helices
52 across the membrane occur in the two aqueous half-channels which open on the two opposite IMM
53 sides. These helices are arranged along the membrane and their axis is oblique with respect to the
54 membrane (Hahn et al. 2016, 2018; Guo et al. 2017; Srivastava et al. 2018). Most likely, the H⁺
55 translocation mechanism works properly providing the adequate transmembrane direction, due to
56 amazing microstructural strategies. The *c*-ring is embedded in the IMM and concave, seen laterally
57 from the membrane. Glu-59 is inserted in this concavity, on the outer C-terminal α -helices of *c*
58 subunits (Symersky et al. 2012). The *c*-ring hourglass shape facilitates the contact between *c* and *a*
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1 subunits, namely proton transfer between *c*Glu-59, which acts as H⁺ binding site and other aminoacid
2 residues in *a* subunits. The locally hydrated environment of *a*-*c* interface (Pogoryelov et al., 2010)
3 allows H⁺ movement. Noteworthy, the localization of crucial aminoacids such as *a*Glu-145 on the
4 matrix side and *a*Glu-203 on the lumen side, which translocate H⁺ in the long helices of *a* subunit
5 arranged along the IMM, allows a closer interaction with the *c*-ring H⁺ binding sites (Fig. 2) with
6 respect to a transmembrane arrangement of *a* subunit helices as hypothesized on the basis of the
7 overall movement of protons. Additionally, the *c*-ring size varies in different organisms, but,
8 curiously, it appears to be constant in a given organism (von Ballmoos et al. 2008; Nesci et al. 2013).
9 Therefore, in adjacent *c* subunits the H⁺ binding sites, namely *c*Glu side chains, are differently spaced
10 according to the *c*-ring diameter. The calculated distances between *a*Glu-145 or *a*Glu-203 residues
11 and *a*Arg-159, pointed out as green straight lines in Fig. 3, cast light on the presumptive spatial
12 arrangement which allows the accommodation of differently sized *c*-rings (Watt et al. 2010) and is
13 are consistent with the architecture of *a* subunits. Accordingly, it was reported that the protonated
14 *c*Glu-59 carboxyl groups of adjacent *c*-subunits can attain a distance around 14Å for the smaller *c*₈-
15 rings (8 *c* subunits) and 11.1Å for the larger *c*₁₅-ring (15 *c* subunits) (Kühlbrandt and Davies 2016).
16 In all the ATP synthase *a* subunits the crucial aminoacid residues for H⁺ translocation are conserved
17 and spaced at 2.5 helix turns from each other. This spacing well correlates with the distance between
18 the H⁺ binding sites of adjacent *c* subunits in differently sized *c*₈₋₁₅-rings (Kühlbrandt and Davies
19 2016). So, the arrangement of *a* subunit helices along the IMM shown in Fig. 3, which illustrates the
20 *a* subunit viewed from two opposite sides and points out the reciprocal position of the aminoacids
21 involved in H⁺ transport, is fully consistent with the available structural and calculated data and well
22 compatible with differently-sized *c*-rings.
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31 *Chemical strategies*

