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Review

The role of subunit epsilon in the catalysis and regulation of F_OF_1-ATP synthase

Boris A. Feniouk [a,*], Toshiharu Suzuki [a,b], Masasuke Yoshida [a,b]

[a] *ATP System Project, Exploratory Research for Advanced Technology, Japan Science and Technology Corporation (JST), 5800-3 Nagatsuta, Midori-ku, Yokohama 226-0026, Japan*
[b] *Chemical Resources Laboratory, Tokyo Institute of Technology, JST ERATO ATP System Project, Nagatsuta 4259, Midori-ku, Yokohama 226-8503, Japan*

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Abstract

The regulation of ATP synthase activity is complex and involves several distinct mechanisms. In bacteria and chloroplasts, subunit epsilon plays an important role in this regulation, (i) affecting the efficiency of coupling, (ii) influencing the catalytic pathway, and (iii) selectively inhibiting ATP hydrolysis activity. Several experimental studies indicate that the regulation is achieved through large conformational transitions of the α-helical C-terminal domain of subunit epsilon that occur in response to membrane energization, change in ATP/ADP ratio or addition of inhibitors. This review summarizes the experimental data obtained on different organisms that clarify some basic features as well as some molecular details of this regulatory mechanism. Multiple functions of subunit epsilon, its role in the difference between the catalytic pathways of ATP synthesis and hydrolysis and its influence on the inhibition of ATP hydrolysis by ADP are also discussed.

Keywords: ATP synthase; Regulation; Epsilon; Inhibition; ADP; Protonmotive force

1. Introduction

H^+-F_OF_1-ATP synthase is a ubiquitous membrane enzyme present in bacterial plasma membrane, chloroplast thylakoid membrane and mitochondrial inner membrane. Despite some discrepancy in the subunit composition, amino acid sequence and functional aspects, enzymes from various organisms share the same general structure and basic catalytic mechanism; the differences influence the efficiency and the regulatory features.

The primary function of H^+-F_OF_1-ATP synthase is converting the energy of electrochemical potential of protons ($\Delta\tilde{\mu}_{H^+}$) into chemical energy of ATP phosphoanhydride bonds [1]. However, the reverse process, namely generation of $\Delta\tilde{\mu}_{H^+}$ at the expense of ATP hydrolysis, is also vitally important in many bacteria. In some cases ATP-driven proton pumping plays a certain role in mitochondria [2–4].

It should be noted that Na^+-F_OF_1-ATP synthase is found in several bacteria. Structurally the Na^+-transporting enzyme is highly homologous to its proton-transporting counterpart; it is also fully capable of both $\Delta\tilde{\mu}_{Na^+}$-driven ATP synthesis and ATP hydrolysis coupled to Na^+-pumping (see [5] and the references therein for details). Moreover, in the absence of Na^+ the enzyme can operate on protons [6]. It is therefore most probable that the regulatory features of the H^+-F_OF_1-ATP synthase described in this review are also relevant to the operation of the Na^+-transporting enzyme.

Structurally F_OF_1-ATP synthase is composed of two distinct portions connected by two "stalks". The hydrophilic F_1-portion (in the simplest bacterial enzyme a complex of five types of subunits in stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$) protrudes ~ 100 Å from the membrane and is responsible for the catalysis of ATP synthesis/hydrolysis. The hydrophobic proton (or Na^+) transporting F_O-portion (in most bacteria a complex of three types of subunits in stoichiometry $a_1b_2c_{10-15}$) is anchored in the membrane. Centrally located γε-subunits complex bound to the ring-shaped oligomer of *c*-subunits and peripheral b_2-dimer connecting subunit *a* to the $\alpha_3\beta_3\delta$-complex compose the stalks (see [7–12] for more detailed information on the F_OF_1 structure).

There are three catalytic and three non-catalytic nucleotide-binding sites on the $\alpha_3\beta_3$-hexamer. Non-catalytic sites are involved in the regulation of the enzyme [13–16].

* Corresponding author. Tel.: +81 45 924 5891/5238; fax: +81 45 924 5239.
E-mail address: fboris-ra@bio.res.titech.ac.jp (B.A. Feniouk).

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During ATP synthesis or hydrolysis the three catalytic sites cyclically change their affinities to ATP, ADP and phosphate in a complex and co-operative manner. The basic principle of the catalytic mechanism is known as "binding change mechanism" that was proposed by Boyer [17]. On the molecular level the catalytic mechanism is realized by rotation of $\gamma\varepsilon c_{10-15}$ subunit complex (rotor) relative to the rest of the enzyme (stator) [18–29]. During ATP synthesis the ion transport through F_O powers the rotation of the ring-shaped *c*-oligomer, and the rotation is passed into F_1 via γε-complex, where it drives the conformational transitions necessary for the catalytic sites affinity changes. Vice versa, the binding and hydrolysis of ATP can induce the conformational transitions in $\alpha_3\beta_3$-hexamer that result in rotation of subunit γ which is passed to the *c*-ring and drives the proton (or Na^+) transport.

The mechanism of ATP synthesis and hydrolysis is complex and even the catalytic events in F_1-portion are not completely clear, despite the vast amount of structural and functional experimental data (see [30–36] for reviews). It has also been noticed that ATP synthesis is not a mere reversal of hydrolysis, but follows a distinct catalytic pathway (discussed in detail by Vinogradov and co-authors in [37]). To make the matter even more complicated, ATP synthase has a complex regulation with several mechanisms to modulate its activity.

A well-known regulatory mechanism present in bacterial and chloroplast enzyme is the inhibition of ATP hydrolysis by subunit ε (reviewed in [38–41]). Rapid progress achieved in the studies of subunit ε in the last few years has clarified several enigmatic aspects of this regulation. This review summarizes recent experimental data on subunit ε, evaluates them in light of the earlier functional experiments, and compares the functions of this small protein in ATP synthases from different organisms. The molecular details of the regulation and the probable role of subunit ε in conformational transitions of ATP synthase upon switching between ATP synthesis and hydrolysis are also discussed.

2. Structure of subunit ε and its location in the ATP synthase

Subunit ε is present in all F_OF_1-ATP synthases. Unfortunately, due to historical reasons the mitochondrial homologue of bacterial/chloroplast subunit ε was named δ. To avoid confusion, we will use subunit nomenclature of bacterial/chloroplast ATP synthase throughout this review, so any reference to merely "subunit ε" presented here is related to mitochondrial δ, in contrast to "mitochondrial subunit ε", which is a much smaller protein that has no homologues in the bacterial and chloroplast ATP synthase.

