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Inhibition of ATP Hydrolysis by Thermoalkaliphilic F_1F_o-ATP Synthase Is Controlled by the C Terminus of the ε Subunit

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The F_1F_o-ATP synthases of alkaliphilic bacteria exhibit latent ATPase activity, and for the thermoalkaliphile *Bacillus* sp. strain TA2.A1, this activity is intrinsic to the F_1 moiety. To study the mechanism of ATPase inhibition, we developed a heterologous expression system in *Escherichia coli* to produce $TA2F_1$ complexes from this thermoalkaliphile. Like the native F_1F_o-ATP synthase, the recombinant $TA2F_1$ was blocked in ATP hydrolysis activity, and this activity was stimulated by the detergent lauryldimethylamine oxide. To determine if the C-terminal domain of the ε subunit acts as an inhibitor of ATPase activity and if an electrostatic interaction plays a role, a $TA2F_1$ mutant with either a truncated ε subunit [i.e., $TA2F_1(\varepsilon^{\Delta C})$] or substitution of basic residues in the second α-helix of ε with nonpolar alanines [i.e., $TA2F_1(\varepsilon^{6A})$] was constructed. Both mutants showed ATP hydrolysis activity at low and high concentrations of ATP. Treatment of the purified F_1F_o-ATP synthase and $TA2F_1(\varepsilon^{WT})$ complex with proteases revealed that the ε subunit was resistant to proteolytic digestion. In contrast, the ε subunit of $TA2F_1(\varepsilon^{6A})$ was completely degraded by trypsin, indicating that the C-terminal arm was in a conformation where it was no longer protected from proteolytic digestion. In addition, ATPase activity was not further activated by protease treatment when compared to the untreated control, supporting the observation that ε was responsible for inhibition of ATPase activity. To study the effect of the alanine substitutions in the ε subunit in the entire holoenzyme, we reconstituted recombinant $TA2F_1$ complexes with F_1-stripped native membranes of strain TA2.A1. The reconstituted $TA2F_oF_1(\varepsilon^{WT})$ was blocked in ATP hydrolysis and exhibited low levels of ATP-driven proton pumping consistent with the F_1F_o-ATP synthase in native membranes. Reconstituted $TA2F_oF_1(\varepsilon^{6A})$ exhibited ATPase activity that correlated with increased ATP-driven proton pumping, confirming that the ε subunit also inhibits ATPase activity of $TA2F_oF_1$.

F_1F_o-ATP synthases are membrane-bound enzymes found in mitochondria, chloroplasts, and bacteria that couple the transport of protons down an electrochemical gradient to the synthesis of ATP (2, 47, 61). Alternatively, the enzyme can work as an ATPase, hydrolyzing ATP to generate an electrochemical gradient of protons ($\Delta\mu_{H^+}$). F_1 contains the catalytic sites of the enzyme and is water soluble, whereas F_o is embedded in the membrane and translocates protons across the membrane. Bacterial F_1 consists of five subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$, in which the catalytic nucleotide-binding sites are on the β subunits and the noncatalytic nucleotide-binding sites are located on the α subunits (1, 36, 57). F_o comprises 3 subunits, 1 a subunit, 2 b subunits, and a ring of 10 to 15 c subunits.

In eukaryotic organisms, the ATPase activity of F_1F_o-ATP synthases is subject to regulation. In mitochondria, ATPase activity is regulated by the natural inhibitor protein IF_1, which binds to the ATP synthase in a pH-dependent manner. Cross-linking studies suggest an interaction between IF_1 and the C-terminal region of the β subunit (21). No homologue of IF_1 has been found in either chloroplasts or bacteria. ATPase activity of the chloroplast F_1F_o-ATP synthase (CF_1F_o) is sub-

ject to complex regulation to prevent wasteful ATP hydrolysis under dark conditions. Under such conditions, the enzyme is inhibited by Mg^{2+}-ADP (8), the ε subunit (38, 39, 42), and the oxidation/reduction state of the γ disulfide bond (37). Exposure of thylakoid membranes to light results in the generation of a $\Delta\mu_{H^+}$ that stimulates the release of inhibitory bound ADP and relieves inhibition by the ε subunit (23). Reduction of the γ disulfide bond further simulates ATP synthesis (37).

