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REVIEW

Directed proton transfer from F_o to F_1 extends the multifaceted proton functions in ATP synthase

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Abstract

The role of protons in ATP synthase is typically considered to be energy storage in the form of an electrochemical potential, as well as an operating element proving rotation. However, this review emphasizes that protons also act as activators of conformational changes in F_1 and as direct participants in phosphorylation reaction. The protons transferred through F_o do not immediately leave to the bulk aqueous phase, but instead provide for the formation of a pH gradient between acidifying F_o and alkalizing F_1. It facilitates a directed inter-subunit proton transfer to F_1, where they are used in the ATP synthesis reaction. This ensures that the enzyme activity is not limited by a lack of protons in the alkaline mitochondrial matrix or chloroplast stroma. Up to one hundred protons bind to the carboxyl groups of the F_1 subunit, altering the electrical interactions between the amino acids of the enzyme. This removes the inhibition of ATP synthase caused by the electrostatic attraction of charged amino acids of the stator and rotor and also makes the enzyme more prone to conformational changes. Protonation occurs during ATP synthesis initiation and during phosphorylation, while deprotonation blocks the rotation inhibiting both synthesis and hydrolysis. Thus, protons participate in the functioning of all main components of ATP synthase molecular machine making it effectively a proton-driven electric machine. The review highlights the key role of protons as a coupling factor in ATP synthase with multifaceted functions, including charge and energy transport, torque generation, facilitation of conformational changes, and participation in the ATP synthesis reaction.

Keywords Proton transport · Oxidative phosphorylation system · F_1F_o ATP synthase · H^+ ions · Protein conformational changes

Introduction

ATP synthases are macromolecular machines that couple soluble bulk phase bioenergetics, based on macroergic pyrophosphate bonds of water-soluble ATP molecules, with membrane bioenergetics, based on the formation of transmembrane electrochemical ion potentials. Accordingly, ATP synthase consists of two bound subunits (factors): intramembrane F_o and hydrophilic F_1. The basic principles of ATP synthase functioning were established quite a long time ago and are based on P. Mitchell's proton-motive force theory (Mitchell 1961) and P. Boyer's binding change mechanism (Boyer et al. 1973) supported by high-resolution structures obtained by J. Walker group (Abrahams et al. 1994). However, there are still many blank spots regarding the details of this amazing molecular machine's functioning. While it is generally accepted that the proton plays a key role in the membrane part of F_1F_o synthase functioning, it is given much less importance when considering the functioning of F_1. The pH of the mitochondrial matrix and chloroplast stroma is usually about 8, which is near optimum for the hydrolysis reaction (Wakai et al. 2005; Rücker et al. 2008). Shift of equilibrium in a reversible reaction toward hydrolysis should impede the ATP synthesis reaction, which is the main reaction for these bioenergetic organelles. Additional H^+ is needed for charge balance in ATP synthesis reaction.

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More specifically, the proton directly participates in the reaction as a substrate, forming water by reacting with the OH^- group cleaved from the ADP molecule (Chen et al. 2006). This is in full agreement with experiments showing the alkalization of the media during ATP synthesis by the suspension of isolated mitochondria. It brings to the point that to prevent self-inhibition of the enzyme due to the developing alkalization, there should be a system of rapid proton delivery to F_1 from the environment.

It is known that proton exchange between the protein-water and lipid-water interphase can be rate-limiting (Mulkidjanian et al. 2006; Nesterov et al. 2022a), so the explanation that protons come from the volume of water, especially with an alkaline pH, is clearly insufficient if we are talking about the optimal conditions for the enzyme. Taking into account the fact that during ATP synthesis, F_1 works in pairs with F_o, which translocates protons into the matrix/stroma just at the docking interface with F_1, it seems natural that F_1 can use these protons from the interface. P. Mitchell noted that a protonmotive potential difference of about 300 mV will induce $\Delta pH \sim 5$ across the active center of F_1 (due to acidification of F_o exit channel) (Mitchell 1975). However, this was not fully reflected in Boyer's mechanism, in which the electrochemical potential is considered only an energy source for torque generation by the F_o subunit. It should be highlighted that the opposite effect on the local pH (F_1 alkalizes and F_o acidifies their docking interface) is an additional factor that makes the docking of F_1 and F_o advantageous and symbiotic. However, the role of this coupling by proton flux is usually ignored because this issue was shadowed by the mechanochemical coupling mechanism of F_oF_1. Numerous reviews exist on the mechanisms of ATP synthase, detailing various aspects of its evolution, structure, functioning, and regulation (Junge and Nelson 2015; Kühlbrandt 2019; Nirody et al. 2020; Zubareva et al. 2020; Courbon and Rubinstein 2022; Vlasov et al. 2022), but they do not address local changes in pH near F_1 as a significant factor. Our review comprehensively examines the role of this mainly overlooked process in ATP synthase caused by directional proton transfer from F_o to F_1. The aim of this work is, along with a review of current data concerning ATP synthase's operation mechanism, to make a detailed analysis of the proton impact on the components of ATP synthase molecular machine.

Structure and regulation of ATP synthases

In this paper, only ATP synthases of the F_1F_o type are considered, which are similar to each other in their general structure. The key amino acids in the active centers of the F_1 and F_o subunits are highly conserved between different organisms (Junge and Nelson 2015; Vlasov et al. 2022). Figure 1 shows the structure of the chloroplast ATP synthase, providing a general insight into the structural organization of F_1F_o ATP synthases. The key structural feature of this molecular machine is the presence of a stator, which is fixed in the membrane and holds the hydrophilic F_1 factor through the peripheral stalk, and a rotor, which performs a rotation around the central axis of the protein and affects the conformation of the catalytic center. Differences in the number and names of subunits, the structure of central and peripheral stalks, and the tendency to oligomerize are observed in ATP synthases between organisms (Kühlbrandt 2019) which reflect peculiarities of regulation rather than differences in the principal mechanisms of action.

The regulation of ATP synthases in various organisms is oriented toward adapting to specific environmental conditions and metabolic peculiarities. In the regulation of FoF_1 synthases, a general trend can also be identified — it is aimed at preventing ATP hydrolysis under conditions of energy deficiency, which manifests itself as a drop in membrane potential or an increase in the ADP/ATP ratio. The ATP synthase is blocked when ADP (more precisely, Mg-ADP) binds without phosphate to the nucleotide-binding centers, while the presence of a high membrane potential prevents such inhibition by reducing the enzyme's affinity for ADP and increasing the affinity for phosphate (Lapashina and Feniouk 2018). In addition, there are specific regulatory systems. For example, in mammalian mitochondria under hypoxia and some other stresses, ATP synthase is blocked by the inhibitory factor IF_1 (García-Bermúdez and Cuezva 2016). In bacteria, ATP hydrolysis can be blocked by changes in the conformation of the epsilon subunit (Iino et al. 2009), and in chloroplasts, a disulfide bond is formed in the γ-subunit in the dark, preventing rotor rotation (Hisabori et al. 2013; Yang et al. 2020). The mechanisms that prevent uncontrolled ATP hydrolysis during energy deprivation are even redundant (Galkina et al. 2022). A detailed description and consideration of the mechanisms of regulation of ATP synthase activity are beyond the scope of this paper. Here we will focus on the proton-related parts of the ATP synthase molecular machine.