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33 A relevant point to be clarified is the chemistry of the microenvironments involved in H⁺
34 translocation. The H⁺ translocation through the IMM (from the luminal to the matrix half-channel
35 during the ATP synthesis and *vice versa* during ATP hydrolysis) implies the reversible *c*Glu-59
36 protonation/deprotonation (Pogoryelov et al. 2010). However, since the IMM separates two
37 environments featured by different pH values (pH ≈7.0 on the positive side and pH ≈8.0 on the
38 negative side), the H⁺ carrier role of the *c*Glu-59 carboxyl is questioned by its known standard p*K*_a
39 of 5.0 in water. Accordingly, at the physiological pH, the *c*Glu-59 facing the luminal half-channel
40 (positive side) would always be deprotonated, thus preventing the rotation that drives ATP synthesis.
41 Moreover, the *c*Glu-59 facing the matrix half-channel (negative side) only could release H⁺ to the
42 matrix, thus preventing the opposite H⁺ flux (towards the intermembrane space) linked to ATP
43 hydrolysis. Consistently, the H⁺ uptake/release mechanism within the half-channels would require a
44 p*K*_a of *c*Glu-59 around 7.0 at both IMM sides to ensure ATP synthesis/hydrolysis coupled to H⁺
45 translocation (Srivastava et al. 2018). However, since the original Mitchell's work (Mitchell 1961)
46 the real pH at both IMM sides and in microenvironments within the IMM was an intriguing matter of
47 debate. Moreover, the IMM is a dynamic structure and, during the *c*-ring rotation, adjacent aminoacid
48 side chains, which at least in some cases have buffering capabilities, even if limited (Santo-Domingo
49 and Demaurex 2012), by approaching or getting away may continuously change the
50 microenvironmental features. We must assume that, in selected microenvironments, charged or polar
51 residues close to the *c*Glu-59 can locally increase its p*K*_a, thus favoring H⁺ dissociation. The H⁺
52 translocation pathway proceeds between the H5 and H6 helices of the *a* subunits, which accommodate
53 the two half-channels. Consistently, on the H5 of the matrix half-channel and on the H6 of the luminal
54 half-channel "H⁺ transfer groups" (Guo et al. 2017; Srivastava et al. 2018) were identified as *a*Glu-
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145 and *a*Glu-203, respectively. In the rotation mechanism which drives ATP synthesis, the protonated *c*Glu-59 moves through the IMM on the outside of the *c*-ring until it encounters the half-channel on the matrix negative side where, in association with the *a*Glu-145, forms the H⁺-release site. At the opposite IMM side, the *c*Glu-59 by interacting with the *a*Glu-203, becomes an efficient “H⁺ transfer group” at pH around 7.0. Moreover, protons can enter the luminal half-channel via an “intermediate H⁺ binding site”, namely the *a*His-168 on the H5, and so reach the *a*Glu-203 (Srivastava et al. 2018) (Fig. 3). As a result, the protons coming from the aqueous lumen neutralize the *c*Glu-59 and allow the *c*-ring to rotate by pushing the protonated carboxylate in the closed conformation in the hydrophobic IMM environment according to the model depicted by Pogoryelov et al. (2009, 2010). Finally, when the *c*-ring rotation driven by Δp is almost complete, the protonated *c*Glu-59 encounters the proton exit channel; at this stage the hydrophilic environment and the low H⁺ concentration cooperate to change the protonated *c*Glu-59 into its open conformation and this deprotonation, which leads to the (-COO⁻) form, starts again the cycle (Pogoryelov et al. 2009; Guo et al. 2017).

What happens if the mechanism does not work properly: molecular evidence and pathologies

