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Regulatory Mechanisms of Proton-Translocating F_OF_1-ATP Synthase

Boris A. Feniouk[1,2] (✉) · Masasuke Yoshida[2,3] (✉)

[1] ATP System Project, Exploratory Research for Advanced Technology, Japan Science and Technology Corporation (JST), 5800-3 Nagatsuta, Midori-ku, 226-0026 Yokohama, Japan

[2] Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, 226-8503 Yokohama, Japan

[3] ICORP ATP-Synthesis Regulation Project (Japanese Science and Technology Agency), National Museum of Emerging Science and Innovation, 2-41 Aomi, Koto-ku, 135-0064 Tokyo, Japan
feniouk@atp.miraikan.jst.go.jp, myoshida@res.titech.ac.jp

Abstract H^+-F_OF_1-ATP synthase catalyzes synthesis of ATP from ADP and inorganic phosphate using the energy of transmembrane electrochemical potential difference of proton ($\Delta\tilde{\mu}_{H^+}$). The enzyme can also generate this potential difference by working as an ATP-driven proton pump. Several regulatory mechanisms are known to suppress the ATPase activity of F_OF_1:

1. Non-competitive inhibition by MgADP, a feature shared by F_OF_1 from bacteria, chloroplasts and mitochondria
2. Inhibition by subunit ε in chloroplast and bacterial enzyme
3. Inhibition upon oxidation of two cysteines in subunit γ in chloroplast F_OF_1
4. Inhibition by an additional regulatory protein (IF_1) in mitochondrial enzyme

In this review we summarize the information available on these regulatory mechanisms and discuss possible interplay between them.

1 Introduction

H^+-F_OF_1-ATP synthase (also known as F-type H^+-ATPase, or simply F_OF_1) is a multisubunit membrane enzyme. It synthesizes ATP from ADP and inorganic phosphate (P_i) using the energy of transmembrane electrochemical potential difference of proton ($\Delta\tilde{\mu}_{H^+}$). In Eukaryota the enzyme is found in mitochondrial inner membrane and in chloroplast thylakoid membrane; in bacteria F_OF_1 is located in the cytoplasmatic membrane.

The conditions under which the enzyme operates vary significantly between different organisms. In mitochondria the $\Delta\tilde{\mu}_{H^+}$ is constantly generated by respiratory chain enzymes and the chemical composition of the milieu on both sides of the coupling membrane is controlled by the cell, so the enzyme environment is more or less stable. In chloroplasts the $\Delta\tilde{\mu}_{H^+}$ is high during daytime, but during the night the membrane is de-energized so that

no ATP synthesis is possible. The pH on both sides of the thylakoid membrane also varies during the day–night cycle (see (Kramer et al. 1999) and references therein). In bacteria, the conditions are most variable; the cell has a very limited control over the chemical composition of the milieu on the periplasmatic side of the membrane, and the magnitude of $\Delta\tilde{\mu}_{H^+}$ may vary significantly in response to such factors as concentrations of oxygen, nutrients, ions (pH), temperature, etc.

The need to regulate the activity of ATP synthase, primarily to avoid ATPase activity upon decrease in $\Delta\tilde{\mu}_{H^+}$ that may result in wasteful ATP hydrolysis, is evident. Indeed, there are several regulatory features present in F_OF_1. This review summarizes the experimental data on these regulatory features and describes how a common catalytic core of the enzyme was tuned to the specific needs of different organisms.

2
Structure and Rotary Catalysis: a Brief Summary

2.1
Structure

Before proceeding to the regulation of F_OF_1, it is necessary to briefly outline the main structural and functional features of the enzyme.

The enzyme is composed of two distinct portions: membrane-embedded F_O and hydrophilic F_1 that protrudes by more than 100 Å from the membrane plane. Both portions are multisubunit complexes. The F_1 portion is involved in nucleotide and P_i binding/release, while the F_O portion is responsible for transmembrane proton transport. The two portions are connected by two "stalks", one of which is located approximately in the center, and the other is on the periphery of the enzyme (Fig. 2). The two portions can be separated (e.g. by sonication in the absence of Mg^{2+}) and reconstituted back. Isolated F_1 portion can hydrolyze ATP at high rate, and therefore is often named "F_1-ATPase"; isolated F_O portion performs passive proton transport downhill $\Delta\tilde{\mu}_{H^+}$.

The catalytic core of F_1 is capable of high rate ATP hydrolysis and is composed of three kinds of subunits in stoichiometry $\alpha_3\beta_3\gamma$. The structure was solved in 1994 for bovine enzyme by X-ray crystallography (Abrahams et al. 1994). Studies revealed that three $\alpha\beta$ pairs form a spherical hexamer with a cavity in the middle. The cavity is filled by part of the elongated γ subunit; the rest of subunit γ protrudes towards the membrane and composes the central stalk in F_OF_1. The primary structure of subunits α, β and γ is highly conserved in ATP synthases from various organisms. Biochemical data strongly indicate that the catalytic mechanism is also highly conserved.

There are six nucleotide-binding sites located in the clefts between subunits α and β (Abrahams et al. 1994). Only three of them are directly involved

in catalysis (Cross and Nalin 1982; Yoshida and Allison 1986) and reside mostly on β subunits; the other three are located mostly on α subunits and are probably involved in regulation of the enzyme.

Besides the $\alpha_3\beta_3\gamma$, there are other smaller subunits in F_1. One of them (named ε in bacterial and chloroplast enzyme, but δ in the mitochondrial F_OF_1) is part of the central stalk connecting F_O and F_1, and is indispensable for coupling between proton transport and ATP synthesis/hydrolysis. In bacterial and chloroplast enzyme this subunit also has regulatory functions, which are discussed in detail below (for a recent review see Feniouk et al. 2006).

The functional core of the F_O portion is composed of a ring-shaped oligomer of *c*-subunits, and of *a*-subunit located on the periphery of the *c*-ring. Subunit *c* is a small hairpin-like protein with two transmembrane helices and a short hydrophilic loop connecting them. Proton transport occurs on the interface of subunit *a* with the *c*-ring. The central stalk connecting F_O and F_1 is composed of subunits γ and ε that are bound to the *c*-ring. The second, peripheral stalk is composed of other subunits; their number, stoichiometry, and nomenclature differs among bacterial, chloroplast, and mitochondrial enzymes. However, the structure itself is quite similar – a complex with transmembrane helices bound to subunit *a*; a protruding long α-helical stretch reaching the very distant part of F_1 and attached to the latter in part directly, and in part through an additional small F_1 subunit (δ in bacteria/chloroplasts and oligomycin sensitivity-conferring protein, OSCP, in mitochondria).

2.2 Catalytic Mechanism

An enormous contribution to our understanding of the ATP synthase catalytic mechanism was made by Paul Boyer and colleagues. They have demonstrated that the energy-requiring step was not the chemical step of ATP synthesis, but the binding of P_i and the release of the tightly bound ATP from the enzyme (Boyer et al. 1973). Later they found that F_OF_1 showed a strong dependence of catalytic events and product(s) release at one site on the binding of substrate(s) at a second site (Kayalar et al. 1977). This general principle of highly cooperative multisite catalysis was later confirmed by lots of functional and structural evidence and is usually referred to as "binding change mechanism" (see Boyer 1997, 2002; Senior et al. 2002, and the references therein for details).