The structure of subunit ε and its position relative to other ATP synthase subunits were extensively studied by cross-linking and other indirect methods (see [39,40] for detailed reviews). The three-dimensional structure of isolated subunit ε from *E. coli* enzyme (solved by NMR [42,43] and by X-ray crystallography [44]) confirmed most of these earlier findings, showing a protein composed of a 10-stranded β-sandwich and two C-terminal α-helices that form a hairpin. A high-resolution structure of mitochondrial F_1 [9] demonstrated that the structure of purified bacterial subunit ε is strikingly similar to that of its mitochondrial homologue within the F_1-complex. The latter structure (Fig. 1A and B) confirmed some of the earlier results on the cross-linking of *E. coli* subunit ε with γ and *c*-oligomer, but was incompatible with some other results (e.g., on the cross-linking of ε with subunits α and β [45]), suggesting that major conformational changes of subunit ε take place in the active enzyme.

A markedly different three-dimensional structure of subunit ε was obtained from crystallographic study of the isolated γε-complex form *E. coli* [46]. In this structure the two C-terminal helices of subunit ε do not form a hairpin, but are extended along subunit γ towards F_1 (Fig. 1C). As this structure was solved not in the whole enzyme, but just for a complex of two subunits in which the N- and the C-terminal regions of γ were truncated, it does not provide a firm basis to state that such conformation might exist in vivo. However, after the appearance of this structure, a re-evaluation [47] of an earlier *E. coli* F_1 electron density map at 4.4 Å resolution [48] revealed that within F_1 the C-terminus of subunit ε can adopt a similar conformation.

This conformation (Fig. 1C and D) is distinct from the hairpin structure seen in the purified *E. coli* subunit ε and in mitochondrial F_1 (Fig. 1A and B). The greatest difference was found in the position of the first C-terminal α-helix. The low resolution of the second α-helix did not allow mapping of its precise location [47]. However, if the location of this second helix is the same as found in the isolated γε-complex (and as modeled in the 1JNV PDB entry shown in Fig. 1D), it is expected to significantly hinder the activity because upon rotation it is expected to sterically clash with the DELSEED segment of subunit β (Fig. 1C, yellow).

The latter structures clarified some apparent controversies of cross-linking experimental results and provided a stimulus for further fruitful functional studies reviewed below.

3. Comparison of amino acid sequence of subunit ε from different organisms

Fig. 2 shows a multiple alignment of subunit ε sequences for a few mitochondrial, chloroplast and bacterial enzymes. Although the data presented are clearly insufficient for statistically significant information (see [49] for a more thorough sequence homology study), they provide several important hints on the conservativeness of subunit ε domains. As can be seen in Fig. 2, the only region that is conserved in ATP synthases from mitochondria, chloroplasts and bacteria corresponds to *E. coli* residues 16–58 (Fig. 2; Fig. 1, red). The most conservative residues are glycines, leucines and prolines that are presumably important for proper folding. An exception is a histidine (*E. coli* εHis38) that is involved in the interactions with the polar loop of subunit *c* [50,51].

The region corresponding to *E. coli* residues 61–109 that comprises the part of the N-terminal β-sandwich that contacts with subunit γ[1] and the first C-terminal α-helix (Fig. 2; Fig. 1, blue) was found to have similarity among bacterial and chloroplast enzymes only.[2] However, several highly conserved

[1] In mitochondrial ATP synthase, this region is also in contact with the mitochondrial subunit ε (shown transparent white in Fig. 1A).

[2] Even at such a small number of sequences there were 2 exceptions, namely the mitochondrial sequence from *Drosophila melanogaster* was included in the homology cluster, while the sequence from a purple photoheterotrophic bacterium *Rhodobacter capsulatus* was not (See Fig. 2).