From the information provided in the previous section, it is clear that the correct H⁺ movement within the IMM is ensured by a series of specific aminoacids, which reversibly anchor and channel H⁺ along their transmembrane route. Most frequently, an impaired ATP production by mitochondrial F₁F₀-ATP synthase stems from pathogenic mutations in mtDNA, which has a higher mutational rate than nuclear genome (Jonckheere et al. 2012). Several point mutations in the mitochondrial ATP6 gene that encodes the *a* subunit of the F₀ domain are known (Dautant et al. 2018). The number of the discovered mutations associated with mitochondrial diseases is continuously increasing (Guo et al. 2018). Table 1 shows mutations in the ATP6 gene associated with human diseases and Figure 4 illustrates the position of the substituted aminoacids in the helices of *a* subunit. Among these mutations, the most severe is the T8993G transversion, in which the nucleotide substitution results in a missense mutation (*a*Leu156Arg) (Trounce et al. 1994). The clinical pathological phenotype results in severe syndromes known as NARP (Neuropathy, Ataxia and Retinitis Pigmentosa) or MILS (Maternally Inherited Leigh Syndrome), according to the degree of heteroplasmy (Uziel et al. 1997). The missense mutation is localized near the crucial electrostatic barrier of *a*Arg-159 (Fig 4A). The presence of two positive guanidine groups at short distance from each other reduces the H⁺ transmembrane flux (Xu et al. 2015) and, consequently, also ATP synthesis. The T8993G transversion mainly results in an energy deficiency since the F₁F₀-ATPase activity becomes unable to pump H⁺ to re-energize membranes. However, the F₁ catalytic domain and the F₀ translocator domain remain structurally joined, as witnessed by the maintenance of oligomycin sensitivity (Sgarbi et al. 2006). Another mutation in the mitochondrial ATP6 gene associated to NARP and MILS diseases consists in the T9176G transversion. The missense mutation on position 220 of *a* subunit changes a conserved leucine into arginine (*a*Leu220Arg) (Dautant et al. 2018) (Fig. 4B). Since the *a*Leu-220 is near the essential *a*Arg-159, the *a*Leu220Arg change implies steric hindrance and electrostatic repulsions which affect the *a* subunit assembly and stability. The two Arg would act as a positively charged gate, which directly blocks H⁺ flux (Xu et al. 2015). As a result, ATP synthesis decreases, accompanied by a decline in complex IV respiration. In addition, being ATP hydrolysis not coupled to proton translocation, as shown by oligomycin insensitivity, the H⁺ pumping activity of the ATPase cannot build the membrane potential (Kucharczyk et al. 2019). A milder pathological phenotype is caused by T>C transition in the same mtDNA position with resulting

1 Leu156Pro substitution (Kucharczyk et al. 2009). The T8993C transition favors an increased ROS
2 production, even if the *c*-ring slow rotation which drives the H⁺ flux coupled to a poor ATP synthesis
3 is maintained (Baracca et al. 2007; Solaini et al. 2008). It was suggested that proline insertion in
4 replacement of leucine alters the protein secondary structure and indirectly affects proton movement
5 (Xu et al. 2015). Accordingly, due to its 5-membered ring, proline is known to cause a kink in the
6 helices (Schmidt et al. 2016).
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8 A rare mitochondrial disorder characterized by MLASA (Myopathy, Lactic Acidosis and
9 Sideroblastic Anemia) has been recently associated with a novel *de novo* mutation (G8969A) in
10 mtDNA which encodes the ATP6 gene (Burrage et al. 2014). The missense mutation Ser148Asn in *a*
11 subunit of MLASA patients (Skoczeń et al. 2018) is localized at one helix turn from the “H⁺ transfer
12 group” *a*Glu-145 in the aqueous exit channel facing towards the mitochondrial matrix (Srivastava et
13 al. 2018) (Fig. 4C). As a result, the H⁺ translocation linked to -COOH deprotonation of *c*Glu-59 is
14 impaired, because the negative charge of *a*Glu-145 is neutralized by the establishment of bonds with
15 the positive charge of *a*Asn-145 side chain (Skoczeń et al. 2018). In general and as far as we are
16 aware, the mutations in *a* subunit block the torque generation within F₀ required for ATP synthesis
17 by F₁.
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22 Interestingly, for the first time, mutations in *a* subunit were shown to accumulate in some
23 cancer types, although in yeast models. The two mutations *a*Pro153Ser and *a*Lys80Glu (*a*Pro136Ser
24 and *a*Lys64Glu in humans) were found to be associated with carcinogenesis, ascribed to prevention
25 from apoptosis of cancer cells. Indeed, the programmed cell death was suppressed by the inhibition
26 of the permeability transition pore (PTP) in the IMM (Niedzwiecka et al. 2018). Increasing evidence
27 points out the ATP synthase as the structural component of the PTP (Nesci et al. 2018). Proline
28 substitutions by point mutations are known to decrease the helix packing (Schmidt et al. 2016). On
29 the other hand the replacement of Lys by Glu dramatically changes the polarity and acid-base
30 properties of the microenvironment. Thus, it seems likely that cell phenotypes in *a*Pro153Ser and
31 *a*Lys80Glu mutants exhibit a distorted *a* subunit (Niedzwiecka et al. 2018) which would prevent the
32 dimerization motif involved in the ATP synthase assembly/disassembly from forming the lethal
33 channel between the two F₀ monomers (Nesci 2018).
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41 Conclusion

