Regulatory Interplay between Proton Motive Force, ADP, Phosphate, and Subunit ε in Bacterial ATP Synthase*

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ATP synthase couples transmembrane proton transport, driven by the proton motive force (pmf), to the synthesis of ATP from ADP and inorganic phosphate (Pi). In certain bacteria, the reaction is reversed and the enzyme generates pmf, working as a proton-pumping ATPase. The ATPase activity of bacterial enzymes is prone to inhibition by both ADP and the C-terminal domain of subunit ε. We studied the effects of ADP, Pi, pmf, and the C-terminal domain of subunit ε on the ATPase activity of thermophilic Bacillus PS3 and Escherichia coli ATP synthases. We found that pmf relieved ADP inhibition during steady-state ATP hydrolysis, but only in the presence of Pi. The C-terminal domain of subunit ε in the Bacillus PS3 enzyme enhanced ADP inhibition by counteracting the effects of pmf. It appears that these features allow the enzyme to promptly respond to changes in the ATP:ADP ratio and in pmf levels in order to avoid potentially wasteful ATP hydrolysis in vivo.

ATP synthase (FoF1) is a ubiquitous enzyme present in the plasma membrane of bacteria, the thylakoid membrane of plastids, and the inner mitochondrial membrane. The enzyme catalyzes ATP synthesis coupled to transmembrane proton translocation3 driven by the proton motive force (pmf). At low pmf the activity is reversed and the enzyme functions as a proton-pumping ATPase. ATP synthase consists of two distinct regions: the hydrophobic Fo, which is embedded in the membrane, and the hydrophilic F1 that protrudes ~100 Å from the membrane bilayer. F1 contains three catalytic and three non-catalytic nucleotide-binding sites and is responsible for both ATP synthesis and hydrolysis reactions. F1 performs transmembrane proton transport. The simplest subunit composition is found in bacteria (e.g. Escherichia coli or thermophilic Bacillus PS3; see Refs. 1 and 2 for reviews), where F1 is a complex of five types of subunits at a stoichiometry of αβγδε, and Fo is a complex of three types of subunits at a stoichiometry of αβγε.

It is now widely accepted that the enzyme operates according to the “binding change mechanism” (see Ref. 3 and the references therein). Briefly, proton translocation is coupled to the rotation of the c-ring oligomer (4) together with the γe complex relative to the rest of the enzyme (see Refs. 5–7 and references therein for details). Rotation of the γ subunit inside the αβ3 hexamer causes sequential conformational changes to the catalytic sites. These sequential conformational changes result in substrate binding, the chemical step, and product release (8–10). This “rotary binding change mechanism” is usually considered reversible, and numerous demonstrations of ATP-driven proton pumping support this assumption. However, several observations indicate that this is not the case. Many factors (e.g. products/substrates of catalysis, inhibitors, inorganic anions, etc.) affect ATP synthesis and the hydrolysis activities of the enzyme (see Ref. 11 for a detailed discussion). One of the most well known anisotropic regulatory factors is ADP; it not only serves as a substrate for ATP synthesis but also inhibits ATPase activity of the enzyme in a non-competitive manner. ADP inhibition is observed for mitochondrial (12–15), chloroplast (16–19), and bacterial (20, 21) ATP synthases. It is observed not only in the whole enzyme or F1 portion but also in the αβγε complex (22, 23), indicating that this regulatory mechanism is embedded in the catalytic core of F1. It is well established that ADP inhibition is caused by the tight binding of MgADP (without Pi) at a high affinity catalytic site (15, 21, 24, 25). Single molecule experiments revealed that ADP inhibition results in long pauses in enzyme turnover (23) and that the rotation of subunit γ, caused by either external forces or thermal fluctuations, relieves ADP inhibition (26). It was also demonstrated that the tightly bound inhibitory ADP can be expelled by pmf (19, 27–30). This phenomenon underlies the so-called “activation by pmf,” that is, the increase in ATPase activity of the enzyme after membrane energization (31–36). In view of the single molecule data, it is conceivable that activation by pmf is caused by pmf-driven rotation of the γ subunit.