The molecular implementation of the binding change mechanism in F_OF_1 involves rotation of subunit γ inside the $\alpha_3\beta_3$ hexamer. Such a rotary mechanism was predicted from the structural data (Abrahams et al. 1994) and later got support from the biochemical (Duncan et al. 1995) and biophysical (Sabbert et al. 1996) studies. Finally, ATP-driven rotation of subunit γ was directly visualized in the $\alpha_3\beta_3\gamma$ complex from *Bacillus* PS3 in single-molecule experiments (Noji et al. 1997). More single molecule data followed, demonstrating

ATP-driven rotation in F_OF_1 that was sensitive to the F_O-inhibitor tributyltin (Ueno et al. 2005), and ATP synthesis driven by mechanical rotation of subunit γ in immobilized F_1 (Itoh et al. 2004; Rondelez et al. 2005). The results of single-molecule FRET experiments with *E. coli* F_OF_1 incorporated into liposomes suggested that rotation of subunit γ also occurs during ATP synthesis driven by artificially imposed $\Delta\tilde{\mu}_{H^+}$ (Diez et al. 2004; Zimmermann et al. 2005).

Combination of the data from single-molecule experiments with structural information from X-ray crystallographic studies allowed reconstruction of a rather detailed molecular mechanism of ATP hydrolysis in isolated F_1. Hydrolysis of one ATP molecule drives a 120°-unit rotation of subunit γ and, therefore, hydrolysis of three ATP molecules is required for the one complete 360° revolution (Yasuda et al. 1998). Analysis of rotation with a high speed camera (Yasuda et al. 1998; Shimabukuro et al. 2003), a slow-hydrolysis mutant F_1 (Shimabukuro et al. 2003; Nishizaka et al. 2004), and direct observation of binding/release of fluorescently labeled nucleotide during rotation (Nishizaka et al. 2004) suggest the following reaction sequence as a plausible model (Fig. 1; see Adachi et al. 2007; Ariga et al. 2007 for more details). Three β subunits are designated as β_I, β_{II}, and β_{III}. When F_1 is waiting for ATP, it is assumed that the catalytic sites of β_I, β_{II}, and β_{III} contain none, ATP, and ADP/P_i, respectively (states I and V in Fig. 1). The angular position of the subunit γ in this state is set to be 0°:

1. ATP binds to an empty catalytic site of β_I (Fig. 1, transition I → II).
2. Binding induces an 80° rotation of subunit γ. This rotation leads to simultaneous release of ADP from the catalytic site of β_{III} (Fig. 1, transition II→III).
3. Two catalytic events, each with a lifetime of ~1 ms, occur at the 80° position. One of these is hydrolytic cleavage of ATP into ADP and P_i at a catalytic site of β_{II} (state III in Fig. 1). The other event is not known but we assume it to be P_i release from β_{III} (state IV in Fig. 1). The order of the two events is not determined (in Fig. 1 ATP hydrolysis precedes P_i release, but the opposite event sequence is also probable).
4. A 40° rotation occurs to complete one 120° rotation (Fig. 1, transition IV → V). ATP binds to the newly emptied catalytic site of β_{III}, and the cycle repeats.

In this model, all three β subunits participate to drive a 120° rotation (active β subunits are marked as filled in the cartoon representation of Fig. 1), and catalytic turnover of one particular ATP molecule needs 360° rotation; the events on β_I are ATP-binding at 0°, ATP-cleavage at 200°, ADP-release at 240°–320°, and P_i release at 320°. Recent crystal structure of yeast F_1 with two catalytic sites occupied by AMPPNP and one occupied with P_i (Kabaleeswaran et al. 2006) may represent state III in Fig. 1, blocked on the level of ATP hydrolysis in β_{II}.

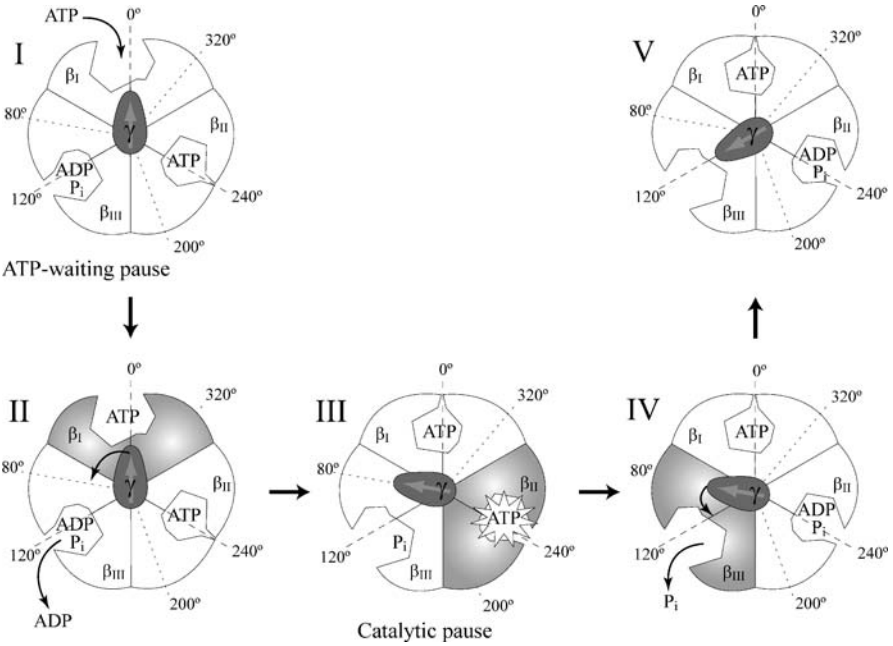

Fig. 1 Hypothetical catalytic mechanism of rotary ATP hydrolysis. F_1 is depicted as seen from the membrane; only the three catalytic nucleotide binding sites are shown. The *filled* αβ-pairs represent the power stroke step that presumably drives subunit γ rotation. Only 1/3 of full γ subunit revolution corresponding to hydrolysis of one particular ATP molecule is shown; state *V* is identical to state *I* (just rotated by 120°). See details in text (Sect. 2.2)

In the whole F_OF_1, subunit γ is bound to the ring-shaped oligomer of *c*-subunits. In the case of ATP synthesis the proton flow driven by $\Delta\tilde{\mu}_{H^+}$ powers the rotation of the *c*-ring with subunit γ (and with subunit ε in bacterial and chloroplast F_OF_1, or with δε complex in the mitochondrial F_OF_1) relative to other subunits. This rotation induces the cyclic conformational changes of the catalytic sites on F_1 that result in ATP synthesis. Although hypothetical mechanisms of proton translocation and torque generation by F_O were proposed (Junge et al. 1997; Vik et al. 1998), the experimental evidence supporting them is still insufficient. It is likely that F_O operates as an entropic machine, as proposed by Junge and collaborators (Junge et al. 1997). This model and its later modifications (Dimroth et al. 1998; Elston et al. 1998) correspond well to the experimental data. A detailed study on *Rhodobacter capsulatus* membranes confirmed that the rotary model can quantitatively describe the proton transport through isolated F_O (Feniouk et al. 2004).

The coupling between the F_O and F_1 is rather tight. For example, DCCD (*N*,*N*-dicyclohexylcarbodiimide), a specific inhibitor of F_O, blocks > 75% ATPase activity of F_OF_1 from *E. coli* (Fillingame 1975) or *Bacillus* PS3 (Suzuki

et al. 2002); an even higher degree of inhibition is observed in other organisms. No detectable proton leak was observed through *Rb. capsulatus* F_OF_1 in the presence of $\Delta\tilde{\mu}_{H^+}$ under conditions where the F_1 portion was blocked, e.g., by specific inhibitors (Feniouk et al. 2001) or in the absence of nucleotides in the medium (Feniouk et al. 2005).