Several bacterial ATP synthases exhibit low or inhibited ATPase activity while being competent in ATP synthesis activity (13–15, 17, 35), but the mechanisms responsible for this latency are not understood. It is noteworthy that these bacteria are aerobic microorganisms that are faced with the challenge of blocking ATP hydrolysis activity when the cells become limited for oxygen and the $\Delta\mu_{H^+}$ decreases. Studies with the ATP synthase from *Paracoccus denitrificans* have revealed that the trapping of Mg^{2+}-ADP at a high-affinity inhibitory site is responsible for the low ATPase activity of this enzyme (62).

The strongest candidate for a regulator of ATPase activity in bacterial ATP synthases is the ε subunit. The ε subunit acts as an inhibitor of ATP hydrolysis activity in F_1 alone, as well as in the entire F_1F_o enzyme in bacteria (24, 25, 31, 48). The ε subunit from F_1F_o-ATP synthases is a two-domain protein which consists of an N-terminal part that forms a flattened 10-stranded β-sandwich structure and a C-terminal domain that forms two α-helices running antiparallel to one another (53, 58, 59). Furthermore, it is apparent that the ε inhibitory effect is caused by the C-terminal α-helical domain of this subunit (25, 29, 60). Two high-resolution structural arrange-

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          89                        116 119 123 127
          |                          |   |   |   |
. . .DVERAKKAKARHETILKRLDKTDKDYLRHKRALERAEVRLQVAN-SKS      Bacillus sp. TA2.A1
. . .DVDRARAAKERAESRLNSTKQDAVDFKRAELALKRAINRLDVTG---K      B. pseudofirmus OF4
. . .DVEARSAKERAEKRLQQAKQENIDFKRAELSLRRATNRLDVAG-R         B. halodurans
. . .DVARAEEAKKRAEMRLDS-KQDDVDVKRAEIALKRAVNRLDISQ--RKF     B. megaterium
. . .DKERAEAARQRAQERLNS-QSDDTDIRRAELALQRALNRLDVAG---K      B. subtilis
. . .DVLRAKARKS-GRTPLQS-QQDDIDFKRAELALKRAMNRLSVAE-MK       G. stearothermophilus
. . .DVLRAKAAKERAERRLQS-QQDDIDFKRAELALKRAMNRLSVAE-MK       Bacillus sp. PS3
. . .DEARAMEAKRKAEEHISSSH-GDVDYAQASAELAKAIAQLRVIELTKKAM    E. coli
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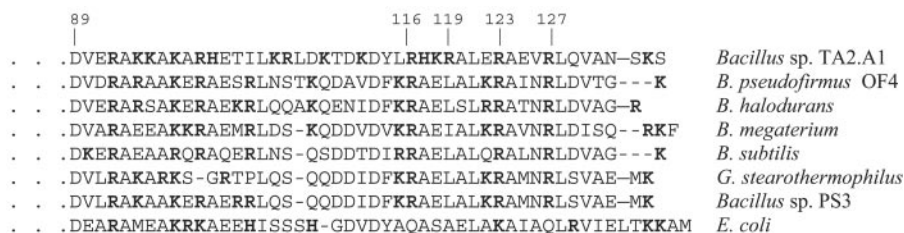
FIG. 1. Alignment of the C-terminal domain of the ε subunit from *Bacillus* species and *E. coli*. Basic residues are shown in boldface. Sources of the amino acid sequences were as follows: *B. pseudofirmus* OF4 (20), *Bacillus halodurans* (accession number NC_002570), *Bacillus megaterium* (3), *Bacillus subtilis* (46), *Geobacillus stearothermophilus* (accession number D38060), *Bacillus* sp. strain PS3 (40), and *E. coli* (54).

ments have been reported for the bovine mitochondrial central stalk in F_1 and an *Escherichia coli* complex of γ and ε (10, 43). Tsunoda et al. (52) have been able to trap conformational changes of the ε subunit in *E. coli* using cross-linking studies, thus demonstrating that at least two distinct conformations of the ε subunit exist in the enzyme complex of *E. coli*. In the ε conformation with the C-terminal domain facing toward F_1 (up state), ATP hydrolysis is strongly inhibited but ATP synthesis is not affected. In the other conformation of the ε subunit with the C-terminal domain towards the F_o (down state), the enzyme operates with equal efficiency in either direction (i.e., ATP synthesis or hydrolysis). Hara et al. (12) have shown that the electrostatic interaction between the positive charges in the C-terminal α-helical domain of the ε subunit and the negative charges in the DELSEED motif in the β subunit is essential for the inhibitory effect of the ε subunit.