Another regulatory mechanism found in chloroplast and bacterial ATP synthase is the inhibition of ATPase activity by subunit ε (see Refs. 37–39 for reviews). Subunit ε is composed of two domains: the N-terminal β-sandwich that is bound to both the c-ring and subunit γ, and two mobile C-terminal α-helices. Only the C-terminal domain appears to be responsible for inhibition of ATP synthase (40–45). In the last few years, it was demonstrated that in both the E. coli and Bacillus PS3 enzymes, the C-terminal domain adopted two conformations: “contracted”, with the two helices forming a hairpin structure close to the c-ring, and “extended”, with the helices stretched toward the F1 region (46, 47). Cross-linking experiments with

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2 Sodium ion-translocating FoF1 ATP synthase is found in certain bacteria; see Ref. 54 for details.
3 The abbreviations used are: pmf, proton motive force; transmembrane difference of electrochemical proton potential; TFγε, ATP synthase from thermophilic Bacillus PS3; EFγεF1, ATP synthase from E. coli.
the Bacillus PS3 enzyme revealed that the extended conformation correlates with the inhibition of ATPase activity, but not of ATP synthesis. The contracted conformation inhibited neither synthesis nor hydrolysis (47). In this work, we investigated the factors necessary to maintain bacterial ATP synthase activity during ATP hydrolysis. We measured ATPase activity in wild-type enzymes from thermophilic Bacillus PS3 (TF0F1) and from E. coli (EF0F1) and in a mutant of TF0F1, where the C-terminal domain of subunit ε was truncated, TF0F1(εΔC). We demonstrated that energization of the membrane (pmf) not only stimulated initial ATPase activity (activation by pmf) but also counteracted ADP inhibition during steady-state ATP hydrolysis. The latter phenomenon was dependent upon the presence of P, and was opposed by the C-terminal domain of subunit ε. A model describing the regulatory interplay between ADP inhibition, P, affinity, subunit ε conformational transitions, and membrane energization is proposed.

**EXPERIMENTAL PROCEDURES**

**Preparation of Inverted Membrane Vesicles, TF0F1 and TF1**

E. coli cells expressing His-tagged TF0F1 or EF0F1 were grown as previously described (48). The plasmid pTR19-ASDS was used for expression of wild-type TF0F1. The TF0F1(εΔC) mutant of TF0F1, containing a stop codon after Asp-87 of subunit ε, was expressed in the same plasmid modified as described previously (49). Plasmid pFV2 (provided by Prof. S. Vik from Southern Methodist University, Dallas, TX) was used for expression of His-tagged EF0F1 (50). All proteins were expressed in DK8 E. coli cells (bglR, thi-1, rel-1, HfrPO1, Δ(uncB-uncC), ilvTn10) lacking the wild-type EF0F1 operon. Inverted membranes were obtained using a French press treatment (48). No further purification of the EF0F1 enzyme was performed. TF0F1 and TF1 were purified by nickel-nitriolactoacetic acid affinity chromatography as previously described (47, 48). The enzymes were frozen in liquid nitrogen and stored in aliquots at −80 °C until use. Inverted membrane vesicles stripped of F1 were prepared by sonication in 0.5 mM EDTA at pH 8.0. After sonications, the suspension was centrifuged (190,000 × g, 45 min, 4 °C) and resuspended in 0.5 mM EDTA, and the procedure was repeated twice. Then, the membranes were resuspended in buffer containing 10 mM HEPES-KOH, 5 mM MgCl2, and 10% glycerol, pH 7.5. The stripping efficiency was verified by measurement of residual ATPase activity. Reconstitution of stripped membranes was carried out by mixing with excess purified TF0F1 for 30 min at room temperature. Excess TF0F1 was washed away by the same buffer in two sequential centrifugations (190,000 × g, 45 min, 4 °C). The reconstituted membranes were frozen in liquid nitrogen and stored at −80 °C until use.

**Reconstitution of TF0F1, ATP Synthase into Liposomes**

Phosphatidylcholine (Sigma S-II type) was resuspended to a final concentration of 44 mg/ml in buffer containing 10% glycerol, 5 mM MgCl2, and 10 mM HEPES-KOH, pH 7.5. The suspension was mixed for 30 min at room temperature and then sonicated at maximum power with an Astrason XL2020 ultrasonic liquid processor (Misonix) for 15 s on ice. The liposomes were then divided into 1-ml aliquots, frozen in liquid nitrogen, and stored at −80 °C. For reconstitution, the liposomes were diluted to a concentration of 8 mg/ml in buffer containing 10 mM Tricine, 80 mM NaCl, 2 mM MgCl2, and 0.8% w/v Triton X-100, pH 8.0. Purified TF0F1 was added to a final concentration of 0.1–0.15 mg/ml, and the sample was incubated 1 h at room temperature with gentle agitation. BioBeads (Bio-Rad) that had been pre-soaked in water were then added to a concentration of 320 mg/ml, and the sample was incubated for 2 h at room temperature with gentle agitation. The BioBeads were removed by filtration, and the proteoliposomes were used immediately or kept overnight at room temperature and used the next day. The same procedures were applied for the mutant TF0F1(εΔC).