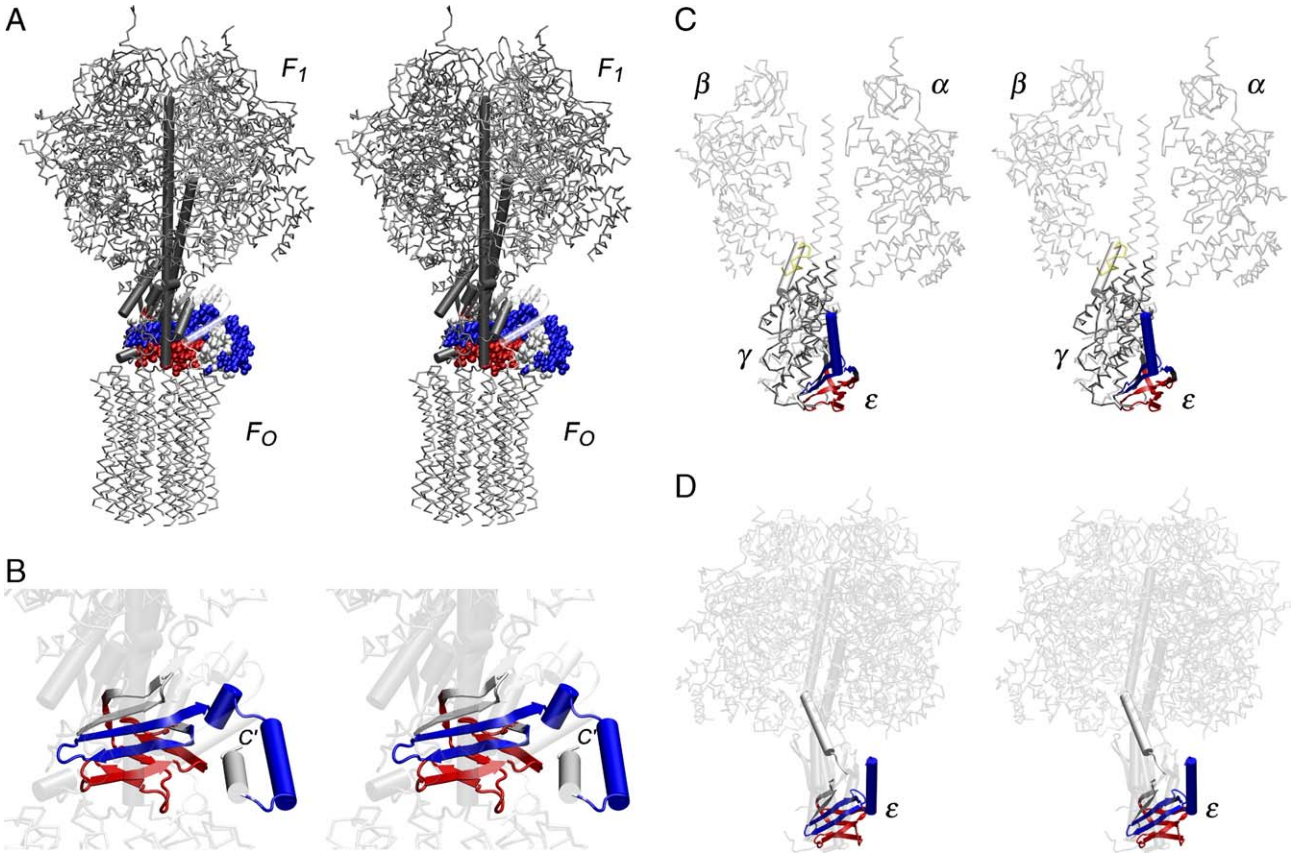

Fig. 1. Structure of subunit ε. (A) Location of subunit ε (mitochondrial δ) in the ATP synthase complex. Coordinates from Protein Data Bank (PDB) entries 1E79 (bovine mitochondrial F_1) and 1QO1 (yeast c_{10}-oligomer) were used to make this cartoon representation. Cα atoms of the N-terminal β-sandwich domain of subunit ε were used for alignment of structures. Subunit ε is shown in color in van der Waals representation: red is the most conservative part of the β-sandwich, blue is the part conserved between bacterial and chloroplasts enzyme only, the rest is white (see Fig. 2 for sequence alignments). Subunit γ is shown in dark grey; $\alpha_3\beta_3$ hexamer and c_{10}-ring are shown as light-gray backbone trace. An additional mitochondrial F_1-subunit that has no homologue in bacteria and chloroplast (mitochondrial ε) is shown as transparent white. (B) Same chimeric structure as in A; subunit ε is shown in cartoon representation (coloring scheme is also the same as in A); its C-terminus is marked as C'. (C) Structure of the isolated *E. coli* γε complex with the ε-subunit in the "partially extended" confirmation (PDB entry 1FS0; subunit γ is dark-grey, subunit ε is colored as in A–C) overlaid with the mitochondrial F_1-portion (light grey; only subunit γ and one pair of α and β subunits are shown; PDB entry 1E79). Cα carbons of subunit γ residues 210–232 (*E. coli* numbering) were used for the alignment. DELSEED fragment of β-subunit is shown in yellow. (D) Extended conformation of subunit ε within the *E. coli* F_1-portion of ATP synthase (Protein Data Bank entry 1JNV). Note that the position of the second C-terminal α-helix of subunit ε is arbitrary because the electron density map was not sufficiently precise to fit the backbone [47]. The image was generated with VMD software package [156].

residues from this part of the β-sandwich domain are found in mitochondrial enzyme as well.

In the region of the first C-terminal α-helix highly conserved amino acid residues were found for chloroplast and also for mitochondrial sequences, but not for bacterial subunit ε (with exception of two alanines that correspond to *E. coli* εAla94[3] and εAla101). Noteworthy, only alanine and leucine residues were conserved between mitochondrial and chloroplast enzymes, while the polar residues were specific for each type of enzyme.

The sequence of the second C-terminal α-helix has several conserved residues in mitochondrial and also in chloroplast enzymes (but only one glutamate conserved between them). No conserved residues were found in this region for bacterial ε.

According to the multiple sequence alignment presented, the N-terminal β-sandwich domain is similar among all ATP synthases; the region facing the *c*-ring seems to be conserved more strictly than the region facing subunit γ. The C-terminal α-helical domain shows much less similarity with only two alanines conserved; however, some similarity is seen within mitochondrial and within chloroplast sequences.

The second C-terminal α-helix of bacterial subunit ε shows particularly high variability, both in length and in amino acid composition. This makes tempting to propose that this region might be involved in fine regulation of the ATP synthase and is responsible for tuning the basic catalytic machinery of the enzyme to variable growth conditions and physiological needs of different bacterial species. In some bacteria the primary role of ATP synthase is not ATP synthesis, but maintenance of $\Delta\tilde{\mu}_{H^+}$ at the expense of ATP hydrolysis, so a markedly different regulatory mechanism can be expected. In line with this speculation, subunit ε from some anaerobic bacteria (e.g., species from *Chlorobium*, *Bacteroides* or *Bifidobacterium* genera) completely lacks both C-terminal α-helices that are primarily responsible for inhibition of ATP hydrolysis. On the contrary, mitochondrial or chloroplast enzyme functions almost exclusively as a synthase inside a living

[3] The important role of the εAla94 is confirmed by the results of site-directed mutagenesis experiments on *E. coli* ATP synthase: a double replacement of the highly conservative εAla94 and non-conservative εLeu128 (second helix) to cysteines completely abolished the inhibitory effect of ε even without cross-linking [52].