Suzuki et al. (51) have investigated the factors that influence the two transitions. In the up state, the two helices of ε are fully extended and insert into F_1. Without an added nucleotide, ε is in the up state and is stabilized by the $\Delta\mu_{H^+}$. ATP addition induces the transition to the down state, and ADP counteracts the action of ATP. Based on these observations, the authors propose that increases in the $\Delta\mu_{H^+}$ and ADP concentration transform the ε subunit into the up-state conformation, thus gearing the enzyme towards ATP synthesis (51). Recent work suggests that the ε subunits of some bacteria are capable of binding ATP, and Yoshida and coworkers propose that the ε subunit acts as a built-in cellular sensor of ATP concentration (26).

A common feature of alkaliphilic F_1F_o-ATP synthases is their specific blockage of the ATP synthase in the ATP hydrolysis direction (7, 15, 17). This feature of the enzyme is more pronounced in the ATP synthase from *Bacillus* sp. strain TA2.A1, and even after reconstitution into proteoliposomes, ATP-driven proton transport or proton-coupled ATP hydrolysis activity could not be detected (7). In contrast, low but measurable ATP hydrolysis activities coupled to proton transport were reported for the ATP synthases from *Bacillus alcalophilus* (18) and *Bacillus pseudofirmus* OF4 (15). We have previously hypothesized that this property of the enzyme may be a necessity to survive at extremes of alkaline pH and temperature under aerobic growth conditions (9). Based on the observation that alkaliphilic bacteria grow under conditions where the $\Delta\mu_{H^+}$ is low due to an inverted pH gradient, the natural tendency of a freely reversible enzyme will be to operate as an ATPase, thus leading to alkalinization of the cytoplasm and hydrolysis of valuable intracellular ATP. This will be further

magnified when these aerobic alkaliphilic bacteria become limited for oxygen and the $\Delta\mu_{H^+}$ collapses. We have demonstrated that the preferential blockage of ATP hydrolysis activity and stimulation by lauryldimethylamine oxide (LDAO) were intrinsic to the F_1 moiety (7). Based on these data, it is tempting to propose that the ε subunit of the ATP synthase from strain TA2.A1 is permanently fixed in a conformation in which the rotational movement in the ATP hydrolysis direction is impaired.

In a previous study, the complete nucleotide sequence of the *atp* operon of strain TA2.A1 was determined (27). Sequence analysis and alignment of the C-terminal domain (residues 89 to 134) of the ε subunit with those from other bacilli and *E. coli* confirmed the presence of a high proportion of positively charged residues with a cluster of four basic residues (i.e., Arg, His, Lys, and Arg) at positions 116 to 119 (Fig. 1). This cluster of basic residues in strain TA2.A1, along with other basic residues, may form a very strong electrostatic interaction with the [383]DELSDED[389] motif in the β subunit of strain TA2.A1 and may effectively anchor the ε subunit in a distinct conformation that contributes to the mechanism of ATP hydrolysis blockage. To address this hypothesis, we have developed a recombinant system to overproduce TA2F_1 complexes in *E. coli* and study the effect of mutations in ε on latent ATPase activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH10B (11) was used for all cloning experiments, and *E. coli* mutant DK8 (28), lacking the ATP synthase genes of the *unc* operon, was used to overproduce the TA2F_1 complexes from the ATP synthase of *Bacillus* sp. strain TA2.A1.