**ATP Hydrolysis Measurements**

ATPase activity was measured by three different methods. **ADP Assay**—ATP hydrolysis was monitored via NADH oxidation enzymatically coupled to the rephosphorylation of produced ADP (51). NADH concentration was measured optically at 340 nm in buffer containing 10 mM HEPES-KOH, 10% glycerol, 100 mM KCl, 2.5 mM MgCl2, 2.5 mM potassium phosphate, 200 μM NADH, 3 mM phosphoenolpyruvate (tricyclohexylammonium salt), 0.1 mg/ml pyruvate kinase, and 0.1 mg/ml lactate dehydrogenase. The final pH was 8.0–8.1. The reaction was initiated by the addition of 1 mM ATP. KCN (3 mM) was present in measurements with sub-bacterial inverted membrane particles to prevent NADH oxidation by respiratory chain enzymes.

**pH Assay**—ATP hydrolysis was measured by following proton release using the pH indicator phenol red at 557 nm (52) in buffer containing 2.5 mM HEPES-KOH, 10% glycerol, 100 mM KCl, and 2.5 mM MgCl2, pH 8.0–8.1. Potassium phosphate (2.5 mM) was present unless otherwise indicated. The response of phenol red was calibrated by the addition of either 10 mM H2SO4 or 10 mM NaOH aliquots. The calibration was verified by measurements of ATPase activity in the same sample by following the concomitant production of either ADP or P. The observed ratio of H⁺:ATP was ~0.85.

**P, Assay**—In this assay, ATP hydrolysis was measured following the production of P, by an enzymatic colorimetric method (53) using an EnzCheck® Phosphate Assay kit (Invitrogen). Samples contained 270 μM MESG (2-amino-6-mercaptop-7-methylpurine riboside), 2 units/ml purine nucleotide phosphorylase, 2.5 mM HEPES, 100 mM KCl, 2.5 mM MgCl2, and 10% glycerol, pH 8.0. Inorganic phosphate produced from ATP hydrolysis reacts with MESG, producing ribose 1-phosphate and 2-amino-6-mercaptop-7-methylpurine. The concentration of the latter product was followed in real time via absorbance at 360 nm.

All measurements were done using a Jasco V-550 spectrophotometer. Activity of TF0F1 was measured at 43 °C; measurements with EF0F1 were done at 36 °C. To analyze changes in ATP hydrolysis rate during the measurements, the experimental traces were differentiated using Origin 7.0 software (Origin-Lab Corp.) and smoothed by averaging 20 adjacent data points. The reliability of this procedure was verified by comparison of the derivatives using the usual linear fits of the experimental data (20 data points/fit).

**Other Methods**

ATP and succinate-driven proton-pumping activity was measured by fluorescence quenching of 0.3 μg/ml 9-amino-6-
**Regulation of Bacterial ATP Synthase**

![Fluorescence changes in ATPase activity](image)

**RESULTS**

**Experimental Setup**—We observed the ATPase activity of $F_{O}F_{1}$ in real time by monitoring 1) production of ADP via oxidation of NADH mediated by lactate dehydrogenase and pyruvate kinase in an ATP-regenerating system (ADP assay), 2) pH change caused by the release of "scalar" protons during ATP hydrolysis via phenol red (pH assay), or 3) production of Pi via enzymatic conversion of ADP by purine nucleotide phosphorylase in a Pi-scavenger system (Pi assay). In the ADP assay, the ATP concentration decreases while concentrations of both ADP and Pi increase as the reaction proceeds. As the ADP concentration increases, so does the possibility of ADP inhibition. The Pi assay keeps the concentration of Pi negligibly small throughout the reaction, while the ATP and ADP concentrations change reciprocally during the reaction. All three assay methods provided time resolution in the range of seconds. We plotted the data as differentials of the original kinetic traces that represent ATPase activity, to directly see the changes in ATPase activity over time.

In this report, we measured the $F_{O}F_{1}$ ATPase activity in both reconstituted proteoliposomes and inverted membrane vesicles prepared from *E. coli*. The reactions were initiated by the addition of ATP to final concentration of 1 mM. As ATP hydrolysis proceeded, ATP was built up across membranes and acted as "back-pressure" that limited the rate of ATP hydrolysis. Valinomycin and nigericin, which collectively act as an uncoupler (in the presence of K+), dissipated the pmf. Hereafter, we refer to the valinomycin-nigericin mixture as "uncoupler".