Such tight coupling ensures that factors affecting the proton transport function of the enzyme also affect the ATP synthesis/hydrolysis and vice versa.

3
ADP-Inhibition: a Common Regulatory Mechanism

As mentioned above, ATP synthase is capable of both $\Delta\tilde{\mu}_{H^+}$-driven ATP synthesis and ATP-driven $\Delta\tilde{\mu}_{H^+}$ generation. In mitochondria, chloroplasts, and aerobic/photosynthetic bacteria the former activity is primary (but see Matsuyama et al. 1998; St Pierre et al. 2000; Lefebvre-Legendre et al. 2003, for

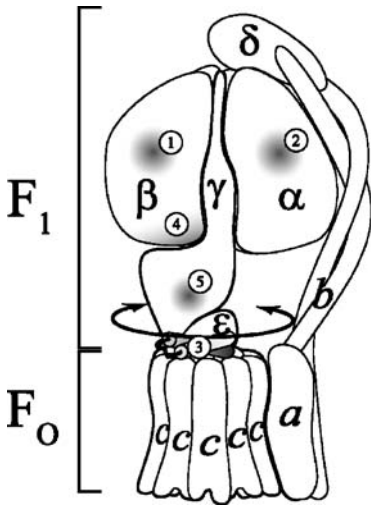

Fig. 2 Cartoon representation of bacterial/chloroplast F_OF_1. Zones involved in regulation are marked:

1 Catalytic sites occlude MgADP without P_i and the enzyme lapses into ADP-inhibited state (Sect. 3)
2 Binding of ATP or pyrophosphate to non-catalytic sites counteracts ADP-inhibition (Sect. 3.2)
3 Subunit ε C-terminal α-helical domain is responsible for inhibition of ATPase activity (Sect. 4)
4 Acid residues of βDELSEED are involved in inhibition exerted by subunit ε C-terminal domain (Sect. 4)
5 In chloroplast F_OF_1 oxidation/reduction of a special cysteine pair modulates the enzyme activity (Sect. 5)

the importance of the reverse activity in mitochondria). The universal way to modulate the ATP synthesis activity is by changing the magnitude of $\Delta\tilde{\mu}_{H^+}$. It is well documented that $\Delta\tilde{\mu}_{H^+}$ above a certain thermodynamic threshold is necessary for ATP synthesis, and that further increase in $\Delta\tilde{\mu}_{H^+}$ results in acceleration of ATP production (Graber and Witt 1976; Slooten and Vandenbranden 1989; Junesch and Graber 1991; Turina et al. 1991; Pitard et al. 1996). Therefore, regulation of ATP synthesis activity can be achieved via regulation of $\Delta\tilde{\mu}_{H^+}$ magnitude either by modulation of respiratory/photosynthetic $\Delta\tilde{\mu}_{H^+}$-generating protein complexes or by changing the proton permeability of the membrane.

There are several regulatory mechanisms controlling ATP hydrolysis (Fig. 2); most of them are aimed at blocking the ATPase activity of F_OF_1 upon decrease in $\Delta\tilde{\mu}_{H^+}$, decrease in ATP concentration, or decrease in the ATP/ADP ratio. This is hardly surprising for aerobic/photosynthetic organisms, where such mechanisms are essential to protect the cellular ATP pool from wasting upon membrane de-energization. However, in many bacteria the primary function of F_OF_1 is ATP-driven proton pumping that provides $\Delta\tilde{\mu}_{H^+}$ necessary for ion transport, flagella rotation, and other vital processes. Nevertheless, certain regulatory features limiting the ATPase activity of F_OF_1 are present in these organisms as well.

3.1 Mechanism of ADP-Inhibition

One of the most well-known unidirectional regulatory factors influencing the activity of F_OF_1 is MgADP: it not only serves as a substrate for ATP synthesis, but also inhibits ATPase activity of the enzyme in a non-competitive manner. Such inhibition (denoted hereafter as "ADP-inhibition") is described for F_OF_1 from chloroplasts (Carmeli and Lifshitz 1972; Dunham and Selman 1981b; Feldman and Boyer 1985; Zhou et al. 1988; Creczynski-Pasa and Graber 1994), mitochondria (Minkov et al. 1979; Fitin et al. 1979; Roveri et al. 1980; Drobinskaya et al. 1985), and bacteria (Yoshida and Allison 1983; Hyndman et al. 1994), and is clearly distinct from simple product inhibition. It is observed not only in the whole enzyme or F_1-portion, but also in the $\alpha_3\beta_3\gamma$ complex (Jault et al. 1995; Hirono-Hara et al. 2001), indicating that this regulatory feature is embedded in the very catalytic core of F_1.

Numerous biochemical studies indicate that ADP-inhibition is caused by tight binding of MgADP without P_i at a high-affinity catalytic site (Minkov et al. 1979; Fitin et al. 1979; Smith et al. 1983; Drobinskaya et al. 1985; Milgrom and Boyer 1990; Hyndman et al. 1994). It is noteworthy that the presence of ADP without P_i in the tight binding catalytic site is not inhibitory by itself, but is a prerequisite for slow transition into the ADP-inhibited state, which probably includes an additional conformational change that is affected by Mg^{2+} (Bulygin and Vinogradov 1991).

Single-molecule experiments on $\alpha_3\beta_3\gamma$ complex from *Bacillus* PS3 revealed that ADP-inhibition results in long pauses in ATP-driven rotation of subunit γ (Hirono-Hara et al. 2001). These pauses occur with subunit γ blocked in the angular position of 80° relative to the "ATP-waiting" state. Spontaneous re-activation occurs in the tens of seconds time scale, but was completely abolished if the angular position of subunit γ was fixed at 80° by external forces. Therefore, it was proposed that spontaneous activation is due to stochastic rotational fluctuations of subunit γ. This proposal was strongly supported by the finding that forced rotation of subunit γ by $> 40°$ in the hydrolysis direction relieved ADP-inhibition (Hirono-Hara et al. 2005). Numerous experimental studies on F_OF_1 from various organisms demonstrated that the tightly bound inhibitory ADP can be expelled by $\Delta\tilde{\mu}_{H^+}$ (Strotmann et al. 1976; Graber et al. 1977; Shoshan and Selman 1979; Sherman and Wimmer 1984; Creczynski-Pasa and Graber 1994; Feniouk et al. 2005). This phenomenon underlies the so-called "activation by $\Delta\tilde{\mu}_{H^+}$", or increase in the ATPase activity of the enzyme after brief membrane energization (Carmeli and Lifshitz 1972; Baltscheffsky and Lundin 1979; Turina et al. 1992; Galkin and Vinogradov 1999; Fischer et al. 2000; Zharova and Vinogradov 2004). In view of the single-molecule data, it is conceivable that such activation is caused by $\Delta\tilde{\mu}_{H^+}$-driven rotation of the γ subunit (see below for a detailed discussion).

ADP-inhibition is likely to be a common feature of all ATP synthases. However, there are many factors that influence ADP-inhibition. As a result, the ATPase activity of F_OF_1 is finely regulated to match the needs of different cells at various physiological conditions.

3.2 Factors Affecting ADP-Inhibition

Phosphate

The role of P_i in the regulation of F_OF_1, as well as the details of P_i binding/release during catalysis, has many unclear aspects. In a pioneer study on P_i binding it was revealed that the mitochondrial F_1 (with ADP bound at a catalytic site and two nucleotides in the non-catalytic sites) reversibly binds a single P_i anion with a high affinity (K_d of 80 μM) (Penefsky 1977). Many factors such as pH, Mg^{2+}, inorganic anions, and nucleotides affected the binding. It was also documented that nucleotide-free mitochondrial F_1 binds P_i poorly, and that binding of P_i requires the presence of tightly bound ADP in the same catalytic site (Kozlov and Vulfson 1985).