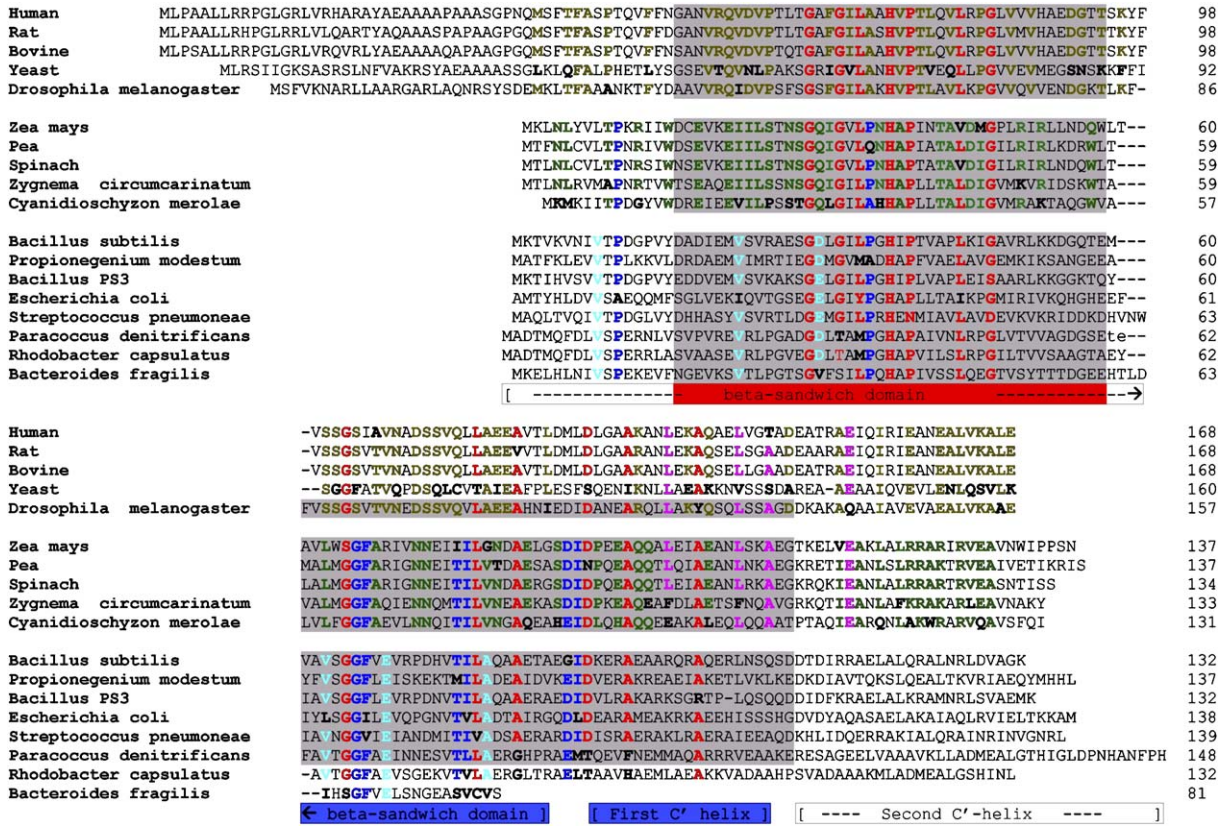

Fig. 2. Sequence alignment of ATP synthase subunit ε (mitochondrial δ) from different organisms. Five mitochondrial (top), five chloroplast (middle) and eight bacterial sequences (bottom) were used for alignment. The sequences were arbitrary chosen from the GenPept Databank; some of them include precursor regions or N-terminal methionines that are absent in the mature proteins. Residues shaded in grey show the conserved blocks found by Macaw multiple alignment software using BLOSUM62 scoring matrix with pairwise score cut-off of 31. Residues colored red are conserved in all organisms; residues colored magenta are conserved between mitochondrial and chloroplast enzymes; residues colored blue are conserved between chloroplast and bacterial enzymes; residues colored in brown, green and cyan are conserved only within mitochondrial, chloroplast or bacterial enzyme, respectively.

cell in a much more uniform and regulated environment, so it is probable that in this case the C-terminal region is conserved as a "best fit" optimized for each enzyme type.

4. Functional role of subunit ε

4.1. Assembly of F_OF_1

It is well documented that in ATP synthase from *Bacillus PS3* or from *E. coli* subunit ε is critically important for the binding of F_1 to F_O [53,54]. Without ε, the enzyme fails to assemble in the cell and no membrane-associated ATPase activity is observed [53,55,56]. Similar effect was found for the enzyme from yeast mitochondria [57]. In chloroplasts the enzyme does assemble without ε, but the coupling between catalysis and proton transport is lost [58,59].

Studies on the *E. coli* enzyme indicated that the N-terminal β-sandwich domain is critically important for F_OF_1 assembly [60,61]. In contrast to the N-terminal domain, the two C-terminal α-helices of ε do not play a role in the assembly of F_OF_1 [56,62–65].

4.2. Influence on catalysis

Subunit ε is also required for the coupling of ATP synthesis/hydrolysis and proton pumping. Several experimental results indicate that subunit ε is not only a passive linker that binds subunit γ to the *c*-ring, but has a more complicated role in catalysis and intra-enzymatic energy transfer. In *E. coli* ATP synthase some conformational changes of subunit γ found in the whole enzyme during activity are lost without subunit ε [66–68]. It was demonstrated that subunit ε is required for ATP synthesis driven by rotation of subunit γ by external force in F_1 [28]. Recent experiments on *Bacillus PS3* enzyme indicate that during ATP synthesis β-subunits adopt markedly different conformations in mutant enzyme lacking the C-terminal domain of subunit ε [69].

It was also demonstrated that the C-terminal α-helices of subunit ε are important (although not indispensable) for the efficient coupling between ATP hydrolysis and proton pumping [70,71]. The details of subunit ε role in the catalysis and coupling are unclear.

4.3. Inhibition of ATPase activity

Perhaps the most well-known role of the ε subunit in bacterial and chloroplast ATP synthase is the inhibition of ATPase activity. Discovered initially in the chloroplast F_1 [72], this phenomenon was later demonstrated [73] and described in detail [74,75] for *E. coli* enzyme. In *E. coli* the inhibitory effect is best seen on the isolated F_1-portion; it results in 5- to 7-fold decrease in the rate of ATP hydrolysis [76,77]. In chloroplast enzyme it is even more pronounced: figures of up to 16-fold are reported [59]. The inhibition can be relieved by several methods, e.g., heat treatment

[58,72,74] or addition of alcohols [59,78] that lead to dissociation of ε from F_1, addition of detergents [77,79–82] that reversibly perturbs the inhibitory interactions without dissociation of ε from F_1, trypsin treatment [83–85] that cleaves the C-terminal region of ε. In the whole F_OF_1-ATP synthase from *E. coli* the inhibitory effect of subunit ε was a doubted issue [86,87], in part because the ε-less F_1 dissociates from F_O [55,56], so it was difficult to experimentally study the properties of the ε-less F_OF_1. Moreover, as the ATPase activity of the whole F_OF_1 is higher than that of the ε-inhibited F_1 (see [82] and references therein), a marked increase in the activity is observed upon binding of F_O to the ε-inhibited F_1. This phenomenon is known as "relieving of ε-inhibition upon binding to F_O" [82,87]. However, in the enzyme from thermophilic *Bacillus PS3* the inhibition can be clearly seen in the whole F_OF_1 at 50 μM ATP [88]. Several experiments pointed out, that there is an inhibitory effect of subunit ε on the whole *E. coli* and chloroplast F_OF_1 as well [52,70,85–91].

The leading role of the C-terminal region of the protein in ATP hydrolysis inhibition was first reported for *E. coli* enzyme, where deletion of up to 60 C-terminal amino acid residues resulted in loss of inhibitory properties without major effect on the ATP synthesis [56]. Same effect was observed on the chloroplast enzyme [64].

Several studies revealed that the ε C-terminus is highly mobile and can be cross-linked with residues in subunits α, β and γ [43,45,84,85,92–94]. Genetic fusion of 12–28 kDa proteins to the C-terminus of subunit ε from *E. coli* ATP synthase resulted in loss of inhibitory effect in all mutant enzymes [70]. It was proposed that some kind of subunit ε conformational transitions are responsible for the inhibition [39,95].

The two different conformations of subunit ε described above (Fig. 1) provided a clue to the structural basis of these transitions. The results of subsequent cross-linking experiments in *E. coli* enzyme indicate that both conformations can be observed in the whole enzyme [96]. It was proposed that the transition of subunit ε C-terminus from contracted hairpin conformation (Fig. 1A and C) to extended one (Fig. 1D) is responsible for the inhibition of ATP hydrolysis [96]. This proposal was in accordance with the above mentioned experimental evidence on the essential role of the ε C-terminus in the inhibition.

A very interesting result was obtained in the functional studies of the cross-linking effects. It turned out that if subunit ε is trapped the extended conformation shown in Fig. 1D, the enzyme retained the ATP synthesis activity, but ATP hydrolysis was dramatically inhibited [96].