**Effect of Uncoupler and ADP on the ATPase Activity of Wild-type $F_{O}F_{1}$ in Proteoliposomes**—We measured ATPase activities of wild-type $F_{O}F_{1}$ and mutant $F_{O}F_{1}(e^{ac})$ reconstituted into proteoliposome membranes in the presence of 2.5 mM Pi (Fig. 1). With the ADP assay, the wild-type $F_{O}F_{1}$ exhibited low hydrolysis activity that gradually increased, reaching a higher steady-state level compared with that in the absence of uncoupler (Fig. 1A, red trace). The apparent time constant ($\tau$) of this activation (hereafter denoted as “initial activation”) was $\sim 40$ s. In the presence of uncoupler, this initial low activity increased at a slow rate ($\tau$ $\sim 60$ s), reaching an $-2$-fold higher steady-state level compared with that in the absence of uncoupler (Fig. 1A, blue trace). This higher steady-state activity was due to the abolishment of the pmf back-pressure. The stimulating effect of uncoupler was also observed when uncoupler was added during the reaction (Fig. 1A, yellow trace).

Similar measurements were done using the pH assay (Fig. 1C). In this assay, the initial ADP concentration was $\sim 5$ mM (due to contamination of the ATP preparation used), gradually increasing to $\sim 100$ mM after several minutes of ATP hydrolysis. The time course in the absence of uncoupler was almost identical to that seen with the ADP assay (Fig. 1C, red trace). In the presence of uncoupler, ATPase activity reached a maximum after initial activation ($\tau$ $\sim 10$ s). However, in contrast to the ADP assay, the activity slowly decreased (Fig. 1C, blue trace).
This slow decrease can be attributed to ADP inhibition caused by the increase in ADP concentration. The addition of uncoupler during the reaction induced a steep increase in activity that was followed by a rapid decay (Fig. 1C, yellow trace).

As mentioned, F₀F₁ is readily inhibited by ADP. Consequently, ATPase activity was very low when measured in the presence of 640 µM ADP (Fig. 1E, red trace; note the expanded y-axis scale). During the assay, ATPase activity increased slowly (τ ~ 60 s). However, activity in the presence of uncoupler was not stimulated and remained low (Fig. 1E, blue trace). We noted that activity in the presence of uncoupler was lower than that in the absence of uncoupler. Similarly, addition of uncoupler during the reaction induced an immediate decrease in activity (Fig. 1E, yellow trace). Thus, the effect of uncoupling on the ATPase activity of wild-type TFOF₁ was reversed in the presence of ADP; ATPase activity was inhibited rather than stimulated by uncoupler. These results suggest that pmf assists TFOF₁ in recovery from ADP inhibition.

Effect of Uncoupler and ADP on the ATPase Activity of TFOF₁(ε<sup>AC</sup>) in Proteoliposomes—To determine the contribution of the two C-terminal helices of the subunit ε to ATPase activity, we examined the activity of the TFOF₁(ε<sup>AC</sup>) mutant, which lacks the C-terminal region of subunit ε. As reported previously (42), in the ADP assay, the mutant enzyme immediately hydrolyzed ATP at a maximal, steady-state rate without apparent initial activation (Fig. 1B, red trace). Uncoupler added either prior to or during the reaction immediately increased the activity, but again slow initial activation was not observed in the TFOF₁(ε<sup>AC</sup>) mutant. These results suggested that the C-terminal domain of subunit ε was responsible for the low initial ATPase activity. It was likely that the relief of subunit ε inhibitory effect, but not pmf, caused the initial activation of the wild-type TFOF₁ in the ADP assay.

With the pH assay, a similar trace was obtained in the absence of uncoupler (Fig. 1D, red trace). Steady-state activity was reached without obvious initial activation. However, activity in the presence of uncoupler measured with the pH assay was very different from that measured with the ADP assay; the initial activity was high and it immediately started to decay (Fig. 1D, blue trace). The decay continued below the level of activity in the absence of uncoupler. The addition of uncoupler during the reaction induced a similar response (Fig. 1D, yellow trace). The decay was likely due to progressive inhibition by accumulated ADP as ATP hydrolysis proceeded. The reasons for the high initial activity were not clear.