There are two points concerning the data above. First, during normal catalysis P_i is likely to be bound/released at an open, not high affinity catalytic site. Second, in a living cell the enzyme is always in the medium with a millimolar concentration of nucleotides. Therefore, the measurements of P_i binding

in the presence of ADP and ATP are more physiologically relevant. In the case of mitochondrial F_1, 150 μM of each nucleotide inhibited the high affinity P_i binding by approximately 50% (Penefsky 1977). It is worthy of note that non-hydrolyzable ATP analog AMP-PNP was a markedly stronger inhibitor of P_i binding, confirming that P_i was bound in the position where the γ-phosphate of ATP resides.

However, a detailed study of mitochondrial F_1 revealed that there is a second binding site for P_i with K_d of ~5 mM (Kasahara and Penefsky 1978). Recently Penefsky confirmed that *E. coli* F_1 also has two P_i-binding sites with K_d in the range of 0.1 mM (Penefsky 2005). This result contradicts the earlier failure to observe P_i binding to *E. coli* enzyme (al Shawi and Senior 1992) and was supposedly due to a rapid dissociation of the bound P_i during the the centrifuge column separation procedure. Studies of chloroplast F_OF_1 incorporated into liposomes also provided evidence for existence of two P_i binding sites on the enzyme (Grotjohann and Graber 2002).

Until recently the high resolution structures of F_1 solved by X-ray crystallography have not revealed any bound P_i. However, a short time ago Walker's group solved the X-ray structure of yeast F_1 that has a phosphate (or sulfate) bound at an "empty" catalytic site. The location of the anion is close to the expected position of ATP γ-phosphate, indicating that P_i might be bound in the empty catalytic site (Kabaleeswaran et al. 2006).

As a substrate of ATP synthesis, P_i was demonstrated to have K_m in the range 0.2–10 mM in enzymes from various sources (Kayalar et al. 1976; Hatefi et al. 1982; McCarthy and Ferguson 1983; Junge 1987; Strotmann et al. 1990; Perez and Ferguson 1990a,b; Richard et al. 1995; al Shawi et al. 1997; Etzold et al. 1997; Grotjohann and Graber 2002; Tomashek et al. 2003). However, P_i in millimolar concentrations does not significantly inhibit the ATPase activity of the enzyme, suggesting that the affinity to P_i is different for ATP synthesis and for uncoupled ATP hydrolysis. Indeed, the affinity of F_OF_1 to P_i is strongly enhanced in the presence of $\Delta\tilde{\mu}_{H^+}$ (Kayalar et al. 1976; Hatefi et al. 1982; McCarthy and Ferguson 1983; al Shawi et al. 1997), in line with the suggestion of Boyer et al. that binding of P_i is one of the main energy-requiring steps during ATP synthesis (Rosing et al. 1977; Rosen et al. 1979).

Interestingly, many experimental studies documented a higher ATPase activity of F_OF_1 in the presence of P_i (Carmeli and Lifshitz, 1972; Melandri et al. 1975; Moyle and Mitchell 1975; Dunham and Selman 1981a; Turina et al. 1992; Zharova and Vinogradov 2004). A pioneering study by Carmeli and Lifshitz on chloroplast F_OF_1 provided evidence that such an increase occurs because P_i counteracts ADP-inhibition (Carmeli and Lifshitz 1972). Later, it was found that P_i also relieves ADP-inhibition in isolated mitochondrial (Drobinskaya et al. 1985; Kalashnikova et al. 1988) and bacterial (Bald et al. 1999; Mitome et al. 2002) F_1, although the concentration of P_i necessary to relieve inhibition was rather high: > 20 mM for *Bacillus* PS3 (Mitome et al. 2002) and > 5 mM for the mitochondrial F_1 (Drobinskaya et al. 1985).

The mechanism of such inhibition relief is not completely clear. It is likely that the presence of P_i in the same site where ADP is bound prevents conformational transition to the ADP-inhibited state. Indeed, it has been demonstrated that in the high-affinity catalytic site ATP is in equilibrium with ADP+P_i, so if P_i can bind to the high-affinity site having ADP, it is expected to keep the enzyme in the active state.

It should be noted that the experimental evidence available is insufficient to determine if P_i can facilitate the re-activation of the enzyme once it has lapsed into ADP-inhibited form, or if P_i only prevents ADP-inhibition of the active enzyme. We find the latter possibility more likely, since in the case of mitochondrial F_1 the P_i concentration necessary to relieve ADP-inhibition (5 mM) matched the experimentally estimated affinity of the second P_i-binding site (Kasahara and Penefsky 1978), which is distinct from the high-affinity catalytic site.

Binding of Nucleotides or Pyrophosphate to Non-catalytic Sites

As mentioned above, there are six nucleotide-binding sites on F_1. Three of them can rapidly exchange nucleotides with the medium, while the other three exhibit slow nucleotide exchange rates, and were named "non-catalytic sites" (Cross and Nalin 1982). The details of nucleotide/pyrophosphate binding to the non-catalytic sites are not completely clear. Early studies have revealed that in mitochondrial F_1 all three non-catalytic sites can be occupied with ATP (Kironde and Cross 1987). The crystal structure confirmed this finding showing AMP-PNP (an ATP analog) in all non-catalytic sites (Abrahams et al. 1994). Experiments with chloroplast F_1 (activated by heat treatment at 60 °C, since the non-activated chloroplast F_1 has almost no ATPase activity) also indicated that all three sites can be filled with ATP, but that ADP is able to fill only two (Milgrom et al. 1991). Several other studies have pointed out that the three non-catalytic sites differ in their binding properties. Experiments with nucleotide-depleted *E. coli* enzyme indicated that F_1 binds a maximum of two ATP, ADP, or GTP molecules at non-catalytic sites, whereas all three sites can be occupied only by a mixture of nucleotide di- and triphosphates (Hyndman et al. 1994). However, a study by Weber and coworkers on the mutant *E. coli* F_1 yielded occupancy of 2.8 and 2.6 non-catalytic sites by MgATP and MgADP, respectively (Weber et al. 1994). In chloroplast F_1 that was not heat-treated, one non-catalytic site was found to tightly bind ADP, while the other two could bind both ADP and ATP, albeit with different affinities (Malyan and Allison 2002). The dissociation of ADP from the latter two sites was much faster than that of ATP.

In chloroplasts, binding of F_1 to F_O was demonstrated to significantly modify the nucleotide occupancy of the non-catalytic sites, decreasing the ATP/ADP ratio for bound nucleotides (Malyan, 2006). Magnesium ions were

also found to influence the nucleotide binding to the non-catalytic sites (Weber et al. 1994; Malyan 2005).

Experimental studies revealed that the occupancy of the non-catalytic sites has a marked effect on the activity of F_OF_1. It was demonstrated on isolated F_1 from mitochondria, chloroplasts, and *Bacillus* PS3 that binding of ATP to these sites stimulates the ATPase activity of the enzyme (Milgrom et al. 1990; Jault and Allison 1993; Jault et al. 1995). This stimulation is due to attenuation of ADP-inhibition: binding of ATP to the non-catalytic sites facilitates the release of the inhibitory ADP from the high-affinity catalytic site (Muratalievand Boyer 1992; Milgrom and Cross 1993; Jault et al. 1995). Binding of pyrophosphate to the non-catalytic sites has a similar effect (Kalashnikova et al. 1988; Jault et al. 1994). In contrast to ATP and pyrophosphate, ADP was demonstrated to promote hysteretic inhibition of mitochondrial F_1 when bound to non-catalytic sites, presumably by blocking the binding of ATP to these sites and thereby preventing the activation mentioned above (Jault and Allison 1994).