Further insights came from the experiments on ATP synthase from thermophilic *Bacillus PS3*. It was demonstrated that subunit ε can extend its C-terminus even further inside the $\alpha_3\beta_3$-hexamer up to the N-terminus of subunit γ (see Fig. 3). This fully-extended confirmation of ε has the same anisotropic effect on the enzyme activity: ATP hydrolysis was inhibited by approximately 80% without significant effect on the ATP synthesis rate [97]. These results strongly supported the proposed role of the ε C-terminus transition in the inhibition.

It was recently doubted if the full extension of the ε C-terminal helices is physiologically relevant and if the second helix actually enters into the $\alpha_3\beta_3$-hexamer up to the N-terminus of subunit γ in vivo [98]. The experiments on *E. coli* ATP synthase showing a minor role of the second α-helix in inhibition of hydrolysis [56,63] and the same level of inhibition with the two ε C-terminal α-helix fixed by a cross-link in the hairpin conformation [52] also make the importance of such fully extended conformation doubtful. An indirect functional evidence for the role of the fully extended conformation of subunit ε came from the chloroplast enzyme, where it was shown that deletion of 8–20 amino acid residues from the N-terminus of subunit γ lead to decrease in the inhibitory effect of subunit ε [99]. However, as it is still unclear, whether partially and fully extended conformations both exist in vivo and whether they play different roles in the regulation of the enzyme, we will hereafter refrain from distinguishing them and will use the term "extended" for both.

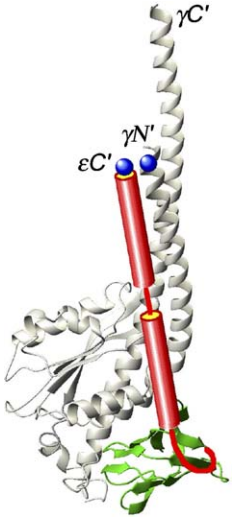

Fig. 3. A cartoon illustrating the hypothetic fully-extended state of subunit ε (modified from [97]). N-terminal domain of subunit ε is colored green; C-terminal α-helices are red. Subunit γ is shown in grey. Blue spheres indicate the residues that can be cross-linked.

The movement of the ε C-terminus per se does not provide information on what kind of interactions might lead to the inhibitory effect. The site-directed mutagenesis experiments on ATP synthase from *Bacillus PS3* shed some light on that. It was demonstrated that the basic, positively charged residues on the second C-terminal α-helix of subunit ε and the negatively charged acid residues of the DELSDED[4] segment of subunit β play an important role in the inhibition [100].

Replacements of the basic residues in the ε C-terminus by alanines led to dramatic decrease of the inhibitory effect. Similar effect was documented in chloroplast ATP synthase from spinach: marked loss of subunit ε inhibitory effect was observed upon truncation of the tenth C-terminal residue (Arg), while the truncation of the previous nine (non-basic) residues had a much less pronounced relative effect [101]. It should be noted that the conservative AXLAL(R/K)RAXX**R** motif in the second C-terminal helix of ε seems to be shared by the enzymes from chloroplast and from the bacteria of *Bacillus* genera (Fig. 2).

[4] DELSEED in most other organisms; corresponds to the *E. coli* ^{380}DELSEED^{386} of subunit beta.

Together with the similar functional role of the last Arg residue in this motif mentioned above, this suggests a common mechanism of ATPase activity inhibition mediated by subunit ε in chloroplast and in *Bacillus PS3*. This suggestion is further supported by experiments where ATP synthase of *Bacillus PS3* with chimeric ε containing the chloroplast C-terminal α-helices was functional and exerted similar level of inhibition on ATP hydrolysis [102].

Loss of inhibition by subunit ε was also observed upon replacement of the acidic residues in the βDELSDED to alanines [100]. Noteworthy, in *E. coli* ATP synthase, the replacement of the first glutamate in the βDELSEED to cysteine also led to a similar increase in the ATPase activity [103].

The role of the βDELSEED acidic residues is most probably similar in all ATP synthases, for they are highly conserved in bacterial, mitochondrial and chloroplast enzyme. The universal role of the positive charges in the second C-terminal α-helix of ε in propagating the inhibitory effect is less certain. Although a conservative motif can be found between the enzymes from chloroplasts and from *Bacillus* sp., in general this region is conserved neither in length nor in its amino acid composition even among bacteria (Fig. 2).

Moreover, in subunit ε from *E. coli* ATP synthase, the deletion of the second C-terminal α-helix alone does not have a detectable effect on the inhibition, and only the deletion of both helices leads to a pronounced reduction of the inhibition [56,63,104].

It should be noted that in both published structures of subunit ε in the extended conformation (Fig. 1D and E) only the very end of the first C-terminal α-helix is close enough to the βDELSEED segment to allow electrostatic interaction. In contrast, the whole second helix can easily be in close proximity to the βDELSEED. Taken together with the inhibitory effect of the first helix alone, this implies that besides the movement of the C-terminus of ε, some other marked conformational changes of the enzyme might be involved in the inhibition of ATP hydrolysis. Electron microscopy (EM) studies of *E. coli* ATP synthase revealed that some large-scale changes take place in response to different nucleotide composition in the medium [105]. Other EM studies demonstrated that the *E. coli* and bovine mitochondrial ATP synthase can undergo a marked contraction so that the distance between F_1 and F_O changes by 15–20 Å [39,106]. Single-molecule FRET experiments also indicate that such contraction might take place [29]. However, it cannot be excluded that the inhibitory mechanism of subunit ε C-terminus includes other factors besides the electrostatic interactions with βDELSEED.

The extent to which subunit ε exerts its inhibitory effect on ATP hydrolysis and the details of the inhibitory interactions are significantly different in ATP synthases from various bacteria. For instance, isolated subunit ε from *Bacillus PS3* binds ATP (but not ADP or GTP) with high affinity and the C-terminal domain is critically important for the binding [107,108]. The apparent dissociation constant is 1.4 μM at 36 °C, but increases to millimolar range at 65 °C (the optimal growth temperature for thermophilic *Bacillus PS3*) [108]. Similar binding, but with an apparent dissociation constant of 2 mM (at 25 °C), was recently observed for *Bacillus subtilis*, suggesting that the role of subunit ε as an ATP-sensor might be a common feature for ATP synthases from different organisms [109]. ATP binding might stabilize the contracted hairpin conformation of subunit ε and therefore serve as an auxiliary means to relieve inhibition. Bearing in mind the similarity of the second C-terminal helices of ε in enzymes form chloroplasts and from *Bacillus* genera bacteria (Fig. 2), it is most interesting to investigate if such binding takes place in the chloroplast enzyme. Further studies are also necessary to clarify the details and the role of such binding on the level of the whole enzyme. The properties of the isolated subunit ε might significantly differ from those of subunit ε within F_1 or F_OF_1.