Construction of expression plasmids. In a previous study, the genes encoding the F_1 subunits were cloned into the expression vector pTrc99A (Amersham Biosciences) under the control of the strong tryptophan promoter to create plasmid pTrcF$_1$ (50). Plasmid pTrcF$_1$ε$^{\Delta C}$, lacking the coding sequence for the C-terminal domain (residues 90 to 135) of ε, was constructed by digesting pTA2F$_1$ (50) with AccI and ClaI and cloning this 4.7-kb fragment into pTrc99A digested with AccI. To construct the ε mutant plasmids, pTrcε^{4A} and pTrcε^{6A}, two 0.23-kb ClaI-HindIII fragments containing codons for four and six alanine substitutions in the C-terminal domain of ε, respectively, were created by the overlap extension PCR method (16). These fragments were then cloned simultaneously with a 4.7-kb EcoRI-ClaI fragment, containing the remainder of the F_1 genes, into pTrc99A digested with EcoRI and HindIII. To create the first ClaI-HindIII fragment, two pairs of primers (viz., atpCfwd2 with atpCmutantP1 and atpCmutantP2 with pUC8rev) (Table 1) and plasmid pA3 (27) as the template were used to generate mutated DNA fragments. These overlapping fragments were then used for overlap extension PCR with the external primers atpCfwd2 and pUC8rev, and the product obtained was digested with ClaI and HindIII. To create the second ClaI-HindIII fragment containing codons for a total of six alanine substitutions, the same procedure as above was used except that plasmid pTrcε^{4A} served as a template and the primer pairs atpCfwd2 with atpCmutantP3

TABLE 1. Mutant primers used for overlap extension PCR

Primer	Sequence (5′–3′)[a]	Description or mutations	Direction
atpCfwd2	CACCTGTCCGCATTAAGCAA	5′ external primer upstream from ClaI	Forward
pUC8rev	CACACAGGAAACAGCTATGA	3′ external primer downstream from HindIII	Reverse
pTrc99Arev	CCGCTTCTGCGTTCTGATTT	3′ external primer downstream from HindIII	Reverse
atpCmutantP1	CAGGGCAgcCgcGgcCgcCAGGTAATCTTTATCTGTCTT	R116A, H117A, K118A, R119A	Reverse
atpCmutantP2	TACCTGgcGgcCgcGgcTGCCCTGGAACGGGCCGAAGTT	R116A, H117A, K118A, R119A	Forward
atpCmutantP3	CAGGgcAACTTCGGCCgcTTCCAGGGCAGC	R123A, R127A	Reverse
atpCmutantP4	GAAgcGGCCGAAGTTgcCCTGCAGGTGGCAAACAGC	R123A, R127A	Forward

[a] Lowercase letters indicate specific changes in the primer for site-directed mutagenesis.

and atpCmutantP4 with pTrc99Arev (Table 1) were utilized. The PCRs were carried out with the Phusion high-fidelity PCR kit (Finnzymes) according to the manufacturer's instructions. The region amplified by PCR in the ε mutant plasmids was confirmed by DNA sequencing.

Expression and purification of recombinant TA2F₁ complexes. The plasmids were expressed in *E. coli* DK8 by growth in 2× YT medium (45) containing 2 g/liter glucose and 100 μg/ml ampicillin at 37°C with shaking to an optical density at 600 nm of 0.35 to 0.4. An inducer, isopropyl β-D-thiogalactopyranoside (IPTG) was added (1 mM final concentration), and incubation continued for 5 h. Cells were harvested, washed with precooled 100 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 8.0), and resuspended in MOPS-Mg buffer (100 mM MOPS, pH 8.0, 2 mM MgCl₂, 10% glycerol). Phenylmethylsulfonyl fluoride (PMSF) was added to 0.1 mM, and the cells were disrupted by two passages through a French pressure cell at 20,000 lb/in². Pancreatic DNase was added to 0.1 mg/ml, and the mixture was kept on ice for 1 h or until viscosity decreased. Lysate was cleared of debris by centrifugation at 8,000 × g for 10 min, and the membranes were pelleted from the supernatant at 180,000 × g for 1 h at 4°C. The supernatant was retained and incubated at 60°C for 30 min to denature *E. coli* proteins, and these were removed by centrifugation at 180,000 × g for 20 min at 4°C. The heat-treated supernatant was then brought to 5 mM MgCl₂ and 0.5 M NaCl before application to a 50-ml column of chelating Sepharose Fast Flow (Amersham Biosciences). Before applying the TA2F₁ samples, the chelating Sepharose column was loaded with 80 ml of 0.3 M CuSO₄, washed with five column volumes of distilled water, and equilibrated with five column volumes of 20 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl, 5 mM MgCl₂, and 10% glycerol. After the TA2F₁ samples (120 ml) were loaded, the column was washed with three column volumes of the same buffer. The bound TA2F₁ complexes were eluted with one column volume of the same buffer containing 100 mM imidazole. The eluted TA2F₁ complexes were dialyzed (molecular weight cutoff [MWCO], 6,000) against 20 mM Tris-HCl (pH 8.0) containing 1 mM MgCl₂ and 10% glycerol to remove excess salt. The samples (approximately 200 mg protein) were applied to a 60-ml POROS HQ 50 column (Applied Biosystems), which was equilibrated with the dialysis buffer. Bound proteins were eluted by 12 column volumes of a 0 to 600 mM linear gradient of NaCl in the same buffer. The ATPase-containing fractions were pooled and concentrated to 30 mg/ml using Amicon Ultra centrifugal filter devices (MWCO, 100,000). The ATPase samples (1 ml) were then applied in aliquots of 0.2 ml to a GPC Superose 6 column (Amersham Biosciences) and fractionated with 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 100 mM NaCl buffer. The peak between 370 to 380 kDa was pooled and immediately used for ATPase assays and reconstitution studies.