Boyer's binding change mechanism of F_1 functioning

One of the most important achievements in the study of ATP synthase, which was awarded the Nobel Prize, is the binding change mechanism discovered by P. Boyer and his co-workers (Boyer 1998) and structurally confirmed by J. Walker and his co-workers (Walker 1998). This mechanism was revolutionary for enzymology. According to it, ATP synthesis in the catalytic center of the enzyme occurs almost isoenergetically, and most of the external energy is expended at the

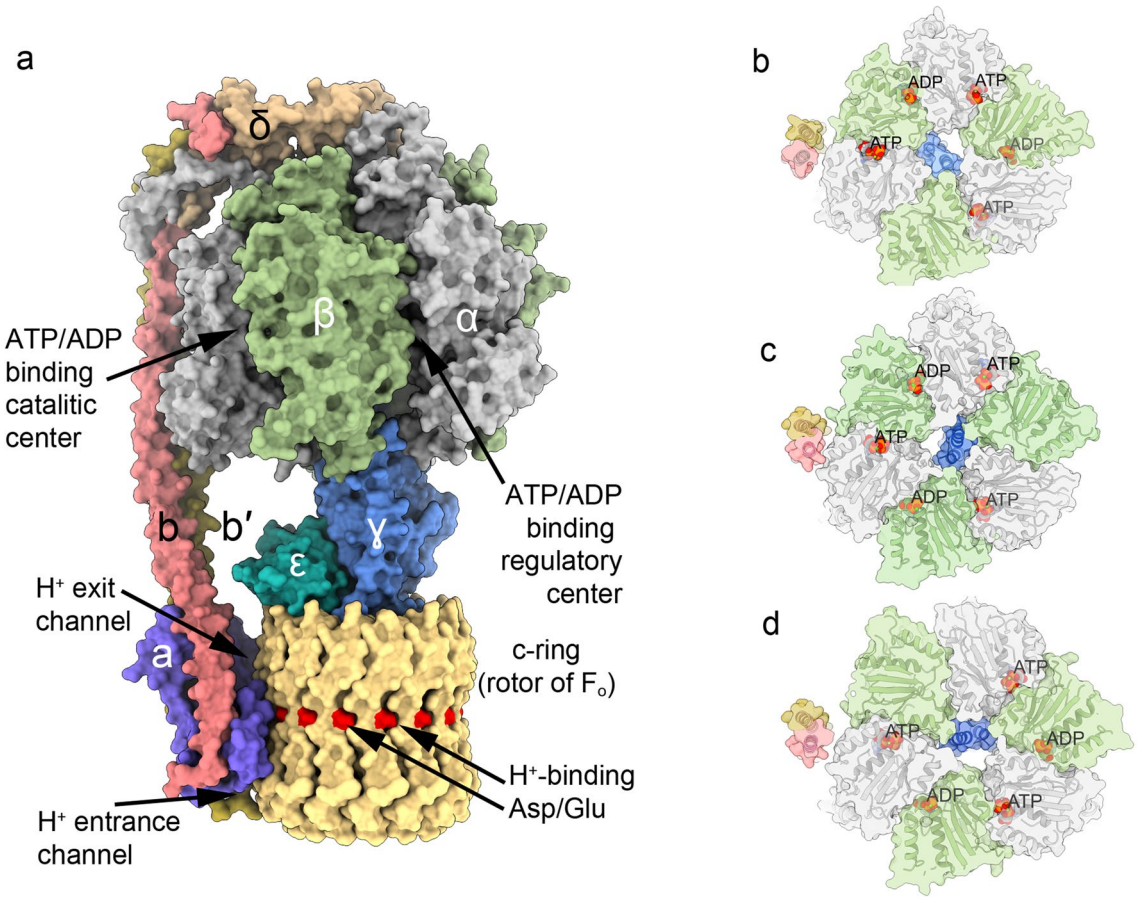

Fig. 1 Demonstration of the ATP synthase structure is shown through the example of spinach chloroplasts ATP synthase, which was obtained by single particle cryo-EM in lipid nanodiscs (Hahn et al. 2018). (a) General view displays the main subunits and functional parts (PDB 6FKF). (b, c, d) Slices through the nucleotide-binding centers of F_1 show three different positions of the rotor subunit γ varying on 120°. (b) Slice of PDB structure 6FKF. (c) Slice of PDB structure 6FKH. (d) Slice of PDB structure 6FKI. The beta subunit, which is tightly connected to the rotor (γ-subunit, blue color), does not bind the nucleotide (open conformation) in accordance with mechanochemical coupling model

stage of ATP detachment from the catalytic center (Boyer 1993). By measuring exchanges of phosphate oxygens with water oxygens catalyzed by ATP synthase with ^{18}O probes, it was shown that reversible synthesis and hydrolysis of tightly bound ATP are independent of proton-motive force and also proceeds in other ATP-binding enzymes such as myosin (Boyer 1998). Structural studies show mechanochemical coupling between F_o and F_1, according to which rotation of the ATP synthase rotary central stalk (γ-subunit) induces conformational changes in the active center, changing the affinity of nucleotides. The rotation of ATP synthase subunits during ATP hydrolysis has even been visualized (Noji et al. 1997; Sambongi et al. 1999) by attached actin filament with fluorescent mark. Using an attached magnetic particle in vitro, it was also confirmed that rotation of the gamma subunit is enough to induce ATP synthesis (Itoh et al. 2004) and that this rotation leads to a change in nucleotide affinity for the enzyme in full agreement with P. Boyer's concept.

Binding change mechanism highlights that ATP synthase is not a simple chemical catalyzer but is a sophisticated molecular machine (Boyer 1997) transforming the electrochemical energy into the changes of ATP/ADP affinity to the enzyme (binding changes). Briefly, according to the binding-change mechanism, four main stages are sequentially realized in the active center of the F_1 subunit during ATP synthesis (Fig. 2). Firstly, the open active center releases any nucleotides. Then during the process of conformational change to the loose form, ADP, magnesium ion, and inorganic phosphate (P_i) bind to the active center. Thirdly, the transition to a tightly bound conformation occurs, in which the energy of ATP binding is approximately equal to the energy of the pyrophosphate bond. At this stage, reversible ATP synthesis-hydrolysis is possible (Fig. 2, forms 2a–2b). Fourthly, the transition to the open conformation occurs, in which the affinity of ATP to the enzyme drops sharply and the nucleotide is displaced. The important point is that