$\Delta\tilde{\mu}_{H^+}$

Corresponding to thermodynamic considerations, in well-coupled membranes $\Delta\tilde{\mu}_{H^+}$ acts as a back-pressure that limits the rate of ATP hydrolysis catalyzed by F_OF_1. This effect is documented in many experimental studies demonstrating stimulation of ATPase activity by uncouplers. But, $\Delta\tilde{\mu}_{H^+}$ is also known to stimulate ATP hydrolysis by F_OF_1. This phenomenon was first documented in chloroplasts, where the enzyme has only traces of ATPase activity (albeit competent in ATP synthesis) (Jagendorf and Avron 1958; Avron and Jagendorf 1959), but can be activated by $\Delta\tilde{\mu}_{H^+}$ (Kaplan et al. 1967; Schwartz 1968; Carmeli and Avron 1972; Bakker-Grunwald and Van Dam 1974; Smith et al. 1976; Komatsu-Takaki 1986). A similar increase in the ATPase activity induced by $\Delta\tilde{\mu}_{H^+}$ was also documented for mitochondrial and bacterial enzymes (Turina et al. 1992; Galkin and Vinogradov 1999; Fischer et al. 2000; Pacheco-Moises et al. 2000; Zharova and Vinogradov 2004).

Stimulation of F_OF_1 ATPase activity by $\Delta\tilde{\mu}_{H^+}$ combines two distinct phenomena. First, $\Delta\tilde{\mu}_{H^+}$ promotes the release of the tightly bound ADP from the enzyme (Strotmann et al. 1976; Graber et al. 1977; Sherman and Wimmer 1984; Feniouk et al. 2005) and therefore relieves ADP-inhibition (Sherman and Wimmer 1984; Zharova and Vinogradov 2004). In view of the single-molecule experiments described in Sect. 3.1, it is highly conceivable that the enzyme is relieved from ADP-inhibition by $\Delta\tilde{\mu}_{H^+}$-powered rotation of subunit γ. Second, the steady-state ATPase activity is also stimulated by $\Delta\tilde{\mu}_{H^+}$ (Turina et al. 1992; Zharova and Vinogradov 2004; Feniouk et al. 2007), although this phenomenon is partially masked by suppression of ATP hydrolysis by $\Delta\tilde{\mu}_{H^+}$ back-pressure. Interestingly, the latter stimulation (unlike

$\Delta\tilde{\mu}_{H^+}$-driven release of inhibitory ADP) is observed only in the presence of P_i (Zharova and Vinogradov 2004; Feniouk et al. 2007). In a recent study we investigated this phenomenon and found that the P_i-dependent stimulation of the steady-state ATPase activity by $\Delta\tilde{\mu}_{H^+}$ in F_OF_1 from *Bacillus* PS3 is due to relief of ADP-inhibition (Feniouk et al. 2007). It is likely that such stimulation occurs because $\Delta\tilde{\mu}_{H^+}$ induces an increase in the affinity of F_OF_1 to P_i (Kayalar et al. 1976; Hatefi et al. 1982; McCarthy and Ferguson 1983; al Shawi et al. 1997). In turn, P_i binding protects the enzyme from ADP-inhibition, as described above. A scheme illustrating such regulatory interplay between ADP-inhibition, $\Delta\tilde{\mu}_{H^+}$, and P_i (and other factors discussed below) is presented in Fig. 3.

As already mentioned, a prerequisite for ADP-inhibition is ADP bound at a high-affinity catalytic site without P_i (**D**-state in Fig. 3). Because the order of ATP hydrolysis product release is unclear, we include both possible pathways for ADP and P_i liberation from a catalytic site: **DP** → **D** → **O** and **DP** → **P** → **O**. In the latter pathway ADP-inhibition requires binding of ADP to the opened site, since the **D**-state does not occur.

A high P_i concentration or increased affinity of the enzyme to P_i caused by $\Delta\tilde{\mu}_{H^+}$ can increase the rate of ATP hydrolysis by increasing the probability of the **DP**→**P** → **O** transition that excludes transition to the **DI** state. P_i binding to the **D** state (in the case of both high and low affinity catalytic sites) is expected to accelerate the **D** → **DP** transition and therefore also prevent the enzyme from lapsing into the **DI** state.

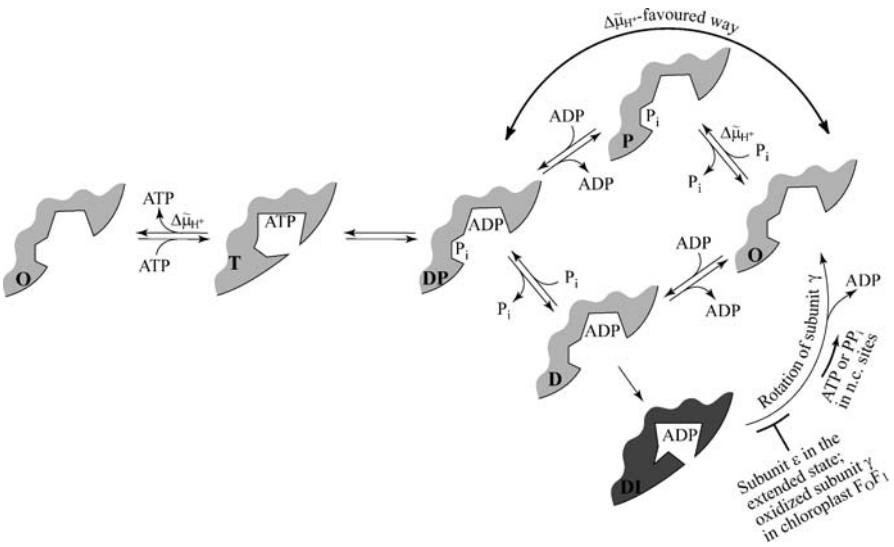

Fig. 3 Scheme of ATP hydrolysis regulation for bacterial/chloroplast F_OF_1 (extended from Feniouk et al. 2007) See text for details (Sect. 3.2)

If ATP is the nucleotide bound to the empty site after release of ADP and P_i, then ATP hydrolysis proceeds. However, binding of ADP (**O** → **D** transition) might lead to ADP inhibition. Again, a high concentration of P_i or an increased affinity to P_i diminishes the probability of the **O** → **D**-transition (and therefore, of ADP inhibition) by biasing the reaction towards the **O** → **P**-transition.

It is probable that ATP or pyrophosphate binding to the non-catalytic (n.c.) sites might destabilize the ADP-inhibited state. Structurally such destabilization might be achieved by facilitating the rotation of subunit γ inside the $\alpha_3\beta_3$ hexamer. Further studies are necessary to clarify this point.

Extending this rationale, one could presume that factors stabilizing the angular position of subunit γ corresponding to the ADP-inhibited state would enhance ADP-inhibition. Below we discuss such factors in detail.