42 Calculations and literature data are consistent in describing how H⁺ pass across the IMM by a stepwise
43 protonation/deprotonation mechanism which involves specific aminoacids in the *a* subunit of the
44 F₁F₀-ATP synthase. Functionally relevant aminoacid residues spaced from the positive charge of the
45 electrostatic barrier (*a*Arg-159), which acts as watershed, translocate H⁺ across the IMM and, at the
46 same time this translocation makes the *c*-ring rotate, thus generating the torsional mechanism which
47 allows ATP synthesis. Quite surprisingly, the passage of H⁺ across the IMM is ensured by the
48 arrangement of α -helices in the *a* subunit along the IMM. Even if apparently tortuous, this model is
49 quite realistic, also on the basis of the distance between the aminoacid residues involved, and
50 shouldered by the documented evidence that mtDNA missense mutations, which affect the outlined
51 H⁺ pathway, are associated with deleterious mitochondriopathies. As underlined by Xu et al (2015),
52 the effects of the known mutations in the ATP6 gene are fully consistent with the role of *a* subunit to
53 provide a half-channel for H⁺ access to the *c*Glu carboxylate as well as a link between the
54 intermembrane space and the matrix.
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1 Mitochondrial dysfunctions produce severe and up to now poorly treatable pathological
2 symptoms, up to now mainly counteracted by symptomatic approaches. The relevance of these
3 aminoacid residues in building the route for H⁺ transfer across the IMM stimulates further studies to
4 improve the knowledge of the molecular mechanisms which allow the transmembrane H⁺ transfer.
5 Hopefully, an improved knowledge of the H⁺ movements across the IMM may help to find the
6 molecular tools to counteract the pathological aminoacid substitutions in *a* subunit, for instance
7 through post-translational modifications designed to mimic the replaced aminoacid and allow its role
8 maintenance. Once again, the amazing enzyme complex of the ATP synthase, and especially the
9 membrane sector F_O, reveals its potential exploitation as drug target (Pagliarani et al. 2016; Nesci et
10 al. 2016).
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18 **Compliance with ethical standards**

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20 **Conflict of interest** The authors declare that they have no conflict of interest.

21
22 **Ethical statement** The manuscript complies to the ethical rules applicable for the journal and the
23 research does not involve data regarding humans or animals.
24