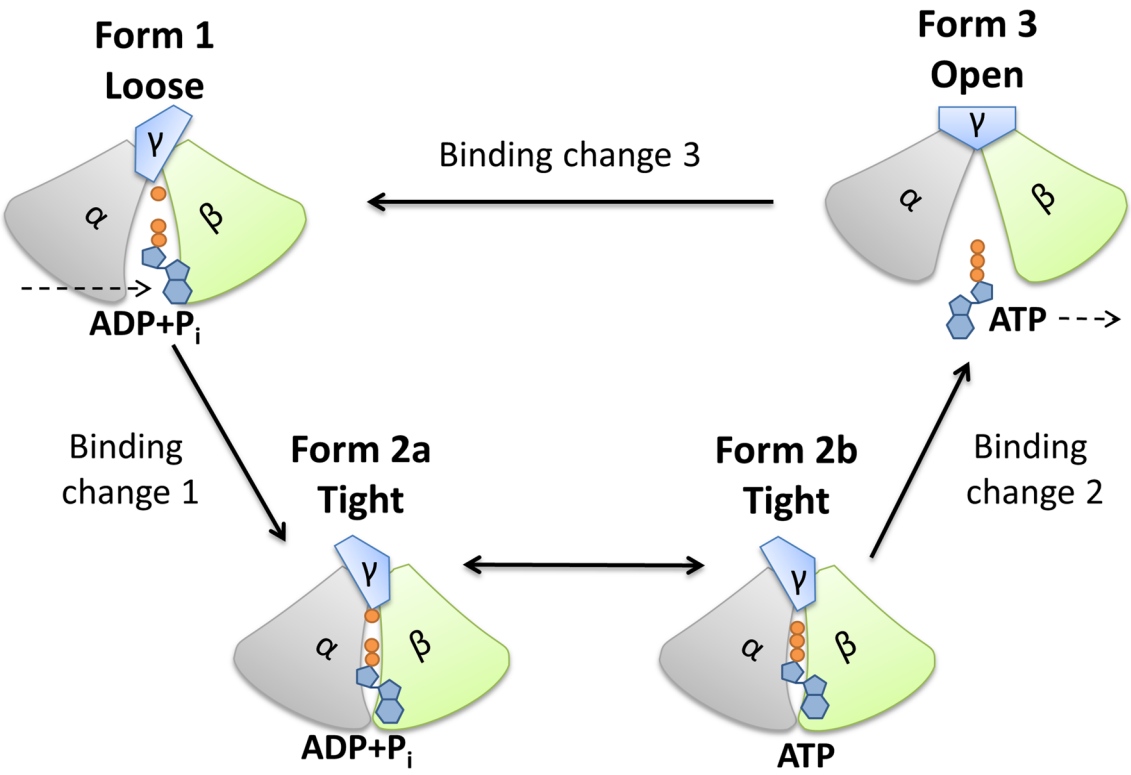

Fig. 2 The binding change mechanism (Boyer et al. 1973). For clarity, it is shown only one direction of catalysis — ATP synthesis reaction. The transition between forms 2a and 2b goes isoenergetically

changes occur in three active enzyme centers at once, each of which is in a different state from the others at a selected moment. The energy for the conformational changes in ATP synthesis mode comes from the F_o subunit, whose rotation is induced by the transmembrane proton transfer along the electrochemical gradient.

The open state of the β-subunit of ATP synthase is formed when it is in close contact with the γ-subunit (Fig. 1(b, c, d)). It has now been shown that there are also additional substeps in 120° rotations (Sobti et al. 2021). According to a recent cryo-EM study, as many as 27 unique conformations of active hydrolyzing and Mg-ADP inhibited F_1F_o ATP synthase were discovered, and it was confirmed that ATP hydrolysis or synthesis in the F_1 region requires three catalytic and three binding dwell conformations (Guo and Rubinstein 2022). It significantly adds to the scheme initially proposed by Boyer. It is now considered such an order of ATP synthase states in the catalytic cycle: state $1_{binding} \rightarrow$ state $1_{catalytic} \rightarrow$ state $2_{binding} \rightarrow$ state $2_{catalytic} \rightarrow$ state $3_{binding} \rightarrow$ state $3_{catalytic}$. For a detailed description of the updated scheme, we refer to the original articles describing additional ATP synthase dwells (Sobti et al. 2021; Guo and Rubinstein 2022).

H^+ participation in ATP synthesis reaction in the catalytic center

One of the rarely available high-resolution structures of the catalytic center with simultaneously bound ADP and phosphate (Sobti et al. 2021) is shown in Fig. 3. Magnesium ions play a key role in the catalytic center of the β subunit of F_1 factor of ATP synthase. First, the magnesium ion enables the formation of the transition state by polarizing the substrates ADP and P_i, both of which are bound to the magnesium cation in the active center (Ko et al. 1999). Second, without magnesium, all three catalytic sites of F_1 bind ATP with equal affinity, which is several orders lower than their affinity to Mg-ATP (Weber et al. 1996). That is, without magnesium, the binding change mechanism cannot be realized. Magnesium also interacts with the P-loop of F_1 (Ko et al. 1999), which is a universal and conserved nucleotide-binding domain and a weak catalyst of phosphate detachment (Ko et al. 1999; Priya et al. 2011). According to the scheme proposed by Pedersen and coauthors (Chen et al. 2006), the approach of the P-loop alanine methyl group (Ala160 in Fig. 3) to phosphate reduces the dielectric constant and facilitates the detachment of the water molecule from ATP.

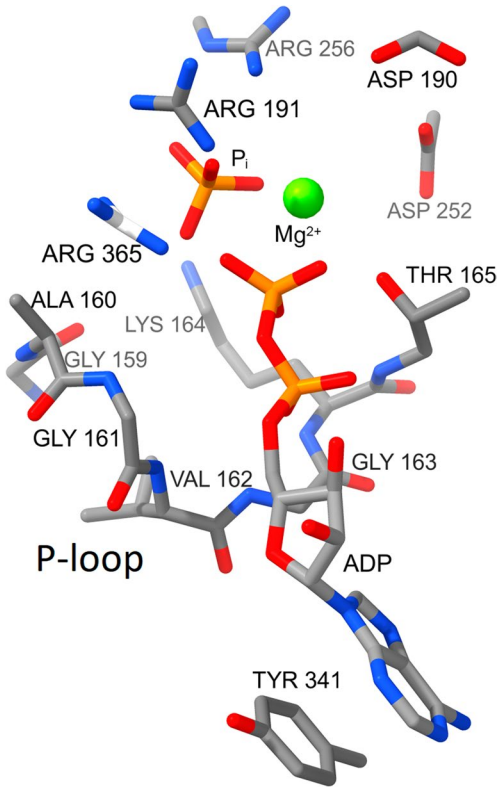

Fig. 3 The structure of the catalytic center in the transitional state, binding ADP, Mg^{2+}, and P_i, is illustrated using the example of Bacillus sp. PS3 F_1 subunit (PDB 7L1Q). The P-loop (from Gly159 to Thr165) and some other charged residues in the vicinity of ADP and phosphate binding sites are shown. The positions of water molecules are not identified. Oxygen is red, phosphorus orange, nitrogen blue, carbons of β and α subunits are gray and white correspondingly

In this reaction, one of the carboxyl groups in the catalytic center acts as a proton donor (Asp190/252 in Fig. 3).