We find probable that in bacterial and chloroplast ATP synthases both C-terminal helices of subunit ε might be involved in the inhibition, with the first helix providing a "basic" level of inhibition, and the second helix enhancing it. The latter effect might be absent in enzymes where prompt prevention of ATPase activity is less important (e.g., in bacteria where ATP synthase primary function is ATP-powered generation of $\Delta\tilde{\mu}^{+}_{H}$).

In chloroplast enzyme the inhibitory effect of subunit ε is intertwined with another regulatory mechanism absent in bacterial and mitochondrial ATP synthases that involves oxidation/reduction of a cysteine pair in subunit γ (reviewed in [110,111]). Oxidation of this cysteine pair results in formation of a S–S bond that results in a pronounced inhibition of both ATP synthesis and hydrolysis [112–114]. It was shown that this redox regulation is markedly influenced by subunit ε [102].

5. What causes subunit ε C-terminus transitions?

The data reviewed above indicate that the inhibitory effect of subunit ε is correlated to the large conformational transitions of its C-terminal α-helices from a hairpin, contracted conformation (Fig. 1A and C) to a partially (Fig. 1D) or fully extended state (Fig. 3). In this section, we address the causes of those transitions.

Several pieces of experimental evidence from the cross-linking experiments cited above indicated that ADP and ATP influence the conformation of subunit ε C-terminus. It was also reported that the conformational changes of the ε-subunit occur upon energization in chloroplasts [90,115,116].

The experiments done in our group on ATP synthase from thermophilic *Bacillus PS3* further clarified the role of these factors on the position of subunit ε C-terminus. It was shown by cross-linking that in the presence of ATP subunit ε adopts the contracted conformation, while ADP promotes an extended one [97]. Remarkably, $\Delta\tilde{\mu}_{H^+}$ also promoted extended conformation of the ε C-terminus. These results suggested that the two conformations of subunit ε might have different physiological roles, the extended being responsible for ATP synthesis and the contracted — for maintenance of $\Delta\tilde{\mu}_{H^+}$ at the expense of ATP hydrolysis. At the moment it is unclear if the conformation of subunit ε that causes inhibition of ATPase activity is the same as the conformation prevailing during ATP synthesis.

It is noteworthy that the cross-linking experiments require cautious interpretation because even if a certain enzyme conformation has a very low probability or lifetime, it still can be trapped by cross-linking in the minutes time scale. Therefore, experiments where only one possible cross link product are much less reliable than those where two or more different cross-linking products can

be formed. But even the latter might provide information irrelevant to the enzyme functioning if the efficiency or rate of the cross-linking formation varies significantly for different products.

To avoid these complications, we investigated the details of the ε C-terminal transitions by fluorescence resonance energy transfer (FRET) in the isolated F_1-portion of *Bacillus PS3* ATP synthase [108]. This technique allows to optically monitor the transitions in real time. The results confirmed that ATP induced the contracted conformation, while ADP favored the extended one. Moreover, it was also demonstrated that the contracted form is induced by non-hydrolysable ATP analogue AMP-PNP, and by ADP in the presence of azide or of aluminum fluoride.

The kinetics of the transition from extended to contracted conformation upon addition of ATP was relatively slow: the half-time was ~100 s at 30 °C and decreased to ~7 s at 45 °C. The reverse transition induced by quick transformation of ATP to ADP by hexokinase was more than one order of magnitude slower: the half-time was ~1300 s at 30 °C and decreased to ~130 s at 45 °C. Such high half-times indicate that the transitions do not occur during each catalytic cycle, but rather reflect a slow switching between the conformations. The ATPase activity of the F_1-portion was found to correlate with the conformation of subunit ε under all experimental conditions tested (different ATP concentration, temperature). The kinetics of subunit ε conformational transition was also similar to that of the change in ATPase activity.

It remains unclear how exactly does the nucleotide binding affect the conformation of subunit ε. The non-catalytic nucleotide binding sites are not critically important for conformational transitions of subunit ε, since the mutant enzyme deficient in these sites also exhibited similar behavior [108]. However, it is not determined whether direct binding of ATP to subunit ε or whether the overall conformation of the F_1 induced by nucleotide binding to catalytic sites plays a critical role in the C-terminus transitions. Noteworthy, it was shown in *Bacillus PS3* ATP synthase that when the fully-extended conformation of subunit ε is fixed by a cross-link, the enzyme loses the ability to bind ATP at the high affinity catalytic site, although the ADP binding is not affected [97]. Further studies are necessary to elucidate how exactly do ATP and ADP influence the position of subunit ε C-terminus.

A recent hypothesis suggested that the conformation of subunit ε might be determined by the direction of subunit γ rotation. It was proposed that rotation in hydrolysis direction induces the contracted conformation, while if rotation is stopped or reversed, the transition to the extended conformation occurs [117]. However, the data from the FRET experiments described above indicate that at least in *Bacillus PS3* enzyme on the level of F_1-portion ADP+ azide, aluminum fluoride, and non-hydrolysable ATP analogue AMP-PNP induce the contracted state [108], although under such conditions the rotation is inhibited.

It seems more probable that the position of subunit ε C-terminal domain is determined by overall F_1 conformation and by the angular position of γ rather than by the direction of its rotation.

It was shown in the single-molecule rotation experiments that there are at least two distinct sub-steps in the process of ATP hydrolysis [118,119]. One sub-step is related to ATP binding: after the release of ADP and P_i the enzyme "waits" for the next ATP to be bound to the empty site. This state corresponds to a certain angular position of subunit γ relative to the $\alpha_3\beta_3$-hexamer (traditionally designated as 0°). The dwell time for this state is dependent on the concentration of ATP [118]. Another sub-step is related to the ATP cleavage and probably to the release of ADP and P_i. This sub-step is preceded by "catalytic dwell" in the conformation with subunit γ rotated by 80° relative to the preceding "ATP waiting" conformation [120]. If ADP is present in the assay, the enzyme tends to be blocked in this 80°-position [119,121].

It seems most probable that these two distinct conformations (0° and 80°) are favoring different states of subunit ε C-terminus. The structure of the *E. coli* F_1 with subunit ε in the extended state [47] provides a striking evidence for this suggestion. If the structure of *E. coli* F_1 with extended ε is overlaid onto the structure of the mitochondrial F_1 inhibited by DCCD (contracted state of ε) using the backbone of β-subunits for alignment, it is clearly seen that the angular position of subunit ε is indeed shifted by ~80° [47] (see Fig. 4).