In the presence of 640 µM ADP, the steady-state ATPase activity of TFOF₁(ε<sup>AC</sup>) was nearly 2-fold higher than that of wild type under the same conditions (Fig. 1F, red trace). This activity was achieved after a period of initial activation (τ ~ 19 s). In the presence of uncoupler, the activity remained low throughout the reaction (Fig. 1F, blue trace). The addition of uncoupler during the reaction caused a decrease in the activity to the same low level (Fig. 1F, yellow trace). It should be noted that in the TFOF₁(ε<sup>AC</sup>) mutant (i) the steady-state ATPase activity was higher both with and without uncoupler, and (ii) the initial activation in the coupled sample was markedly faster than in the wild-type. These results indicate that the C-terminal domain of subunit ε in wild-type TFOF₁ increases ADP inhibition by counteracting the effects of pmf.

**Effect of P<sub>i</sub> on the Relief of ADP Inhibition by pmf in Wild-type TFOF₁ and TFOF₁(ε<sup>AC</sup>)—**All the experiments shown in Fig. 1 were done in the presence of 2.5 mM P<sub>i</sub>. To investigate the effect of P<sub>i</sub> on the relief of ADP inhibition by pmf, we compared the activity of TFOF₁ in the presence and absence of P<sub>i</sub> (Fig. 2, A and
Regulation of Bacterial ATP Synthase

C; note that 640 μM ADP was present). Without added P, ATPase activity was unaffected by the presence of uncoupler; in other words, pmf did not relieve ADP inhibition if P was not present.

Next, the same measurements were done for TF0F1(eAC). In contrast to wild-type TF0F1, the effect of pmf was obvious even without added P. Steady-state ATPase activity was significantly higher without uncoupler than in its presence (Fig. 2D). It should be noted, however, that the initial activation was markedly slower when P was omitted (compare red traces in Fig. 2, B and D).

The above-mentioned slow activation could be due to a gradual increase in P, concentration resulting from ATP hydrolysis during the pH assay. To address this possibility, we measured the ATPase activity of TF0F1(eAC) using the P assay (Fig. 2E). In this assay, P produced by ATP hydrolysis is removed instantaneously. The results obtained were markedly different than what was observed with the pH assay (compare Fig. 2, E and D). There was no detectable effect of uncoupler on activity. The same results were observed for the wild-type enzyme using the P assay (not shown), implying that the relief of ADP inhibition by pmf was dependent upon P.

ATP-driven Proton Pumping by Wild-type TF0F1 and TF0F1(eAC) and the Effect of ADP—Proton-pumping activities of proteoliposomes containing either wild-type TF0F1 or TF0F1(eAC) were measured under the same conditions used in the pH assay except that 9-amino-6-chloro-2-methoxyacridine was added instead of phenol red (Fig. 3).

Upon addition of ATP, wild-type TF0F1 began pumping protons after a short lag, whereas TF0F1(eAC) lacked a lag period. The initial kinetics of proton pumping closely reflected the time required to reach steady-state ATPase activity (the initial activation) shown in Fig. 1, C and D. Even though the ATPase activity of TF0F1(eAC) was almost identical to wild type shown in Fig. 1, C and D, the proton-pumping activity of TF0F1(eAC) was significantly higher. The reason for this discrepancy is not known.

In the presence of 640 μM ADP, TF0F1(eAC) pumped protons much more efficiently than did wild type. The mutant also exhibited a much shorter lag period. These observations are qualitatively consistent with ATPase activity shown in Fig. 1, E and F.

Effects of Membrane Pre-energization on ATPase Activity—Studies on chloroplast, bacterial, and mitochondrial F0F1 reported marked stimulation of ATPase activity by pre-energization, i.e. when pmf is imposed across membranes before ATP addition (31, 33–36). To determine whether this is also true for Bacillus PS3 ATP synthase, TF0F1 ATPase activity in inverted E. coli membranes was measured with the assay. Unfortunately, variations in the properties of individual membrane preparations complicated the comparison between wild-type TF0F1 and TF0F1(eAC). To avoid this problem, the inverted membranes were stripped of TF1 by EDTA treatment and reconstituted either with wild-type TF1 or TF1(eAC). In the presence of 640 μM ADP, these inverted membranes showed ATPase activity similar to that observed for proteoliposomes (Fig. 4): the presence of uncoupler lowered this activity. The slow ATP stimulation of wild-type TF0F1 and the rapid stimulation of TF0F1(eAC) were also in good agreement with the results in proteoliposomes.