Biochemical methods. Protein concentrations were determined by using the bicinchoninic acid protein assay kit from Sigma, with bovine serum albumin as the standard. Purified TA2F₁ complexes were analyzed by 13% polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% sodium dodecyl sulfate (SDS) (30), and protein bands were visualized by staining with Coomassie brilliant blue.

Proteolysis of TA2F₁ complexes and TA2F₁Fₒ-ATP synthase. All protease treatments of TA2F₁ complexes and TA2F₁Fₒ were performed in an assay mixture containing 50 mM Tricine [*N*-tris(hydroxymethyl)methylglycine]-NaOH, pH 8.0, 10% sucrose, 10% glycerol, 2 mM EDTA, and 2.5 mM ATP. TA2F₁ and TA2F₁Fₒ preparations (1 mg/ml) were incubated at 30°C with either trypsin (DPCC [diphenyl carbamyl chloride] treated) or α-chymotrypsin (TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone] treated) at a ratio of 1:250 to 1:1,000 (wt/wt). At various time intervals 5-μl aliquots were withdrawn and immediately assayed for ATPase activity in the presence and absence of LDAO. At intervals of 60 to

120 min, proteolysis was stopped by a 15-fold dilution of the sample into buffer (50 mM MOPS [pH 7.5] and 2 mM MgCl₂) containing PMSF (100-fold excess by weight over proteases) and stored at −70°C. The subunit composition was analyzed by SDS-PAGE.

Reconstitution of TA2F₁ complexes with F₁-stripped membranes. Inverted membrane vesicles of strain TA2.A1 were prepared as previously described (7). To strip F₁ from wild-type membranes, approximately 15 mg of washed membranes (500 μl) was resuspended in 100 ml of 5 mM Tris-sulfate buffer (pH 8.0) containing 0.5 mM EDTA and the mixture was incubated for 30 min at room temperature with gentle agitation. The homogenate was subsequently centrifuged at 180,000 × g for 45 min. The pellet was resuspended in 100 ml of 0.5 mM EDTA (pH 8.0) with stirring for 1 h at room temperature, followed by high-speed centrifugation (180,000 × g for 45 min) to harvest the membranes. The pellet was resuspended in 0.5 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 2 mM MgCl₂ and 0.1 mM PMSF. The ATPase activity (LDAO stimulated) of the F₁-stripped membranes indicated that the protocol was effective at removing >95% of the F₁ moiety.

Purified TA2F₁ complexes (1.3 mg) were incubated with freshly prepared F₁-stripped membranes (1.5 mg) in a total volume of 300 μl, and the volume was increased to 5 ml with 50 mM MOPS (pH 7.5) containing 100 mM KCl and 2 mM MgCl₂ (buffer A). The mixture was incubated on ice for 60 min and then centrifuged at 180,000 × g for 45 min. The membranes were washed with buffer A (10 ml) to remove unbound TA2F₁ complexes. This step was repeated three or four times or until the supernatant contained no ATPase activity.