Single-molecule FRET experiments on *E. coli* enzyme incorporated into liposomes provided further support for this proposal. Real-time measurements of the distance between the fluorescent labels on N-terminal domain of subunit ε and on the *b*-dimer that composes the second stalk of the $\alpha_3\beta_3\delta ab_2$-stator revealed that under ATP synthesis/hydrolysis conditions there are three main positions of subunit ε (distances 4.6, 6.3 and 7.8 nm) [29]. However, in the inactive enzyme in the absence of ATP (with or without ADP and phosphate), these distances shifted to ~3.5, 6.4 and 8.3 nm. This might be in part due to the angular shift of the γε-complex from 0° to the 80° position. Electron microscopy studies and cross-linking experiments also support this speculation [45,122].

This favors the notion that not rotation direction, but overall conformation of the F_1-portion and the angular position of subunit γ in the $\alpha_3\beta_3$-hexamer are determining the conformation of ε. In turn, this position is defined by the nucleotide composition of the medium, by presence of inhibitors and by $\Delta\tilde{\mu}_{H^+}$. It is therefore tempting to suggest that under ATP synthesis conditions the conformation with angular position of γ corresponding to the

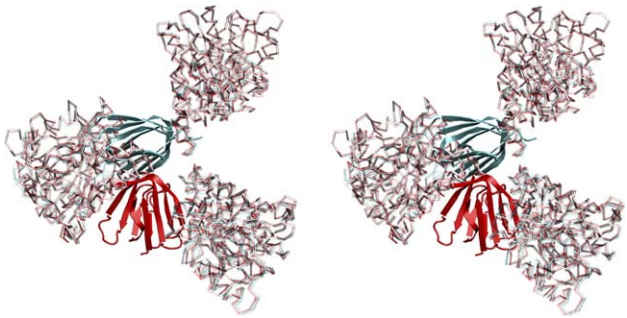

Fig. 4. Angular shift of subunit ε N-terminal domain relative to the $\alpha_3\beta_3$ symmetry axis. Structure of *E. coli* F_1 with subunit ε in the extended state (cyan, PDB entry 1JNV) overlapped with the structure of bovine mitochondrial F_1 (red, corresponding to the contracted state, PDB entry 1E79) using the Cα atoms of β -subunits for alignment. Subunits β are shown as semi-transparent backbone trace; the C-terminal α-helical region of subunit γ and the N-terminal β-sandwich domain of subunit ε are shown in cartoon representation. View from the "top" of F_1 towards F_O along the presumable rotation axis of subunit γ. The image was generated with VMD software package [156].

extended state of subunit ε prevails, while ATP hydrolysis favors the angular position seen in the structures of the mitochondrial F_1 (corresponding to contracted state). If this suggestion is correct, the direction of subunit γ rotation would indeed determine the conformation of subunit ε, but not directly (as has been proposed in [117]), rather then by driving the F_1 into one of the conformations mentioned above.

6. Possible roles of subunit ε C-terminus transitions and of inhibition by MgADP in the difference between ATP synthesis and hydrolysis pathways

The experimental evidence indicating that ATP hydrolysis is not a mere reversal of ATP synthesis reaction is numerous. The rate of ATP hydrolysis by chloroplast enzyme in the dark was found to be less than 5% of the synthesis rate upon illumination [123,124]. Later, it was pointed out by Junge that the forward and reverse reactions catalyzed by the chloroplast enzyme are not in the thermodynamic equilibrium; it was proposed that high $\Delta\tilde{\mu}^{+}_{H}$ was necessary to switch ATP synthase into active state [125,126]. Another argument supporting the different catalytic pathways is that some inhibitors that effectively block the ATPase activity are not inhibiting ATP synthesis [37,127,128].

The simplest explanation for the observed effect proposed in several references cited above is the inhibitory effect of MgADP. Indeed, inhibition by MgADP seems to be a common regulatory mechanism shared by ATP synthases from different organisms. When MgADP without phosphate is bound at the high affinity catalytic site under de-energized conditions, the enzyme is inactivated in terms of ATP hydrolysis [129–137]. Upon membrane energization the tightly bound MgADP is released from F_1, the enzyme switches to the active conformation and ATP synthesis starts [138–141]. The energy that drives this switching presumably comes from $\Delta\tilde{\mu}^{+}_{H}$ and is passed from F_O to $\alpha_3\beta_3\gamma$ via subunit γ rotation.

Such $\Delta\tilde{\mu}_{H^+}$-driven release of the inhibitory MgADP is the most probable cause of increased ATPase activity after temporary membrane energization documented for mitochondrial, chloroplast and bacterial enzymes (so-called "activation by protonmotive force") [140,142–147]. This hypothesis gained further support from single molecule experiments on the $\alpha_3\beta_3\gamma$-complex from *Bacillus PS3*. It was found that inhibition by ADP results in long pauses in the ATP-driven rotation of subunit γ [119]. Further experiments indicated that the re-activation can be achieved by forced rotation of subunit γ from the "ADP-inhibited" position by 40° in the hydrolysis direction; preliminary data indicate that rotation by 160° in the synthesis direction has the same effect [121]. Without forced rotation the spontaneous re-activation from the "ADP-inhibited" state is slowly induced by thermal rotational fluctuations of subunit γ; the average time necessary for such re-activation is ≈30 s (2 mM ATP, room temperature) [121].

The data summarized above suggest that inhibition by MgADP can indeed block ATP hydrolysis, but is readily relieved when $\Delta\tilde{\mu}_{H^+}$ increases and ATP synthesis starts.

The inhibitory effect propagated by subunit ε C-terminus is also anisotropic in a similar way: while ATP hydrolysis is markedly diminished, ATP synthesis rate remains largely unaffected [64,96,97]. It seems probable that the conformational transitions of subunit ε might also be responsible for the difference between the catalytic pathway of ATP synthesis and hydrolysis.

Measurements of the activation energies for ATP hydrolysis catalyzed by *E. coli* ATP synthase revealed that subunit ε significantly alters the catalytic pathway [82,87]. The induction of subunit ε C-terminus transitions by ATP, ADP and energization indicates that the conformation of ε is indeed different under ATP synthesis and hydrolysis conditions. The observation that ATP binding to the high-affinity catalytic site is prevented when ε is in the fully-extended state also strongly suggests that this subunit is involved in the switching between synthesis and hydrolysis [97]. Finally, it was demonstrated recently in our group that in *Bacillus PS3* ATP synthase the absence of subunit ε C-terminus markedly alters the conformations adopted by subunits β during ATP synthesis but not during ATP hydrolysis [69]. These results confirm that ATP synthesis cycle has different intermediate enzyme conformations than ATP hydrolysis and that the difference is dependent on the presence of subunit ε C-terminal domain.