As for ATP hydrolysis, it is even better studied than ATP synthesis because it can be induced in the F_1 subunit without the membranous F_o. It is believed that ATP hydrolysis is catalyzed not by one water molecule, but by the cooperative action of two water molecules, giving rise to rapid nucleophilic attack and proton transfer from the nucleophilic water to ATP (Dittrich and Schulten 2005). The same mechanism has been proven for ATP hydrolysis in kinesin (Parke et al. 2010). The role of the enzyme in the reaction is to create an optimal orientation of water, magnesium, and nucleotide for the reaction to proceed. The energy released as a result of hydrolysis is used to reorient the structure of the protein, inducing a transition to a loose form, i.e., to perform useful work and mechanochemical coupling with F_o.

Torque generation by protons transferred through F_o

A practically separate topic of research is the study of the ATP synthase rotary mechanisms of the c-ring, which is a part of the F_o subunit. According to modern concepts, during ATP synthesis, protons are "pushed" into the ATP synthase entrance channel via the transmembrane electrochemical gradient. The access and exit proton channels of F_o operate via a Grotthuss mechanism (Feniouk et al. 2004) involving a column of single water molecules (Yanagisawa and Frasch 2021), which is consistent with the available cryo-EM data (Murphy et al. 2019; Spikes et al. 2020). Approximately at the middle of the hydrophobic membrane part, there is a carboxyl group of the c-ring, which is protonated through the access channel. At the same time, the carboxyl-H^+ ion pair on the neighboring c-subunit, which is located opposite to the exit channel at that moment, on the contrary, gives a proton to the ATP synthase exit channel that communicates with the other side of the membrane (Fig. 4). During the movement in the hydrophobic part of the membrane, positive protons are compensated by the negative charges of the c-ring carboxyl groups, ensuring the electroneutrality of the process. The long and positively charged arginine residue accompanies the carboxyl group due to electrostatic attraction until it is protonated in the entrance channel. Then, arginine turns again toward the exit channel to the next deprotonated carboxyl group, making an oscillatory motion (Vorburger et al. 2008). Therefore, the enzyme ensures electroneutrality, while no bond is formed between arginine and carboxyl, allowing the rotation almost without friction between the subunits (Pierson et al. 2018). On the membrane side, the low resistance to protein rotation is provided in a similar way by the fact that the negatively charged lipids surrounding the c-ring form only low-energy and short-lived bonds with the lysines of the c-ring (Duncan et al. 2016).

Despite the apparent simplicity of the model and the similarity of F_o in the principle of functioning with the water mill, there is no consensus in the literature on how the released energy of the electrochemical potential provides the rotation itself. One popular explanation is the rotation of the ATP synthase rotor by the mechanism of the Brownian "ratchet" (Ishmukhametov et al. 2010; Watanabe et al. 2013). According to this model, the protonation and deprotonation of the c-ring carboxyl groups are treated as a factor preventing reverse rotation. The movement of a charged hydrophilic amino acid into the hydrophobic lipid layer is energetically disadvantageous, as is the movement of a protonated carboxyl toward the positively charged arginine, which separates the input and output channels

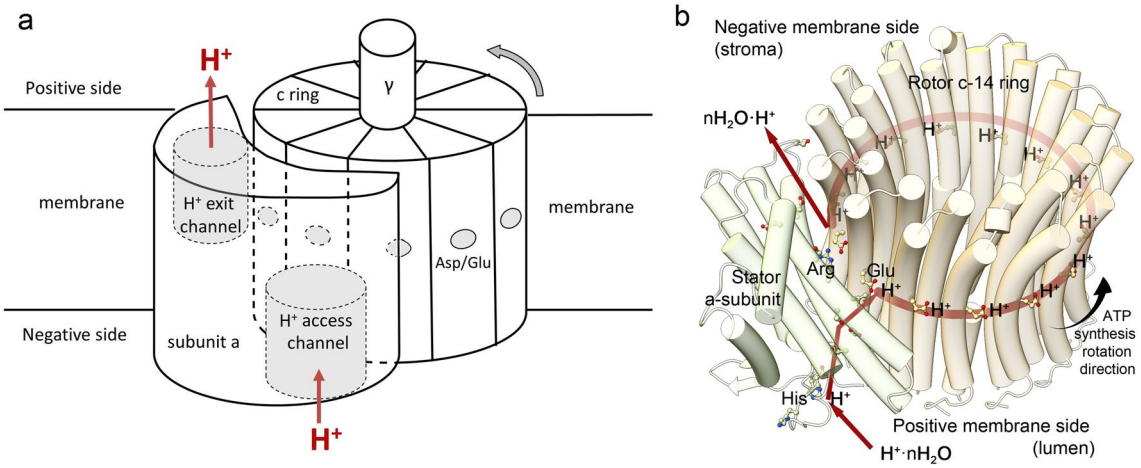

Fig. 4 Proton path within the membrane-embedded F_o factor. **a** Schematic representation. **b** Illustration of proton path in three-dimensional structure (PDB 6FKF). Alpha-helices of the c-ring and a-subunit are shown as tubes and are surrounded by membrane lipids, which are not shown. The proton inside the protein partially loses its hydrate shell and should regain it in or after the exit channel. After binding to the glutamate of c-ring, the proton moves with the rotation of the c-ring to the exit channel

(Kulish et al. 2016). So, proton translocation-dependent step appears to generate torque due to the exclusion of the deprotonated and newly charged carboxyl from the hydrophobic membrane. This suggests that the ATP synthase rotation mechanism is based not only on Brownian ratchet but also on power stroke (Yanagisawa and Frasch 2021). According another view, ATP synthase is an electric field-driven machine (Miller et al. 2013). This model is supported by the experimental observations that the electric potential is necessary for ATP synthase operation and that the conversion of ΔpH to support synthesis must first be done by the electrogenic transport of anions, such as succinate, across the membrane (Kaim 1999). Thus, although ATP synthase may have some ratchet properties, it appears to rely on a combination of electrical and hydrophobic interactions associated with protonation and deprotonation of the carboxyl groups of the c-ring to generate torque as the proton moves through F_o.

It has long been accepted that mechanochemical coupling between the F_o and F_1 subunits is performed by the central stalk of the enzyme, which consists of a γ-subunit and possibly additional subunits, depending on the organism. Simplified, the rotation of the c-ring and γ-subunit results in mechanical pressure on the F_1 α- and β-subunits, leading to changes in their conformation and causing active center rearrangement, including changes in affinity for ATP and ADP. Recent structural and kinetic experiments have significantly expanded the model, adding more details, especially about F_o-F_1 coupling. For example, using single-molecule fluorescence resonance energy transfer, it was shown that during ATP synthesis, the c-ring undergoes sequential steps corresponding to the rotation with a shift by one c-subunit (Düser et al. 2009) or even smaller substeps (Yanagisawa and Frasch 2021). At the same time, the rotation of the rotary γ-subunit was long thought to be discrete, with a rotational angle of about 120° (Watanabe et al. 2013) according to Boyer's three basic F_1 states. As mentioned earlier, recent cryo-EM structures showed the implementation of intermediate turns of about 40° and 80° as well (Sobti et al. 2021; Guo and Rubinstein 2022). In any case, there is a rotational mismatch between F_o and F_1, which raises the question of how the membrane and hydrophilic parts of the rotor are coupled. The rotational mismatch is also in line with the structural symmetry mismatch between the α3β3 heterotrimer and the c-ring stoichiometry (ranging from 8 to 17 subunits in different species (Vlasov et al. 2022)). That fully excludes a cogwheel-like coupling model.