Pre-energization of membranes was achieved by including succinate in the pH assay solution. Succinate oxidation does not alter the pH of the bulk phase, and therefore it does not inter-
regardless of the presence of uncoupler. This decay was more state activities measured with the ADP assay, but decayed using the pH assay, very different kinetics were obtained (Fig. 5). Uncoupler led to a small but clear increase in the ATPase synthase (EFOF1). In the ADP assay, the ATPase activity was highest at the beginning, without initial activation (Fig. 5A). In contrast, there was no significant difference in wild-type kinetics between samples with or without pre-energization (Fig. 4A). These results again suggest that the C-terminal domain of subunit ε helps prevent pnf-assisted relief of ADP inhibition of TF0F1 during pre-energization.

**Effect of ADP, Energization, and Uncoupling on E. coli ATP Synthase (EFOF1) in Inverted Membranes**—To determine the generality of our results for TF0F1, we repeated the experiments using inverted membranes containing E. coli ATP synthase (EFOF1). In the ADP assay, the ATPase activity was highest at the beginning, without initial activation (Fig. 5A, red trace). This finding was closer to the kinetics of TF0F1 (εC) than to wild type TF0F1 (see Fig. 1, A and B). Uncoupler led to a small but clear increase in the ATPase activity both when added prior to ATP addition and when added later during the reaction.

When the same reactions mentioned above were measured using the pH assay, very different kinetics were obtained (Fig. 5B). The initial activities were similar to those of the steady-state activities measured with the ADP assay, but decayed regardless of the presence of uncoupler. This decay was more rapid in the presence of uncoupler than in its absence, and after 1 min the activity was lower in the uncoupled sample (Fig. 5B, blue trace). It appeared that, as ADP concentration increased with time, a larger fraction of the enzyme was inhibited by ADP. The addition of uncoupler during the reaction did not stimulate activity but reduced it to the level of the sample uncoupled prior to the addition of ATP (Fig. 5B, yellow trace).

When the assay solution contained 640 μM ADP, the initial ATPase activity was very low due to ADP inhibition. In the absence of uncoupler, the inhibition was slowly relieved and activity increased ~3-fold after 3 min. In the presence of uncoupler, the inhibition was not relieved and the activity remained at the same low level (Fig. 5C). Thus, the pnf-assisted relief of ADP inhibition is likely a common property among FOF1s from a wide range of bacteria. We note, however, that the contribution of the C-terminal domain of subunit ε to such relief may vary among species.

**DISCUSSION**

**Initial Activation of TF0F1 May Result from a Conformational Transition of Subunit ε**—In the absence of ADP, an initial activation of ATP hydrolysis was observed in wild-type TF0F1, but not in TF0F1 (εC) (Fig. 1), in good agreement with earlier measurements via ADP assay done with 2 mM ATP (42). Cross-linking experiments on TF0F1 (47) and fluorescence resonance energy transfer experiments on TF1 (55) revealed that initial activation correlates with a structural transition of subunit ε from an extended state to a contracted state. It was also shown that in TF0F1, ATP induces the non-inhibitory, contracted conformation of subunit ε, whereas ADP promotes the inhibitory, extended conformation (47). Importantly, the isolated TF0F1 subunit ε can directly bind ATP (56), and this binding was proposed to stabilize the contracted state (55). Taken together, these data suggest that the initial activation shown in Fig. 1A was caused by an ATP-induced transition of subunit ε to the contracted conformation. Interestingly, the initial activation observed in the wild-type TF0F1 was unaffected by uncoupling. Apparently, pnf generated by ATP hydrolysis has no major effect on the ATP-induced transition of subunit ε into the contracted state in the absence of ADP.

**The C-terminal Domain of Subunit ε Helps Stabilize the ADP-inhibited State of TF0F1**—In the presence of ADP, the final steady-state ATPase activity of the wild type was nearly one-half that of the TF0F1 (εC) mutant in both coupled and uncoupled samples (Fig. 1). ATP-driven proton pumping was more readily inhibited by ADP in the wild type than in
Regulation of Bacterial ATP Synthase

TF₆₃F₁(ε⁴ΔC) (Fig. 3). These results suggest that the C-terminal domain of subunit ε enhances ADP inhibition. Indeed, the initial ATPase activity of the pre-energized membrane was suppressed in the case of the wild-type TF₆₃F₁ but was fully stimulated in the case of TF₆₃F₁(ε⁴ΔC) (Fig. 4). As mentioned earlier, the activation of ATP hydrolysis by exposure to pmf prior to ATP addition is caused by the release of the tightly bound inhibitory ADP. It is therefore most likely that the C-terminal domain of subunit ε prevents activation by pmf through stabilization of the ADP-inhibited state of TF₆₃F₁ (57).