It was also proposed that there might be interplay between the inhibition by ADP and by subunit ε. The latter was suggested to serve as a "safety lock", fixing the enzyme in the 80°-state by electrostatic interactions between βDELSEED and basic residues in the ε C-terminus [117]. Such interaction might indeed prevent the spontaneous recovery from ADP inhibition due to stochastic rotational fluctuations of subunit γ, but be readily broken by $\Delta\tilde{\mu}^{+}_{H}$-driven subunit γ rotation. Recent experiments on the effect of subunit ε on subunit γ rotation in the *E. coli* F_1-portion are also in line with this proposal. It was demonstrated that subunit ε increased the length of pauses during rotation by a factor of ~ 1.5 [148]. The effect was not observed with mutated subunit ε that has cytochrome b_{562} fused to its C-terminus. As the ATP concentration in these experiments was 2 mM and therefore was not limiting, the pauses probably occurred in the 80°-position corresponding to the "catalytic dwell" [120] (although a careful analysis of the effect at sub-micromolar ATP concentration is necessary to distinguish the catalytic dwell from "ATP waiting" dwell). It is plausible that the inhibitory interactions of the C-terminal domain of subunit ε take place in this angular position and therefore stabilize the ADP-inhibited state that also corresponds to the 80°-position [119,121].

So far there is no direct evidence for any kind of interplay between the inhibition by ADP and by subunit ε. Experiments that might shed light on that are currently underway in our group.

It should be noted that such interplay could provide the flexibility necessary to tune the basic regulation by inhibitory MgADP common in all ATP synthases to various conditions specific for each organism. Variation of the length and of the amino acid composition of the C-terminal domain of subunit ε might well result in different degree of inhibitory effect. This speculation is in good correspondence with the experimental results described above, with high variability of the C-terminal domain amino acid composition (Fig. 2), and with the very absence of this domain in some anaerobic bacteria where the

generation of $\Delta\tilde{\mu}_{H^+}$ is the main expected role of ATP synthase and inhibition of ATP hydrolysis is unfavorable.

7. Is the inhibition by subunit ε also present in the mitochondrial enzyme?

There is no evidence so far that conformational transitions similar to those of bacterial subunit ε C-terminus take place in the mitochondrial enzyme. Moreover, the conformation of the mitochondrial homologue of bacterial subunit ε might be fixed in the contracted state by an additional small protein that is absent in bacterial and chloroplast enzyme (mitochondrial ε).

There is also an additional regulatory mechanism found for mitochondrial ATP synthase only: inhibition by a special small protein IF_1 (reviewed in [149]). The X-ray crystallographic studies clarified the structure of IF_1–F_1 complex from bovine mitochondria [150]. It turned out that α-helical IF_1 N-terminus can penetrate into $\alpha_3\beta_3$-hexamer between α and β subunits near their C-terminal regions. It should be noted that the mitochondrial enzyme also has the highly conserved βDELSEED sequence, the only known function of which is the propagation of the subunit ε inhibitory effect. As the IF_1 is rich in basic amino acid residues, of which Arg25 and Arg32[5] are only ~6 Å and ~8 Å from the side-chain carboxyls of the βDELSEED in the crystal structure [150], it is likely that similar electrostatic interactions underlie the inhibitory properties of both subunit ε and IF_1. This is also in line with the results of earlier zero-length cross-linking experiments that demonstrated the proximity of IF_1 and βDELSEED in the IF_1–F_1 complex [151].

It remains unclear and is doubted by several research groups [96,150,152] if the mitochondrial homologue of bacterial subunit ε plays any role in the inhibition of the ATPase activity. The C-terminal region of the mitochondrial δ is not particularly basic (Fig. 2) and might be fixed in the contracted state by the mitochondrial ε. However, it was shown that the mitochondrial subunit ε is able to relieve the inhibition of F_1 by the mitochondrial IF_1 inhibitor [153], indicating that this region of the ATP synthase is not static in the mitochondrial enzyme as well.

8. Subunit F of V-type H^+-ATPase might have a function similar to that of ATP synthase subunit ε

It was recently demonstrated that subunit F of prokaryotic V-type ATPase/synthase from *Thermus thermophilus* undergoes large conformational transitions similar to those of bacterial ATP synthase subunit ε. The crystal structure, together with the single-molecule analysis using fluorescence resonance energy transfer, showed that the C-terminus of subunit F exhibits two conformations, a "retracted" form in the absence and an "extended" form in the presence of ATP [154]. Although there is neither sequence nor structure homology between subunit ε and V-type ATPase F-subunit, the results suggest that the roles of both subunits might be similar. However, there is no experimental evidence so far that subunit F has an inhibitory effect on the activity of V-type ATPase. On the contrary, it was shown to stimulate the ATPase activity by a factor of ~1.5 [155]. Ongoing experiments in our group might further clarify the role of subunit F in the vacuolar V-ATPase.

[5] Numeration given for bovine mitochondrial enzyme.

9. Conclusions

Subunit ε plays a dual function in the ATP synthase: it is necessary for coupling of catalysis to proton transport and it regulates the catalytic activity. The N-terminal β-sandwich domain of subunit ε seem to have structural role interconnecting the *c*-oligomer with subunit γ and is indispensable for coupling (and, therefore, normal activity), but does not play a significant role in the regulation. Both its amino acid sequence and its spatial structure are conserved among ATP synthases from bacteria chloroplasts and mitochondria.

The α-helical C-terminal domain seems to be involved in several regulatory features, but is strictly necessary neither for ATP synthesis nor for ATP-driven proton pumping.

In bacterial and chloroplast enzymes, it is responsible for inhibition of ATPase activity of both the F_1-portion and the whole F_OF_1-ATP synthase. The inhibition is at least partially caused by electrostatic interactions of the positively charged residues in the ε C-terminal domain with the negatively charged acid residues of the βDELSEED segment. A prerequisite for these interactions is a large conformational change of the C-terminal domain from a contracted hairpin-like structure that is in close proximity to the *c*-oligomer to the extended state in which the C-terminus of subunit ε reaches $\alpha_3\beta_3$ hexamer. The hairpin contracted state of subunit ε might also be important for efficient coupling of ATP hydrolysis to proton pumping. Direct binding of ATP to the C-terminal domain observed in some bacteria of *Bacillus* genera suggests that subunit ε might act as an ATP sensor.

It is probable that high variability of amino acid composition in the C-terminus of subunit ε is one of the factors that determine different regulation patterns of ATP synthases from different organisms.

The physiological role of inhibition mediated by subunit ε could be in preventing of wasteful ATP hydrolysis by F_1 that is unbound to F_O, or by F_OF_1 upon membrane de-energization. It is likely that in mitochondrial ATP synthase this role is passed to (or shared with) a special small inhibitory protein IF_1 and mitochondrial subunit ε.

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