Based on experimental data, it is hypothesized that the energy is intermediately stored as elastic deformation of ATP synthase subunits (Junge et al. 2001; Vahidi et al. 2016; Kulish et al. 2016; Martin et al. 2018). So, the energy for conformation changes originates not from the kinetic energy of c-ring motion, but from the elastic strain energy of ATPase subunits, which accumulates on the enzyme and releases in discrete rotation steps. Such a mechanism is more appropriately called torsional (Nath 2008), then simply rotational. It should be emphasized that the different affinity of nucleotides to the three beta subunits arises from the asymmetric interactions of the catalytic sites with the single copy subunits of the central stalk. This view is largely supported by modern data showing the binding dwells of ATP synthase (Sobti et al. 2021). It was also convincingly demonstrated that the peripheral stalk exhibits a large bending motion from a left-handed to the right-handed curvature (Guo and Rubinstein 2022). This extends the understanding of the role of the stator subunits in ATP synthase function

and shows that they not only perform the scaffold function but also accumulate elastic energy for coupling of the F_1 and F_o subunits.

Protonation of F_1 subunit after energization

The quantity and quality of hydrophilic groups, as well as the set of its conformational states, can be studied through the analysis of a biopolymer buffer titration curve (Opanasenko et al. 1978). Mitochondrial buffer capacity measurements paradoxically showed that the minimum buffer capacity is achieved exactly at the pH range in which the mitochondrial matrix operates (Mitchell and Moyle 1967). The access of protons to the mitochondrial matrix was provided either by a detergent or by adding an uncoupler with valinomycin. Similar results were obtained in chloroplasts in the dark (Polya and Jagendorf 1969) (Fig. 5). It was also found that under illumination, the buffer capacity of the chloroplast in the pH range of 7–9 increases, and, in the same manner, decreases in the range of 5–7 (Polya and Jagendorf 1969). This effect was attributed to the large change in the pH gradient across the thylakoids on the light, shifting the local pH in which the ionic groups are located (Walz et al. 1974). Using tritium water, it was also shown that the protonation of the ATP synthase F_1 subunit changes upon illumination (Ryrie and Jagendorf 1971). It is now known that the formation of an

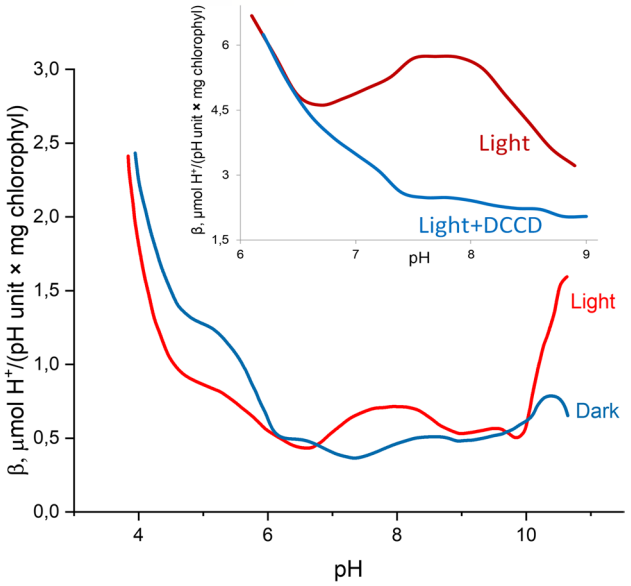

Fig. 5 The buffering capacity β of lettuce leaf chloroplasts was evaluated in both light and dark conditions, utilizing data from (Walz et al. 1974). It is seen that beta increases in the 7–9 pH range under illumination, while simultaneously decreases to a similar value in the 4–6 range. The inset displays the buffering capacity of pea leaves chloroplasts in the light with and without DCCD, based on the data from (Zolotareva et al. 1986)

electrochemical gradient in the light actually switches ATP synthase into an active conformational state that differs from the inactive one in at least two aspects: reduced thiol groups in the γ-subunit and decreased affinity of ADP for nucleotide-binding centers (Malyan and Strotmann 1994).

Similarity in distribution of ionogenic groups in different F_1Fo-type ATP synthases

It is important to note that the amino acid residues of arginine, lysine, histidine, glutamate, and aspartate are ionogenic groups that bind the proton during titration. Tyrosine may also contribute to the buffer capacity, the pK of which may shift to the acidic side under enzyme operating conditions (Malyan and Opanasenko 2018) which explains the light-dependent changes in the chloroplasts titration curve in Fig. 5 above pH 9. As observed from the titrations of mitochondrial buffer capacity and the chloroplast in the dark (Fig. 5), the observed pK of a portion of the glutamate and aspartate residues in ATP synthase is shifted to the alkaline region by almost two units compared to the pK of the free form of the amino acid (pK 3.9–4.1). This effect is due to the proximity of positively charged arginines or lysines to the carboxyl groups (Opanasenko and Makarov 1980). Figure 6 shows the distribution of charged amino acids on the surface of F_oF_1 ATP synthases of different organisms. It can be seen that in all of these structures, the distribution is similar, and the carboxyl groups of aspartate and glutamate are, indeed, adjacent to arginine or lysine in most cases. ATP synthase is practically electrically neutral at pH 7–8. Thus, the structure of bovine ATP synthase 5ARA, shown in Fig. 6, contains 496 negative amino acids (aspartate, glutamate), 482 positive ones (arginine, lysine), and 55 histidines, only a small portion of which can be protonated at pH about 8.

F_o subunit transfers protons to F_1

In the works of Zolotareva et al., the light-induced changes in both buffer capacity and protonation of F_1 were studied in more detail (Zolotareva et al. 1986, 1990). F_1 protonation was shown to be blocked by dicyclohexylcarbodiimide (DCCD) (Fig. 5 inset), which exclusively inhibits H^+ transfer through the c-ring of ATP synthase, consequently preventing rotation of the rotor and ATP synthase activity. DCCD binds to the protonated carboxyl groups of ATP synthase c-rings and does not in any way prevented electrochemical gradient formation associated with photosystem functioning. If the change in buffer capacity by light were only due to the formation of a pH gradient, as previously assumed (Walz et al. 1974), then DCCD should have no effect because it does not eliminate the pH gradient (Opanasenko et al. 1992). However, DCCD prevented both F_1