Subunit ϵ (in Bacterial and Chloroplast F_OF_1)

It was proposed by Feniouk and Junge that in the bacterial and chloroplast F_OF_1 the ADP-inhibition might be enhanced by subunit ε (Feniouk and Junge 2005), which is part of the central stalk in F_OF_1 (see below for details). Single-molecule experiments on cyanobacterial F_1 confirmed that subunit ε blocks the rotation of subunit γ at the same angular position as ADP inhibition does (Konno et al. 2006). Biochemical studies on the F_OF_1 from *Bacillus* PS3 also confirmed that ADP-inhibition is enhanced by ε, presumably because the latter subunit stabilizes the ADP-inhibited state (Feniouk et al. 2007). However, subunit ε affects the ATPase activity of F_OF_1 also in the absence of ADP, so we have summarized the data on the inhibitory role of this subunit in Sect. 4.

4
Subunit ϵ in Bacterial and Chloroplast Enzyme

4.1
Structure of Subunit ϵ

Subunit ε (subunit δ in mitochondrial F_OF_1) is a small protein consisting of the N-terminal β-sandwich domain and the C-terminal domain composed of two α-helices. Structural NMR and X-ray studies revealed that in *E. coli* subunit ε the two C-terminal helices form a hairpin (Wilkens et al. 1995; Uhlin et al. 1997). The location of the subunit within F_1 was also determined in a high-resolution X-ray structure of bovine mitochondrial F_1 (Gibbons et al. 2000). The latter structure demonstrated a striking similarity in the fold of *E. coli* subunit ε and its homolog in bovine mitochondrial F_OF_1 (subunit δ).

Subunit ε plays a dual role in F_OF_1 from bacteria and chloroplasts (for reviews see Capaldi and Schulenberg 2000; Vik 2000; Feniouk et al.

2006). On one hand, subunit ε is indispensable for coupling between proton translocation though F_O and ATP synthesis/hydrolysis in F_1. On the other hand, subunit ε has a regulatory role inhibiting the ATPase activity of the enzyme. These two functions are structurally separated: the N-terminal β-sandwich domain is responsible for the coupling function, while the C-terminal α-helical domain is responsible for inhibition of ATP hydrolysis (but see Cipriano and Dunn 2006, for some evidence on the influence of the C-terminal domain on coupling efficiency in *E. coli* F_OF_1). In this review we discuss only the inhibitory function of subunit ε. We would also like to emphasize that there is no sound evidence for a similar regulatory role of mitochondrial F_OF_1 subunit δ (homologous to the bacterial/chloroplast ε). It is therefore likely that this regulatory feature is present exclusively in bacterial and chloroplast F_OF_1.

4.2 Inhibition of ATP Hydrolysis by Subunit ϵ

In 1972 Nelson et al. reported that subunit ε inhibits ATP hydrolysis in chloroplast F_1 (Nelson et al. 1972). Later, a similar inhibitory effect was documented (Smith et al. 1975) and studied in detail (Smith and Sternweis 1977; Laget and Smith 1979) on *E. coli* F_1. The possibility of performing mutagenesis makes bacteria a powerful experimental system for studies of protein function, and most of the data on subunit ε inhibitory role come from studies on *E. coli* or *Bacillus* PS3 F_OF_1.

It was revealed that the inhibitory effect of bacterial subunit ε is lost upon truncation of its C-terminal domain ($\varepsilon^{\Delta C}$-mutant) (Kuki et al. 1988; Keis et al. 2006; Cipriano and Dunn 2006). However, the details of the inhibitory effect vary among different species. In *E. coli* $F_OF_1\varepsilon^{\Delta C}$ mutation leads to 1.5-fold increase in the ATP hydrolysis rate, and the inhibitory effect is constant in the ATP concentration range from 50 μM to 5 mM (Cipriano and Dunn 2006). Markedly stronger stimulation was observed in $\varepsilon^{\Delta C}$-mutant enzyme from *Bacillus* PS3 (Kato-Yamada et al. 1999): at 50 μM ATP the activity is more than fourfold higher in the mutant. However, at 2 mM ATP the steady-state activity was the same in the $\varepsilon^{\Delta C}$-mutant and in the wild-type enzyme (but the initial lag in the onset of ATPase activity present in the wild type was lacking in the mutant). In F_OF_1 from thermoalkaliphilic *Bacillus* TA2.A1 the inhibition was also dependent on ATP concentration and decreased from a factor of seven at 50 μM ATP to ~three at 2 mM ATP (Keis et al. 2006). These findings indicate that there is a pronounced difference between the inhibitory effects of subunit ε in different bacteria.

In chloroplast enzyme the inhibitory effect of subunit ε C-terminal domain is very strong: at 5 mM ATP the ATPase activity of $\varepsilon^{\Delta C}$-F_OF_1 in spinach thylakoids was more than sixfold higher than that of the wild-type enzyme (Nowak and McCarty 2004).

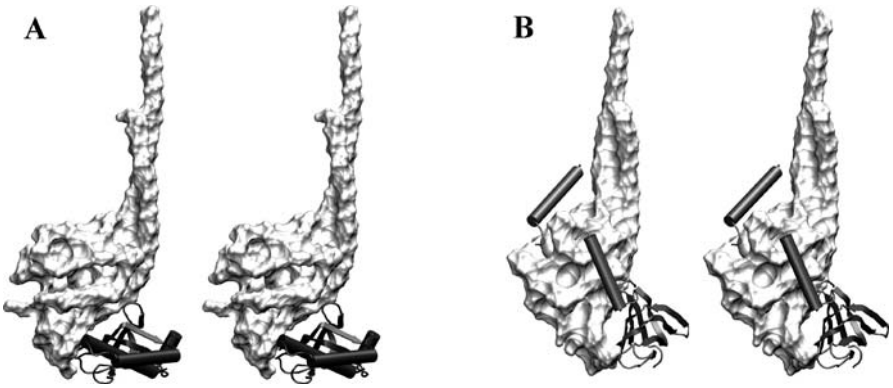

Fig. 4 Two conformations of bacterial F_OF_1 subunit ε C-terminal domain (stereopairs): *A* Contracted hairpin state (bovine mitochondrial F_1, coordinates from PDB entry 1E79). *B* Extended state (*E. coli* F_1, PDB entry 1JNV). The backbone of subunit γ is shown in surface representation (colored *light gray*); subunit ε (mitochondrial δ) is shown in cartoon representation (colored *dark gray*). The image was generated with VMD software package (Humphrey et al. 1996)

4.3 Conformational Transitions of Subunit ϵ C-Terminal Domain

An important advance in the understanding of the molecular mechanism of the inhibitory effect of subunit ε was initiated by a publication reporting the structure of the γε complex from *E. coli* F_OF_1 (Rodgers and Wilce 2000). In this structure the α-helices of subunit ε C-terminus were not folded in a hairpin (A in Fig. 4), but were stretched along subunit γ towards the $\alpha_3\beta_3$ hexamer (B in Fig. 4). The existence of such conformation in the whole F_OF_1 was later confirmed by Tsunoda et al. in cross-linking experiments (Tsunoda et al. 2001).

Similar cross-linking experiments performed in our group demonstrated that in *Bacillus* PS3 F_OF_1 the C-terminus of subunit ε can be stretched even further, reaching the N-terminus of subunit γ (Suzuki et al. 2003). Moreover, it was revealed that in the mutant where both the extended and the contracted hairpin conformations of subunit ε C-terminus could be fixed by a cross-link, the extended conformation prevailed in the absence of ATP, while the contracted conformation was induced by ATP. Functional studies of the mutants with one of the ε conformations fixed by a cross-link revealed that subunit ε in the extended conformation inhibited the ATPase activity of F_OF_1 but had no significant effect on ATP synthesis (Suzuki et al. 2003), in agreement with the results obtained on *E. coli* F_OF_1 (Tsunoda et al. 2001). In the contracted hairpin conformation subunit ε had no effect on either activity (Suzuki et al. 2003). This result explained the earlier data indicating two distinct states of *Bacillus* PS3 subunit ε, of which only one was inhibiting ATP hydrolysis (Kato et al. 1997).