Although we have demonstrated interplay between ADP inhibition and subunit ε, ADP is not a prerequisite for the inhibitory effect of subunit ε. It was demonstrated in E. coli F₁ that, in the absence of ADP (in an ATP-regenerating system), subunit ε significantly altered the catalytic properties of ATP hydrolysis and reduced the rate of product release by 15-fold (58). More recent bulk phase (44) and single molecule (59) experiments in E. coli F₁ in the absence of ADP revealed that subunit ε still inhibited steady-state ATPase activity and slowed down the rotation of subunit γ.

**Contribution of Subunit ε to F₆₃F₁**

**Regulation May Differ between Bacteria**—The kinetic behavior of EF₆₃F₁ is more similar to TF₆₃F₁(ε⁴ΔC) than to wild-type TF₆₃F₁, even though EF₆₃F₁ has a full-length subunit ε. Deletion of the C-terminal domain of subunit ε of EF₆₃F₁ resulted in a small (<1.5-fold) stimulation of ATPase activity at ATP concentrations ranging from 50 μM to 8 mM (44). In contrast, the same mutation in both TF₁ and TF₆₃F₁ caused a 3-fold stimulation at 50 μM ATP and no stimulation at 2 mM ATP (42). The results shown in Fig. 1 confirmed the same activities of both wild-type TF₆₃F₁ and TF₆₃F₁(ε⁴ΔC) at 1 mM ATP. Thus, the inhibitory effect of subunit ε is strong and ATP dependent in TF₆₃F₁ and weak and ATP independent in EF₆₃F₁.

This difference could partly be due to the temperature at which we measured TF₆₃F₁ activity. We carried out measurements at 43°C; it is possible that at temperatures closer to the optimal growth temperature for *Bacillus* PS3 (i.e. 65°C), the inhibitory effect of subunit ε of TF₆₃F₁ could be weaker and less dependent upon ATP concentration. Comparisons of the amino acid sequences in the second α-helix of the C-terminal domain of subunit ε between the two species raise another more interesting possibility. The sequence of *Bacillus* PS3 contains several basic residues that the *E. coli* sequence lacks. These residues might be involved in electrostatic interactions with the DELSDED⁴ region of subunit β and are important for the inhibitory effect (60). They might also be involved in low affinity ATP binding to subunit ε (56, 61) that presumably stabilizes the non-inhibitory, contracted conformation of subunit ε at a high ATP concentration (55). Although large conformational changes of subunit ε are also documented in EF₆₃F₁ (62–64), the absence of these basic residues in *E. coli* subunit ε might explain weak, ATP-independent inhibition.

In general, the sequence of the C-terminal domain is much more variable than that of other regions of subunit ε. In several strictly anaerobic bacteria (*e.g.* *Bacteroides* and *Bifidobacterium*), the entire C-terminal domain of subunit ε is missing (39). Such variation might provide the regulatory flexibility necessary for the “tuning” of the core function of F₀F₁ to particular requirements of different bacteria. For example, strong inhibition of ATP hydrolysis might be disadvantageous in organisms where F₆₃F₁ serves mainly as an ATP-driven pmf generator. It should be noted that the primary habitat of *E. coli* is anaerobic and F₆₃F₁ is involved in the generation of pmf. Importantly, the basic residues in the second C-terminal helix of subunit ε, which enhance its inhibitory power, are also present in the chloroplast enzyme (39) where the inhibitory effect of subunit ε is very strong (65). Such inhibition may help to preserve ATP in chloroplasts in the dark. Recent single molecule experiments on cyanobacterial F₁ confirmed that subunit ε strongly blocks the rotation of subunit γ at the same angular position where ADP inhibition occurs (66).

**ADP Inhibition Is Relieved by pmf under Steady-state Conditions**—When the ATPase activities of wild-type TF₆₃F₁ and TF₆₃F₁(ε⁴ΔC) were measured with the pH assay, ADP inhibition occurred over time in the absence of pmf (+ uncoupler), but not in the presence of pmf (− uncoupler) (Fig. 1, C and D). When TF₆₃F₁ and TF₆₃F₁(ε⁴ΔC) were preincubated with ADP, the ATPase activities in the absence of pmf remained low, whereas a slow activation was observed in the presence of pmf (Fig. 1, E and F). These results indicate that pmf assists both enzymes to escape ADP inhibition during steady-state ATP hydrolysis. This is also the case for EF₆₃F₁. Notably, stimulation of steady-state ATPase activity by pmf in the absence of an ATP-regenerating system was also reported for inverted membranes from *Rhodobacter capsulatus* (33) and *Paracoccus denitrificans* (36). Therefore, it appears likely that the pmf-assisted relief of ADP inhibition under steady-state conditions is a general feature of bacterial F₀F₁.