25 **Informed consent** not applicable.
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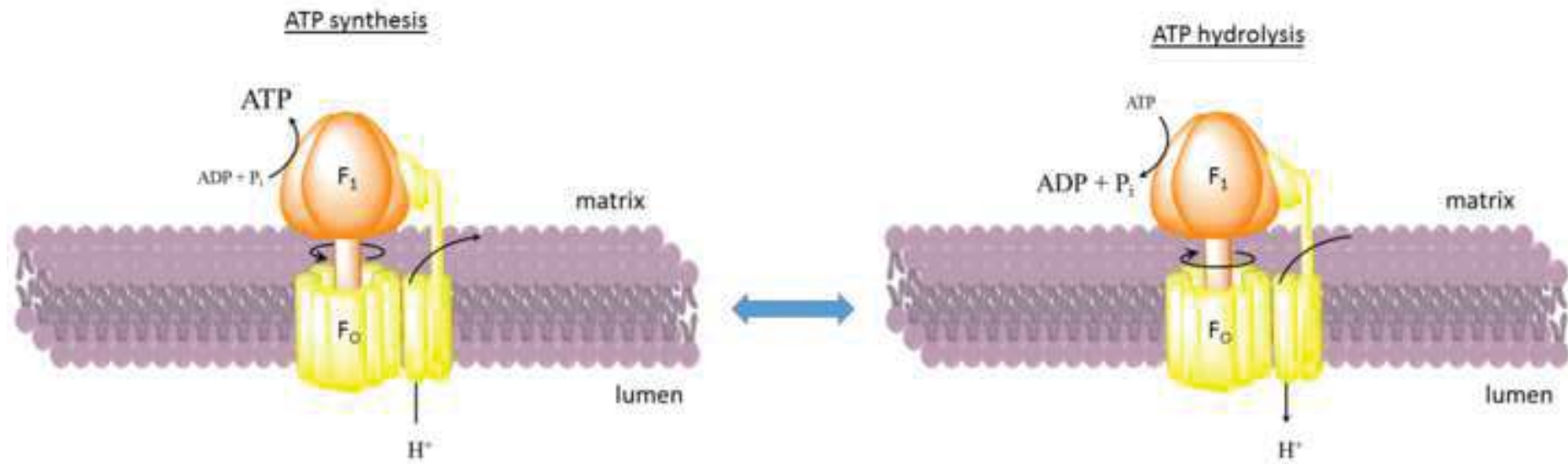
Caption to figures

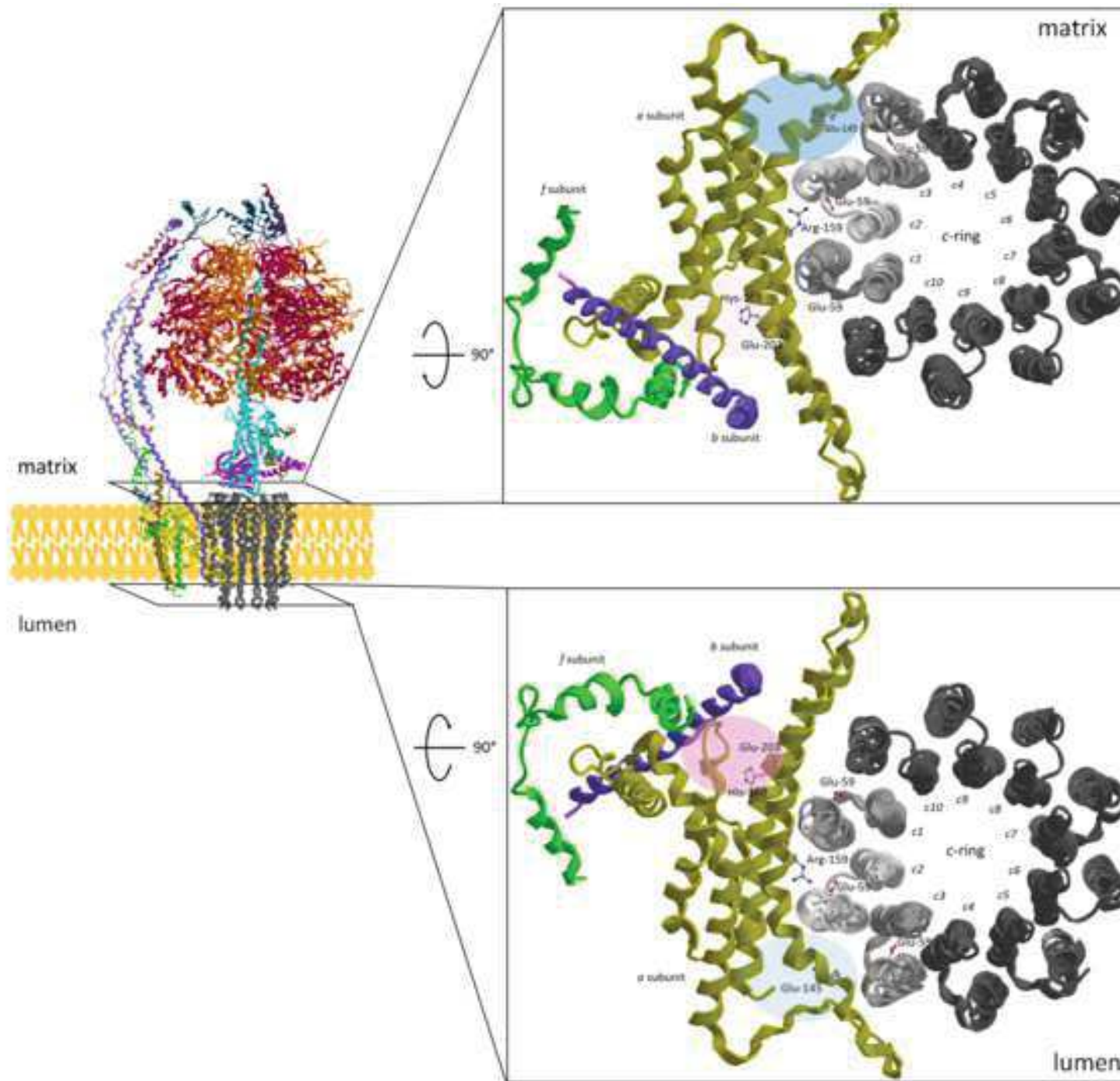
Fig. 1. H⁺ flow across the IMM and rotor rotation according to two opposite directions during ATP synthesis/hydrolysis by the mitochondrial F₁F₀ complex.