Experiments on fluorescence resonance energy transfer between labels introduced in *Bacillus* PS3 F_1 on the N-terminus of subunit γ and on the C-terminus of subunit ε confirmed that the transition from extended to contracted state is induced by ATP and correlates with the increase in the ATPase activity (Iino et al. 2005).

The findings described above indicate that in *Bacillus* PS3 F_OF_1 subunit ε might play a regulatory role, and that the molecular mechanism of the regulation involves large conformational transitions of the C-terminal α-helical domain triggered by ATP. Although no sound evidence on similar transitions in F_OF_1 from other organisms has been published, there are several studies reporting conformational changes of subunit ε in response to nucleotides, P_i, and $\Delta\tilde{\mu}_{H^+}$ in the *E. coli* enzyme (Mendel-Hartvig and Capaldi 1991; Wilkens and Capaldi 1994; Aggeler and Capaldi 1996). $\Delta\tilde{\mu}_{H^+}$-induced changes in subunit ε conformations are also reported for chloroplast F_OF_1 (Richter and McCarty 1987; Komatsu-Takaki 1989; Nowak and McCarty 2004).

4.4 The Role of βDESLEED Region in Inhibition Mediated by Subunit ε

The demonstration of conformational transitions of the ε C-terminus does not provide information on the interactions responsible for the inhibitory effect. The latter issue was partially clarified by a study in our group demonstrating that in *Bacillus* PS3 F_OF_1 the inhibitory effect of ε was dependent on the presence of basic, positively charged residues on the second C-terminal α-helix of subunit ε and of the negatively charged acid residues in the DELSDED[1] segment of subunit β (Hara et al. 2001). Alanine replacements of either basic residues in the ε C-terminus or acidic residues in the βDELSDED segment led to a dramatic decrease of the inhibitory effect. The same effect of alanine replacements in subunit ε was reported in a recent study on *Bacillus* TA2.A1 F_OF_1 (Keis et al. 2006). It should be noted that in *E. coli* F_OF_1 the replacement of the first glutamate in the βDELSEED to cysteine also led to a marked increase in the ATPase activity (Garcia and Capaldi 1998). It is tempting to speculate that interactions of the βDELSEED segment with the C-terminal domain of subunit ε is a common inhibitory mechanism in bacterial and probably chloroplast F_OF_1.

In support of the latter suggestion, a marked decrease in the inhibitory effect of ε was observed in chloroplast enzyme upon truncation of the tenth C-terminal residue (Arg, marked bold in the sequence motif below), while the truncation of the previous nine (non-basic) residues had a much weaker effect (Shi et al. 2001). It should be noted that the AXLAL(R/K)RAXX**R** motif in the second C-terminal helix of ε is present both in chloroplast F_OF_1 and in the enzyme from the bacteria of *Bacillus* genera (Feniouk et al. 2006). It is prob-

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

able that the mechanism of ATPase activity inhibition mediated by subunit ε in chloroplast and in *Bacillus* F_OF_1 is the same. This suggestion is further supported by experiments demonstrating that *Bacillus* PS3 F_OF_1 is effectively inhibited by chimeric ε with the C-terminus replaced by that from chloroplast enzyme (Konno et al. 2004).

Although the enzymes from chloroplasts and from *Bacillus* bacteria share a conservative motif in the subunit ε second C-terminal α-helix, the latter region is conserved neither in length nor in its amino acid composition among bacteria (Feniouk et al. 2006). 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 decrease of inhibition (Kuki et al. 1988; Xiong et al. 1998; Cipriano and Dunn 2006). This implies that the role and the inhibitory power of subunit ε might differ substantially among bacteria. It is likely that the conservative C-terminal positive residues mentioned above are necessary for a strong inhibitory effect in photosynthetic/aerobic organisms, while a less "inhibitory" C-terminus is present in species that use F_OF_1 as an ATP-driven $\Delta\tilde{\mu}_{H^+}$ generator (Feniouk et al. 2006). In line with this hypothesis, the whole C-terminal domain is absent in subunit ε from some anaerobic bacteria (e.g. of *Bacteroides*, *Bifidobacterium*, or *Chlorobium* genera) (Feniouk et al. 2006).

It should be noted that isolated subunit ε from bacteria of *Bacillus* genera can directly bind ATP with $K_d \sim 1–2$ mM at optimal growth temperature, and that the C-terminal domain is critically important for the binding (Kato-Yamada and Yoshida 2003; Iino et al. 2005; Kato-Yamada 2005). Such binding was proposed to stabilize the contracted conformation of subunit ε and thereby prevent the inhibition of ATPase activity (Iino et al. 2005). Recent high resolution crystal structure of *Bacillus* PS3 subunit ε with bound ATP is also in line with this hypothesis (Yagi et al. 2007). It remains unclear if subunit ε has ATP-binding properties in the whole F_OF_1 and if these properties are also present in F_OF_1 from other organisms.

5 Thiol Regulation in Chloroplast Enzyme

Chloroplast F_OF_1 has a distinctive redox regulatory feature absent in bacterial and mitochondrial enzymes (for reviews see Evron et al. 2000; Hisabori et al. 2002, 2003; Richter 2004). Early studies revealed that latent ATPase activity of chloroplasts is markedly stimulated by reduction with thiol reagents (Petrack et al. 1965; Kaplan and Jagendorf 1968). Later study by Mills and Mitchell demonstrated that ATP synthesis was also stimulated by the reduction of the enzyme under conditions of limiting $\Delta\tilde{\mu}_{H^+}$, suggesting that the $\Delta\tilde{\mu}_{H^+}$ required for activation of the chloroplast F_OF_1 is larger than that re-

quired thermodynamically for ATP synthesis (Mills and Mitchell 1982). This suggestion was confirmed by experiments with flashing light excitation of thylakoid membranes showing that the $\Delta\tilde{\mu}_{H^+}$ threshold for release of the inhibitory ADP, for activation of ATP hydrolysis, and for initiation of ATP synthesis was higher than the phosphate potential of the medium, especially in the oxidized F_OF_1 (Hangarter et al. 1987). Increase of ATP concentration from 10 μM to 1.5 mM had no detectable effect on $\Delta\tilde{\mu}_{H^+}$-induced release of the inhibitory ADP from reduced thylakoid membranes, indicating that the phosphate potential has no effect on activation. Assessment of the activation $\Delta\tilde{\mu}_{H^+}$ value done in the same study yielded ~42 kJ/mol and ~51 kJ/mol for reduced and oxidized enzyme, respectively. Experiments with acid–base transitions on thylakoids indicated that the ΔpH necessary for half-maximal activation of reduced F_OF_1 was 2.2, but increased to 3.4 for the oxidized enzyme (Junesch and Graber 1987).