**The pmf-assisted Relief of ADP Inhibition Requires P₃**—The results shown in Fig. 2 clearly demonstrate that the pmf-assisted relief of ADP inhibition during steady-state ATP hydrolysis requires P₃. A P₃ requirement for pmf stimulation of F₆₃F₁ ATPase activity was also noticed in *P. denitrificans* (36). A relieving effect of P₃ on ADP inhibition was documented previously for F₁ (without the F₆₃ region) from *Bacillus* PS3 (67) and for the mitochondrial F₁ (15); the concentration of P₃ necessary was rather high (20 and 5 mM, respectively). However, it was demonstrated for mitochondrial, chloroplast, and bacterial F₆₃F₁ enzymes that pmf increases P₃ affinity (68–71). The results shown in Figs. 1 and 2 provide experimental evidence that there might be interplay between this affinity increase and the relief of ADP inhibition by P₃, as was hypothesized earlier (57).

**A Model of the Regulatory Interplay between ADP, P₃, pmf, and Subunit ε**—The results reported here, as well as those published previously, can be explained in the framework of the following regulatory model (Fig. 6). As already mentioned, ADP inhibition is caused by binding of (or failure to release) ADP at a high affinity catalytic site in the absence of P₃ (D → D₁ transition in Fig. 6). Because it is unclear whether P₃ release precedes ADP release during ATP hydrolysis, we included both possible pathways for product liberation from a catalytic site: DP → D → 0 and DP → P → 0. The latter pathway presumably excludes ADP inhibition because the D state does not occur. Therefore,
both a high Pi concentration and increased affinity of the enzyme to P, caused by pmf are expected to increase the rate of ATP hydrolysis at any given ATP:ADP ratio by increasing the probability of the DP → P → 0 transition. Similarly, P binding to the D state is expected to accelerate the D → DP transition and therefore prevent the enzyme from lapsing into the DI state. If ATP is the nucleotide bound to the empty site after release of ADP and P, then ATP hydrolysis proceeds. However, binding of ADP (0 → D transition) might lead to ADP inhibition. Again, either a high concentration of P or an increased affinity to P diminishes the probability of the 0 → D transition (and therefore, of ADP inhibition) by biasing the reaction toward the 0 → P transition.

Single molecule experiments on \( \alpha_{3}\beta_{2}\gamma \) from Bacillus PS3 (26) revealed that the recovery from ADP inhibition via the DI → 0 transition is caused by rotation of subunit \( \gamma \) driven either by external forces or by thermal fluctuations. Several studies also confirmed that both activation and ADP release can be caused by membrane energization (i.e. by pmf-driven rotation of subunit \( \gamma \)) (27–33, 36). The results shown in Figs. 1 and 4 indicate that the recovery of the enzyme from the DI state is hindered by the C-terminal domain of subunit \( \epsilon \). Such behavior is in line with the “safety lock” role of subunit \( \epsilon \); it was proposed that interactions of the basic residues in the C-terminal domain of subunit \( \epsilon \) with the BDELSEED region stabilize subunit \( \gamma \) in the angular position that corresponds to the DI state (57). The latter suggestion is directly confirmed by recent single molecule experiments on cyanobacterial F1 (66). Noteworthy in the case of TF1F1 is that this safety lock is ATP sensitive; ATP induces and presumably stabilizes the non-inhibitory, contracted conformation of subunit \( \epsilon \) (47).

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FIGURE 6. Model of the inhibition of ATP hydrolysis by ADP. DP denotes the nucleotide binding, catalytic site where ATP hydrolysis takes place, but before product release. Two possible product release pathways (DP → P → 0 and DP → D → 0) are suggested. DI denotes the ADP-inhibited state with ADP occluding the high affinity catalytic site. Rotation of subunit \( \gamma \) leads to the release of the inhibitory ADP and thereby activates the enzyme. The extended conformation of subunit \( \epsilon \) presumably hinders the rotation of subunit \( \gamma \) and therefore stabilizes the ADP-inhibited state. See the last section under “Discussion” for details.

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Regulation of Bacterial ATP Synthase