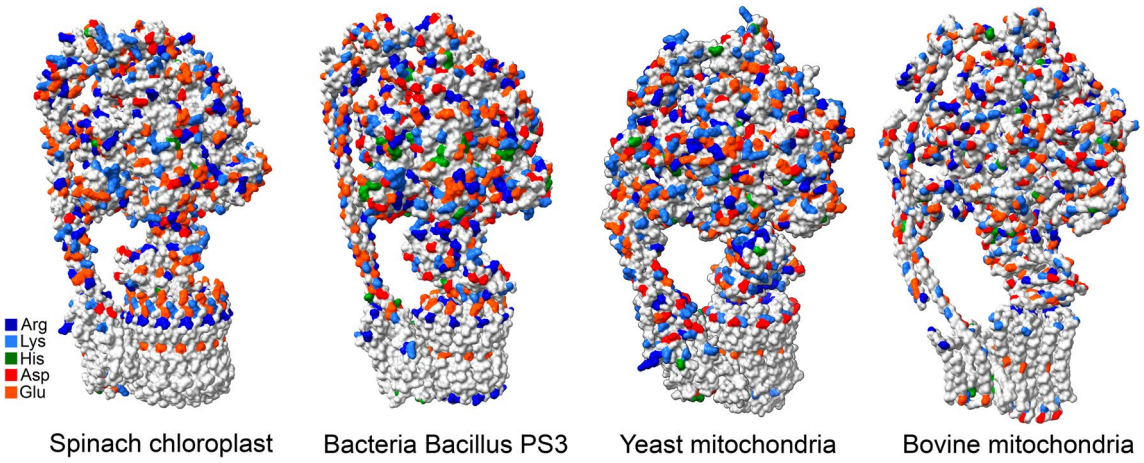

Fig. 6 The comparison of F_1F_o ATP synthases from different organisms is presented. Charged amino acids on the water-accessible surface of the enzymes, which have a significant role in proton binding at varying pH, are highlighted with different colors as per the legend. The ATP synthase structures of spinach chloroplast (PDB 6FKF), thermophilic bacterium *Bacillus* sp. PS3 (PDB 6N2Y), yeast *Saccharomyces cerevisiae* mitochondria (PDB 6CP6), and bovine mitochondria (PDB 5ARA) are displayed

protonation and light-dependent changes in chloroplast buffer capacity (Zolotareva et al. 1986). At the same time, according to Mitchell's notion (Mitchell 1975), the electrochemical proton potential of about 180mV (which is more typical for membranes than the estimate of 300mV given by Mitchell in 1975) is transformed by F_o into $\Delta pH \sim 3$ units between F_o output channel and the bulk phase. This is very close to the experimental pK shift between dark and light (Fig. 5), which is also about 3 units. If the protons from F_o easily diffuse to F_1 and change the effective pH of its environment, it fully explains the effect. Thus, ATP synthase in the synthesis mode is in a locally different pH, which is less than the matrix pH by three units. In fact, the local pH of the F_1 subunit of ATP synthase is nearly equal to the pH of the perimembrane space on the other side of the membrane (the intermembrane space of mitochondria or thylakoid lumen).

It is known that H^+ ions are retained at the interfacial boundaries. A retention of protons has been observed on the surfaces of artificial membranes (Antonenko et al. 1993), and mitochondrial membranes (Yurkov et al. 2005; Moiseeva et al. 2011), as well as on the surfaces of protein proton pumps, such as rhodopsins (Drachev et al. 1984), photosystem (Junge and Ausländer 1974), and cytochrome c oxidase (Salomonsson et al. 2005). These non-equilibrium protons are energy carriers and are used, among other things, for ATP synthesis (Yaguzhinsky et al. 1976). The barrier of proton detachment from the interface can be lowered by the presence of mobile (that can freely diffuse) buffers in the environment (Mulkidjanian et al. 2006; Nesterov et al. 2022a). Together with the data that the buffer capacity of mitochondria and chloroplast is minimal in their operating pH range, this suggests that a local proton fraction with increased activity is formed on the surface of ATP synthase and is weakly exchanged with the environment. The above confirms that the local proton activity (pH) on the surface of the ATP synthase during its functioning must differ significantly from that in the volume.

H^+ ions induce conformational change in F_1

As noted, there are a number of natural regulatory mechanisms, such as Mg-ADP, that reversibly inhibit ATP synthase under energy deficit. To resume the enzyme's functioning after inhibition, it must be activated, i.e., transferred into a conformational state in which all inhibitions are removed. Different inhibitors can be eliminated in various ways. The mechanism of ATP synthase turning on after complete inhibition has been suggested to involve preactivation by "activating protons" before entering the active mode (Valerio et al. 1992; Groth and Junge 1995). It has also been shown that several protons are consumed without synthesis at the beginning of ATP synthase operation (Feniouk et al. 1999). Experiments with tritium water have shown that, in the presence of a potential on the thylakoid membrane under illumination, tritium ions in the amount of about 100 ions per molecule remain bound even after the F_1-factor isolation procedure (Zolotareva et al. 1986). So, the pH shift of the F_1 environment induced by F_o causes conformational changes of F_1, transitioning from the inactive form to the active one, which persist in F_1 for a considerable amount of time, even after uncoupling from F_o. More detailed experiments have confirmed that the inhibition of the chloroplast ATP synthase by the addition of ADP is accompanied by a decrease in protonation of about 100 ionogenic groups of the enzyme

(Gubanova et al. 1994), while reactivation of Mg^{2+}-ADP inhibited F_oF_1 (as well as isolated F_1) is determined by protonation of a limited number of acid–base groups buried in the enzyme molecule (Malyan et al. 1998). To summarize, it was shown that F_1 protonation is needed both for ATP synthase and hydrolase activity of the enzyme. In the inhibited enzyme, about 100 amino acid groups lose the ability to bind protons from the environment. However, only in ATP synthesizing mode the enzyme has an abnormally increased ability for proton retention in the 7–9 pH region by a number of carboxylic groups, which is due to the acidification of F_1 by F_o ($\Delta pH \sim 3$).

More detailed experiments were performed to elucidate the role of transmembrane transport of hydrogen/tritium ions during F_1 protonation, as well as the role of the phosphorylation process. Chloroplasts were incubated for several hours in the dark in tritium medium, after which they were repeatedly washed from this isotope and, at the same time, from endogenous phosphate and nucleotides (Gasparyan et al. 1989). A wash-resistant tritium fraction was found to be firmly bound to lipids and membrane proteins in the chloroplast lumen, but not to ATP synthase. In these preparations, it was not enough to turn on the light to protonate the F_1 factor, but it was also necessary to activate the phosphorylation process by adding ADP and phosphate/arsenate (Zolotareva et al. 1990). This is easily explained by the fact that without the presence of phosphate/arsenate, it was impossible to remove Mg-ADP inhibition of the enzyme by electrochemical potential alone. The induction of tritium ion transport from the thylakoid lumen by the gramicidin protonophore cannot replace the transport through the F_o subunit (Zolotareva et al. 1990). This emphasizes that protonation of F_1 is not the result of a free exchange of ions with the external media or membrane but is strictly associated with conformational changes in the F_oF_1 during its transition to the active form by F_o-derived protons in the presence of ADP and P_i.