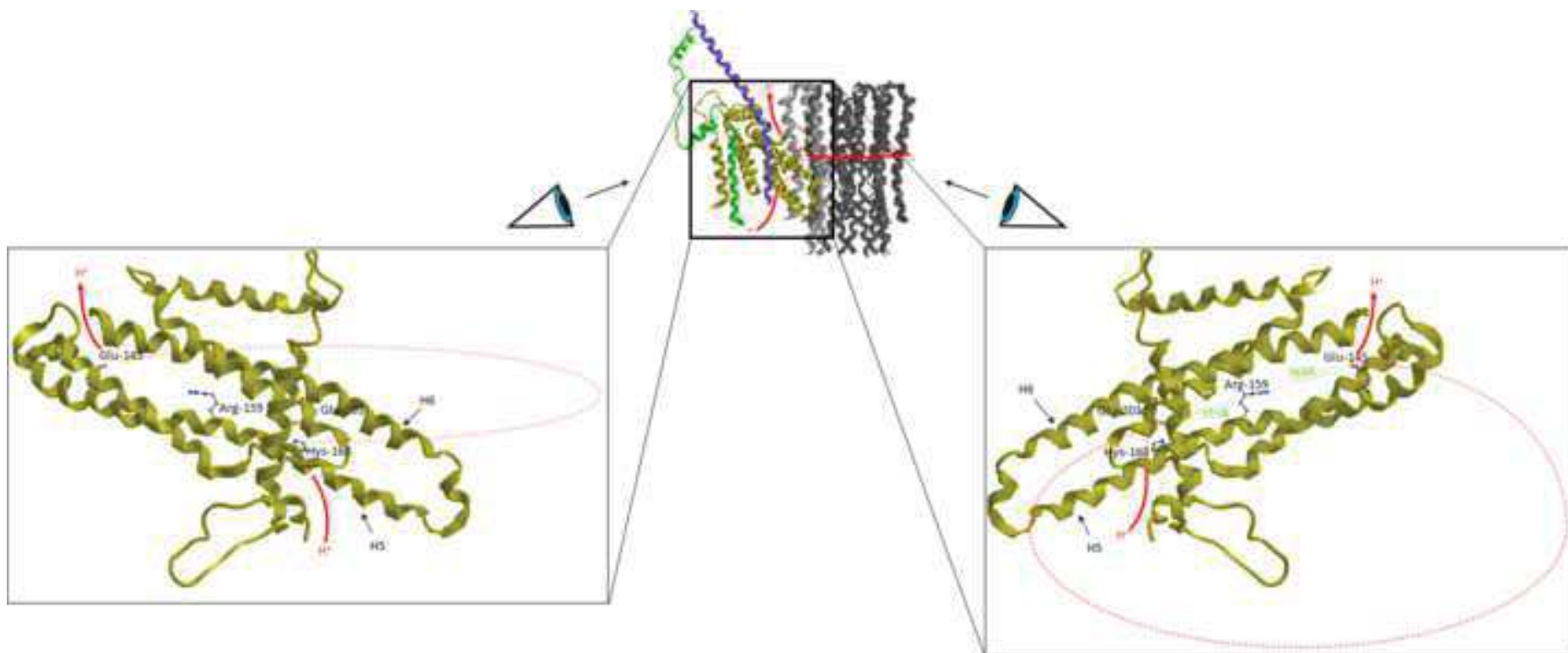
Fig. 2. The transmembrane H⁺ pathway of the ATP synthase. The membrane portion of *f*, *b* and *a* subunits ~~towards~~ viewed from the lumen of the *cristae* in the intermembrane space (lower panel) and the *a* subunit and the outer N-terminal helices of *c2* and *c3* subunits viewed from the matrix (upper panel) form the two half-channels which open to the luminal (rose spheres) and to the matrix (blue spheres) sides of the membrane, respectively. All the aminoacid residues shown as ball and stick models can uptake and release H⁺ and channel them across the membrane, making the *c*-ring (black) rotate. The positive charge of *a*Arg-159 acts as electrostatic barrier. The ATP synthase molecular structure and the membrane H⁺ pathway were drawn by modifying PDB ID codes 6CP6 and 6CP7, respectively, by ChemOffice2017 Software.