The stimulation of chloroplast F_OF_1 ATPase activity correlates with reduction of two cysteine residues in subunit γ (Arana and Vallejos 1982; Nalin and McCarty 1984). These two cysteines specific for chloroplast enzyme are located in a ~30 residue long "regulatory region" in subunit γ that is not found in bacterial or mitochondrial enzymes (Hisabori et al. 2002; Hong and Pedersen 2003). It is probable that the formation of a disulfide bond between these two cysteines markedly elevates the $\Delta\tilde{\mu}_{H^+}$ threshold necessary for release of the inhibitory ADP from chloroplast F_OF_1, and stabilizes the ADP-inhibited state. However, this disulfide bond does not affect ATP synthesis rate at high $\Delta\tilde{\mu}_{H^+}$ (Junesch and Gräber 1985, 1987; Hangarter et al. 1987). Therefore, it is tempting to suggest that the thiol regulation of chloroplast F_OF_1 is also partially due to the modulation of the ADP-inhibition efficiency. It is likely that the formation of the disulfide bond impedes the rotation of subunit γ necessary to expel ADP from the high-affinity catalytic site.

Besides the modulation of ADP-inhibition strength, oxidation/reduction of subunit γ also influences the inhibitory effect of subunit ε on ATPase activity of the chloroplast F_OF_1. It has been demonstrated that reduction of the disulfide bond on subunit γ enhances the dissociation of subunit ε from F_1 (Duhe and Selman 1990; Soteropoulos et al. 1992). In turn, subunit ε protects the SS-bond from reduction when bound to F_1. Noteworthy, the truncated ε lacking the C-terminal domain does not protect subunit γ from reduction (Nowak and McCarty 2004). The influence of subunit ε C-terminal domain on redox regulation in chloroplast F_OF_1 is supported by experiments on the introduction of subunit γ regulatory region into *Bacillus* PS3 enzyme (Konno et al. 2004). It was found that the redox regulation emerged only when the regulatory region was introduced together with the C-terminal domain of chloroplast subunit ε. This finding indicates that specific interactions between the regulatory region of subunit γ and the C-terminal domain of subunit ε might be important for the modulation of chloroplast F_OF_1 activity. It should be noted, however, that chloroplast F_1 lacking subunit ε can still be activated

by reduction (Richter et al. 1984; Duhe and Selman 1990), as well as the mutant $\varepsilon^{\Delta C}$ F_OF_1 (Nowak and McCarty 2004). Therefore, despite some interplay with the inhibition mediated by subunit ε C-terminal domain, the latter is not a prerequisite for inactivation of chloroplast F_OF_1 caused by oxidation of the γ subunit.

From the experiments on chloroplasts it was suggested that in vivo subunit γ is reduced by thioredoxin, which in turn is photoreduced in the chloroplasts by ferredoxin–thioredoxin reductase (Mills et al. 1980). Further experiments supported this suggestion and pointed out that thioredoxin-f rather than thioredoxin-m is responsible for F_OF_1 reduction in chloroplasts (Schwarz et al. 1997). An elegant biophysical study by Kramer and Crofts on leaves of intact plants provided evidence that light-dependent reduction by thioredoxin is indeed involved in the regulation of chloroplast F_OF_1 activity in vivo (Kramer and Crofts 1989). It was revealed that full reduction of F_OF_1 through the thioredoxin system occurs at a light intensity of ~0.2% of the physiologically "normal" value that saturates primary photosynthetic proteins. Therefore, the thiol modulation is likely to be a "day–night" switch rather than being involved into daytime regulation of F_OF_1 activity (Kramer and Crofts 1989).

6 Mitochondrial Inhibitor Protein IF_1

Mitochondrial F_OF_1 has a more complicated subunit composition than bacterial and chloroplast enzymes. A special mitochondrial "inhibitor protein" (IF_1) that reversibly binds to F_OF_1 plays a role in regulation of ATP hydrolysis (for a review see Green and Grover 2000). The inhibitory effect of this small α-helical basic protein on ATPase activity of both isolated F_1 and of submitochondrial particles from beef heart mitochondria was reported in 1963 by Pullman and Monroy (Pullman and Monroy 1963). In the same study it was revealed that IF_1 does not inhibit ATP synthesis and that the inhibition of ATP hydrolysis is pH-dependent and occurs at pH below 8. Later, IF_1 was also found in yeast (Hashimoto et al. 1981) and rat (Cintron and Pedersen 1979) mitochondria. Bovine IF_1 was shown to inhibit F_OF_1 from yeast and vice verse (Cabezon et al. 2002; Ichikawa and Ogura 2003). In the case of yeast, it was reported that two other protein factors with molecular masses of 9 and 15 kDa interact in a complex manner to stabilize the F_1–IF_1 complex (Hashimoto et al. 1983).

The X-ray crystallographic studies clarified the structure of IF_1–F_1 complex from bovine mitochondria (Cabezon et al. 2003). It turned out that α-helical IF_1 N-terminus can insert itself into $\alpha_3\beta_3$ hexamer between the α and β subunits near their C-terminal regions and the βDELSEED region, which is involved in the subunit ε inhibitory effect in bacterial F_OF_1 (see Sect. 4).

The pH dependence of the IF_1-mediated inhibition (Pullman and Monroy 1963; Panchenko and Vinogradov 1985) was reported to correlate with the pH dependence of IF_1 oligomerization (Cabezon et al. 2000). At pH below neutral, IF_1 exists as a dimer that efficiently inhibits the ATPase activity of F_1, while at pH above neutral IF_1 forms a tetramer that has no inhibitory power. Such pH dependence was suggested to provide a feedback mechanism for preserving mitochondrial ATP in case of uncoupling or anoxia. When glycolysis becomes the only source of cellular ATP, it lowers the cytosolic pH, which is transmitted to the matrix and promotes the inhibition of ATP hydrolysis by IF_1 (Cabezon et al. 2000).

As mentioned above, similar to ADP-inhibition and inhibition mediated by subunit ε in bacterial and chloroplast F_OF_1, IF_1 inhibits ATP hydrolysis without detectable effect on ATP synthesis (Pullman and Monroy 1963; Asami et al. 1970; Iwatsuki et al. 2000). It has been demonstrated that IF_1 dissociates from F_OF_1 upon membrane energization (Schwerzmann and Pedersen 1981; Lippe et al. 1988), suggesting that rotation of subunit γ forces the release of bound IF_1. Experiments with mutant yeast strains lacking IF_1 revealed that in mitochondria it is responsible for prompt deactivation of ATP hydrolysis upon uncoupling (Mimura et al. 1993; Iwatsuki et al. 2000).

In vivo, the deletion of IF_1 in yeast does not affect the growth rate on non-fermentable carbon sources, but it is necessary to preserve mitochondrial and cellular ATP under starving conditions (Ichikawa et al. 2001).

7 Conclusions

F_OF_1 cannot be treated as a simple enzyme that merely accelerates a reversible reaction. Several mechanisms (ADP-inhibition, inhibition mediated by subunit ε in bacteria and chloroplasts, oxidation of subunit γ in chloroplasts, and binding of IF_1 in mitochondria) deactivate the enzyme upon dissipation of $\Delta\tilde{\mu}_{H^+}$ and prevent uncoupled ATP hydrolysis. Re-activation from the inhibited state might require $\Delta\tilde{\mu}_{H^+}$ higher than that necessary for ATP synthesis from thermodynamic considerations. Therefore, $\Delta\tilde{\mu}_{H^+}$ is necessary not only to provide energy for ATP synthesis, but also to maintain the F_OF_1 active state. High affinity to P_i in the presence of $\Delta\tilde{\mu}_{H^+}$ is a key feature of the active state maintenance, protecting the enzyme from ADP-inhibition. Such regulation supposedly prevents ATP waste upon membrane de-energization, but allows ATP-driven $\Delta\tilde{\mu}_{H^+}$ generation on well-coupled membranes.

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