Experiments with mitochondria labeled with a pH-sensitive FITC (fluorescein isothiocyanate) probe, covalently bonded to the proteins, showed that the slow stage of H^+ equilibration, which takes approximately 20 min, is the detachment of protons from the immobilized matrix buffer (Krasinskaya et al. 1997). Electrophoresis and F_1 isolation revealed that most of the FITC attached to mitochondria and submitochondrial particles (SMP) was bound to the F_1 (Kozlova et al. 2003). It was also demonstrated that the activation of proton pumps on FITC-labeled mitochondria and SMP (Krasinskaya et al. 1997, 1998) leads to alkalization of the membrane-water interface region where F_1 subunits are located. This alkalization is not a manifestation of $\Delta\mu H$ because it is not eliminated by the addition of an uncoupler, which induces depolarization (Krasinskaya et al. 1997). Only preincubation with uncoupler and without substrate prevents this effect, presumably because it leads to ATP hydrolysis, transforming the ATP synthase to a hydrolytic state and then to an inhibited form (by IF_1 or Mg-ADP). In that state, ATP synthase loses the ability to transfer protons associated with F_1 to other parts of the membrane, including proton pumps, which is in full agreement with the results described earlier for chloroplast ATP synthases. Cumulatively, these data for mitochondria and SMP are well explained by the assumption that, similar to chloroplasts under conditions of membrane energization, a number of protons are bound to F_1 of mitochondrial ATP synthase.

Structural aspects of F_1 conformational changes by H^+ ions

The protonation of approximately 100 negatively charged amino acids during ATP synthase activation will shift the charge equilibrium. This shift should be accompanied by a compensatory attraction of anions from the volume to the enzyme. The excess charge can be compensated by the formation of an electrical double layer of counterions, including phosphate, and ADP anions needing for ATP synthesis and coming from phosphate transporter and nucleotide translocator, which are located next to ATP synthase (Chen et al. 2004). However, the key consequence of protonation is that the salt bridges between negatively and positively charged amino acids will be destroyed. This means that the protein structure will become less rigid, and conformational changes will be facilitated. This is indirectly confirmed by the DCCD-dependent weakening of the bonds between the hydrophilic F_1 subunit and the F_o membrane subunit, induced by the proton gradient (Fielder et al. 1994; Ponomarenko et al. 1999). It appears that the weakening is due to the protonation of some of the carboxyl groups involved in the formation of the salt bridges that hold F_o and F_1 together. In other words, the active (protonated) form of the enzyme becomes less rigid due to breakdown of salt bridges, and the conformational changes induced by the γ-subunit rotation are facilitated.

It is known that the conservative glutamate- and aspartate-rich domain of the β-subunit (DELSEED loop) participates in torque transmission (Tanigawara et al. 2012; Watanabe et al. 2015) and ADP inhibition (Feniouk et al. 2007). We propose that the DELSEED loop may be one of the most prominent targets of protonation. The electrostatic interaction of lysine in the γ-subunit with the DELSEED region of the β-subunit stabilizes the ADP-inhibited state of the enzyme by hindering the rotation of the γ-subunit (Feniouk et al. 2007). Protonation eliminates this interaction by neutralizing the charge of the carboxyl groups of DELSEED

loop. Therefore, protonation of the DELSEED loop through the F_o subunit by protonmotive force is a simple and elegant explanation of the ATP synthase reactivation mechanism.

Opanasenko and Makarov (Opanasenko and Makarov 1980) estimated the conformational transition energy of chloroplast ATP synthase under light during protonation of about 100 carboxylic groups as 100–130 kcal/mol F_1. This energy is roughly equivalent to the energy required to synthesize up to 14 molecules of ATP. Thus, enzyme activation is a significantly energy-consuming process that occurs at the expense of the energy of the electrochemical potential. Such activation, through increased mobility of protein chains, results in significantly greater energy savings. Hydrogen/deuterium-exchange mass spectrometry shows that γ-rotor of ATP synthase is load-dependent destabilized leading to an overall free energy "penalty" of ~15 kkal/mol F_oF_1, which is close to the energy of two ATP molecules hydrolysis (Vahidi et al. 2016). This is another indication that some energy must be expended to convert ATP synthase to the active form. It should be emphasized that the destabilized γ-subunit may be a good conductor of protons between F_o and F_1 because in structurally unstable protein regions water and hydrogen exchange occurs faster (Englander et al. 2007). All functions of proton in ATP synthesing system are summarized in the Fig. 7.

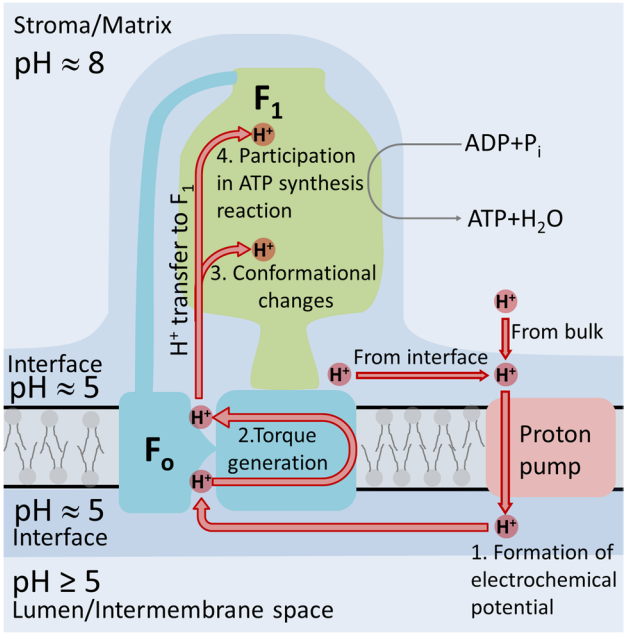

Fig. 7 Proton functioning and cycling in ATP synthesis system. Protons are transferred from F_o to F_1 subunit of ATP synthase in the presence of an electrochemical potential and induce local increase of H^+ activity in the interface

Discussional questions

ATP synthase activation

Although experiments have shown binding of up to 100 protons to ATP synthase, it remains unclear exactly how many of these protons are required to convert the enzyme into the active form. It is possible that only a few protons are necessary for preliminary activation of the enzyme, such as protonation of the DELSEED loop closest to the output channel. Further protonation may occur simultaneously with ATP synthesis coupled rotations, stabilizing the enzyme's active form. If a large number of protons are necessary, a logical question arises: how can protons activate the inhibited form of ATP synthase when conformational changes in F_1 are forbidden and the tightly coupled F_o rotor cannot rotate, thus preventing proton transfer? One possible answer is that ATP synthase can operate in an uncoupled state, in which proton transfer through F_o does not result in ATP synthesis (Evron and Avron 1990; Evron and Pick 1997). In mitochondria, a similar effect can be achieved through reversible pore opening, which partially disrupts the connection between F_1 and F_o (Pinke et al. 2020). In this case, protons can flow directly through the central part of the c-ring, which is typically filled with lipids or isoprenoid quinones (Vlasov et al. 2019). Specific changes in this hydrophobic fraction within the c-ring can be induced by reactive oxygen species (ROS), which are produced when the electrochemical potential rises sharply under conditions of inhibited ATP synthesis (Vyssokikh et al. 2020). This leads to the hypothesis that ROS signaling may be one of the mechanisms providing reactivation of ATP synthesis after energy stress. If such a mechanism occurs, it would offer a new perspective on the mechanisms of coupling between ATP and ROS synthesis in bioenergetic systems.