Fig. 3. Spatial arrangement of the *a* subunit. On the left and right panels, which illustrate the isolated *a* subunit viewed from two opposite directions to point out the relative positions of some crucial aminoacids, the aminoacids involved in H⁺ translocation are drawn as ball and stick models. The F₀ subunits and the *c*-ring-are in the same colors as in Fig. 2. The calculated distances (green line) between the *a*Arg-159 and the aminoacid residues of luminal and matrix side half-channels are 17.1 and 14.0 Å, respectively. The H⁺ pathway is drawn in red. The dotted curved lines in the lower panels show the H⁺ movement during the *c*-ring rotation. The F₀ domain and the *a* subunit were drawn by modifying PDB ID codes 6CP7 and 6B2Z, respectively by ChemOffice2017 Software.

Fig. 4. Structure of *a* subunit and main known point mutations:- Leu156Arg (A), Leu220Arg (B) and Ser148Asn substitution (C) (see the text for details). The aminoacids which are replaced by these mutations are written in red. The *a* subunit was drawn by modifying PDB ID code 6B2Z by ChemOffice2017 Software.







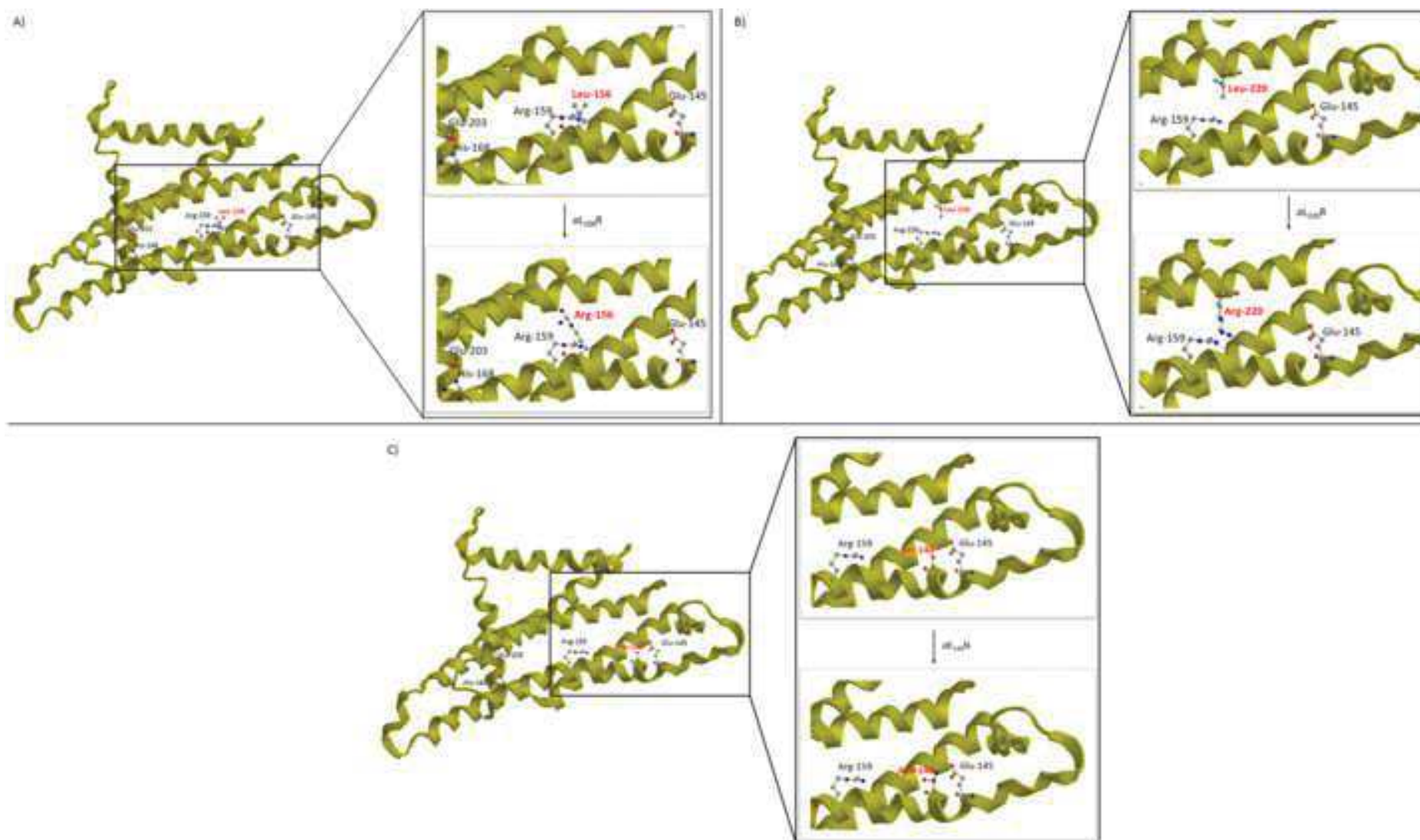


Table 1. Point mutations in the ATP6 gene which affect the H⁺ pathway of the mitochondrial ATP synthase and related human pathologies

Point mutation	Aminoacid substitution	Disease	Reference
T8993G	<i>a</i> Leu156Arg	NARP	Trounce et al. 1994
T9176G	<i>a</i> Leu220Arg	MILLS	Kucharczyk et al. 2019
T8993C	<i>a</i> Leu156Pro		Kucharczyk et al. 2009
G8969A	<i>a</i> Ser148Asn	MLASA	Skoczeń et al. 2018
A8716G	<i>a</i> Pro136Ser	cancer	Niedzwiecka et al. 2018
C8932T	<i>a</i> Lys64Glu		

NARP, Neuropathy, ataxia, and retinitis pigmentosa; MILLS, maternally inherited Leigh syndrome; MLASA, mitochondrial myopathy, lactic acidosis and sideroblastic anemia.