Participation of potassium ions in ATP synthesis

It is worth noting that there are forms of F_1F_o ATP synthases that work on the gradient of sodium ions (Kaim 2001), which have a significantly smaller electron shell than potassium ions. Sodium binding to the c-ring has been confirmed by structural studies showing specific differences in the c-ring (Meier et al. 2009; Schulz et al. 2013). At the same time, the H^+-conducting F_o subunits are extremely proton-selective and practically do not bind sodium ions (Krah et al. 2010) and even less potassium or cesium ions. In this context, the available report on the possibility of F_1F_o ATP synthase operating on potassium ions instead of hydrogen ions (Juhaszova et al. 2022) contradicts most of the available literature data.

The simplest explanation for the experimental observation of ATP synthesis due to the potassium gradient in the absence of an electric potential sufficient for synthesis is the presence in isolated ATP synthase preparations of tightly bound cardiolipin (Mühleip et al. 2019), which have high affinity to the c-ring subunits (Gasanov et al. 2018). Lysocardiolipin, which easily forms from cardiolipin, is known to be a potassium carrier (Medvedev et al. 1977; Evtodienko et al. 1979). In the presence of a potassium gradient in proteoliposomes with ATP synthase, lysocardiolipin transfers potassium, returning to the other side of the membrane in neutral protonated form, thus acting as a K^+/H^+ exchanger and forming a surface proton fraction, as does nigericin on a bilayer lipid membrane (Antonenko et al. 1993). In this case, the observed effect can be interpreted as a uniport of potassium ions by ATP synthase, although there is no direct movement of potassium ions through the c-ring of ATP synthase. It is important to note that such a synthesis mechanism can also be realized under special in vivo conditions, for example, under oxidative stress accompanied by a decrease in the electrochemical proton gradient but preservation of the potassium gradient.

Free-radical mechanism of ATP synthesis

Some experimental data also suggest a free-radical mechanism for ATP synthesis and some other phosphorylating enzymes at very high concentrations of magnesium (Buchachenko and Kuznetsov 2008), but the significance of such a process under physiological conditions is doubtful, at least for plants and animals. Additionally, these results cannot be reproduced universally (Crotty et al. 2011). Even if a free-radical mechanism of ATP synthesis were to be realized, it may be relevant for some microorganisms in high saline environments or for early evolution stages where reactions of non-enzymatic photo-induced ATP synthesis in solutions occurred.

The role of quantum effects and collective excitations

Despite the fact that available models of enzyme functioning, including ATP synthase, provide a fairly clear picture of their functioning, they do not describe phenomena at the atomic level and, in particular, do not consider in detail the process of energy transfer along the amino acid backbone of the protein during conformational changes. One of the problems of this transfer is energy loss due to dissipation. The search for non-dissipative, long-range energy transfer pathways in proteins led to the formulation of Davydov's soliton theory (Davydov 1973), further developed by Scott for more realistic model of proteins (Scott 1991). This theory predicts the possibility of non-dissipative motion of compression strain in the form of a soliton along the alpha helix of the protein. It is important to note that there is still no conclusive experimental evidence for the presence of solitons in protein alpha helices, although some experiments can be interpreted as the presence of self-trapped excitations (Xie et al. 2000; Edler et al. 2004). The involvement of soliton-like excitations is also suggested for the lipid part of the membrane (Heimburg and Jackson 2005), including the process of proton transfer from H^+-pumps to ATP synthase (Nesterov et al. 2022b).

It has recently been calculated that soliton self-localization in a protein is possible only in the presence of a high barrier, which can occur at the contact points of protein subunits (Georgiev and Glazebrook 2022). This observation is relevant in the context of the fact that ATP synthase contains regulatory α-subunits, of which only one arginine may be involved in catalysis (Arg365 in Fig. 3). It is tempting to speculate that the massive alpha subunit could be used to localize the soliton, thus accumulating elastic energy for subsequent transfer to the catalytic center. The role of the alpha subunit remains to be clarified. It is also worth noting theoretical work that shows that due to quantum effects, there can be an increase in the activity of the proton in the catalytic center of the enzyme by several orders of magnitude (Wang et al. 2014). If a similar effect is also realized in ATP synthase, it could significantly facilitate the reaction of ATP synthesis, in which a proton is involved. The connection of localized states with proton transfer has been suggested in a number of papers (Stuchebrukhov 2009; Kavitha et al. 2016). The boldest assumption is that increased proton retention at active F_1 is itself a consequence of self-localized enzyme excitations.

ATP synthase reversibility

It was initially proposed that ATP synthesis and hydrolysis were fully reversible and proceeded through the same set of enzyme states. However, some data is not in full agreement with such reversibility, which is why A.D. Vinogradov proposed a model of two nonequilibrium forms of F_1F_o that unidirectionally catalyze ATP synthesis and/or hydrolysis (Vinogradov 2000, 2019). For a detailed review of this topic, please refer elsewhere (Zharova et al. 2023). The presence of two isoforms can be partially explained by the fact that the direction of γ-subunit rotation determines whether the ε-subunit is in a contracted or expanded form (Feniouk and Junge 2005). Based on the data on synthase protonation given in our review, we can conclude that hydrolytic and synthase states may also differ in the degree of protonation. This could explain quite well a number of differences between the hydrolytic and synthase forms while allowing for rapid switching between enzyme isoforms under the action of protons transported through F_o.

Conclusion

In the present work, the data on the mechanisms of functioning and some structural features of F_1F_o ATP synthases have been systematized and analyzed. It has been shown that the energy of the electrochemical H^+ potential is converted into elastic deformation of the enzyme and then used to change the conformation of the active center and the energy of nucleotide binding, ensuring catalysis of ATP synthesis and then ATP release from the enzyme. Much attention has been paid to less known data on the mechanisms of ATP synthase transition into active form by protonation of a significant number of the enzyme's carboxyl groups. It is argued that, under ATP synthesis conditions, such protonation is provided by the F_o component of ATP synthase, which increases local H^+ activity. Protonation of the carboxyl groups of the enzyme is necessary, first, to break the salt bridges between amino acids of rotor and stator, ensuring the rotation of the γ-subunit. Second, it is needed to ensure the availability of protons for the ATP synthesis reaction. The directivity of proton transfer from F_o to F_1 is determined by the presence of a pH gradient between these subunits, since local alkalinization occurs at the active center of F_1 during the reaction of ATP synthesis. Acidification of F_1 under conditions of H^+ transport through F_o ensures the supply for ATP synthesis reaction. In contrast, the slowdown of electrochemically driven proton transport through F_o leads to F_1 local pH equilibration with the environment (pH≈8) corresponding to the ATP hydrolysis optimum. So, the work shows that ATP synthase is in an alkaline environment only in the hydrolysis mode, while in the synthesis mode, it functions in a locally acidic environment optimal for proton delivery to the catalytic center for ATP synthesis reaction. Thus, the work formulates the mechanism of ATP synthase F_1 protonation through F_o and organically integrates it with the available concepts of this molecular machine functioning. This review emphasizes that the proton is a ubiquitous element in the membrane bioenergetics system. It performs membrane potential formation, energy transfer across the membrane, torque generation in F_o, ensures the withdrawal of the ATP synthase rotation inhibition, and finally proton is one of the substrates in the phosphorylation reaction.

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Declarations

Conflict of interest The authors declare no competing interests.

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