Determination of ATPase and proton-pumping activity. ATP hydrolysis activity was measured at 45°C by using an ATP-regenerating assay. The assay mixture contained 100 mM MOPS (pH 7.5), 3 mM phosphoenolpyruvate, 2 mM MgCl₂, 0.25 mM NADH, 0.57 U/ml pyruvate kinase, 3.2 U/ml lactate dehydrogenase, and 50 μM or 2 mM ATP, as specified in the figure legends. The reaction was initiated by the addition of the sample into 1 ml of assay mixture, and the rate of NADH oxidation was monitored continuously at 340 nm. Nine micrograms of TA2F₁ subunit complexes was used for measurements. The activity that hydrolyzed 1 μmol of ATP per min is defined as 1 unit.

ATP-dependent proton translocation was determined at 45°C by the quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA). The 1.5-ml reaction mixture contained 5 mM potassium phosphate buffer (pH 7.5), 5 mM MgCl₂, 1 μM ACMA, and 150 to 200 μg of inverted membranes or reconstituted TA2FₒF₁ complexes. After the fluorescence signal had stabilized (100%), the reaction was initiated by the addition of neutralized ATP (1.7 mM). Fluorescence was measured with an excitation wavelength of 410 nm and an emission wavelength of 480 nm (slit width, 10 nm). At the times indicated in Fig. 7, 1.5 μM of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added.

RESULTS AND DISCUSSION

Purification and characterization of recombinant TA2F₁ complexes. To study the role of the ε subunit in the ATP synthase of *Bacillus* sp. strain TA2.A1, we developed a heterologous expression system in *E. coli* to produce recombinant TA2F₁ complexes with various mutations in *atpC*. The genes encoding TA2F₁ were overexpressed in DK8 (Δ*atp*) and purified using a three-step procedure. The purity of the recombi-
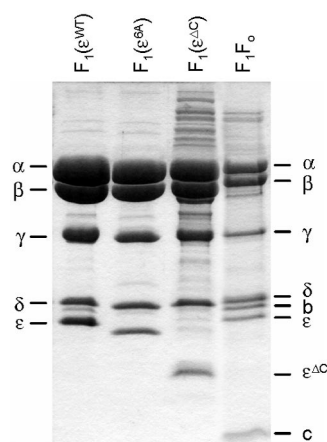
FIG. 2. Analysis of recombinant $TA2F_1$ complexes and $TA2F_1F_o$-ATP synthase by 13% SDS-polyacrylamide gel electrophoresis. Lane 1, wild type F_1; lane 2, F_1 complex containing mutant ε^{6A} subunit; lane 3, F_1 complex containing truncated $\varepsilon^{\Delta C}$ subunit; lane 4, F_1F_o-ATP synthase purified from native membranes. Fifty micrograms of F_1 complexes (lanes 1 to 3 [from the left]) and 50 μg of F_1F_o-ATP synthase (lane 4) were applied.

nant $TA2F_1$ complexes was assessed by SDS-PAGE and compared to the subunit composition of purified $TA2F_1F_o$ from native membranes (Fig. 2). The five protein bands from the recombinant $TA2F_1(\varepsilon^{WT})$ complex, corresponding to the α, β, γ, δ, and ε subunits, coincided with those from the native $TA2F_1F_o$-ATP synthase (Fig. 2, compare lanes 1 and 4). The $TA2F_1(\varepsilon^{\Delta C})$ complex, containing the ε subunit from which the C-terminal domain had been removed (Fig. 2, lane 3), and the $TA2F_1(\varepsilon^{6A})$ complex, where the basic residues in ε (i.e., Arg^{116}, His^{117}, Lys^{118}, Arg^{119}, Arg^{123}, and Arg^{127}) were substituted with nonpolar alanines (Fig. 2, lane 2), exhibited the expected changes at a protein level. The ε subunit of $TA2F_1(\varepsilon^{6A})$ migrated at a slightly lower molecular weight than $TA2F_1(\varepsilon^{WT})$.

To determine whether the recombinant $TA2F_1$ complexes were functional, ATP hydrolysis assays were carried out. Like the native F_1F_o-ATP synthase, the $TA2F_1(\varepsilon^{WT})$ was blocked in

ATP hydrolysis activity with a specific activity of 0.9 units mg $protein^{-1}$. ATPase activity was activated 30-fold by the addition of LDAO (Fig. 3A), and maximal ATP hydrolysis activity was observed at 0.04% LDAO with a specific activity of 28.5 units mg $protein^{-1}$. Purified $TA2F_1F_o$-ATP synthase was also stimulated by LDAO (20-fold, 19.4 units mg $protein^{-1}$), but 10-fold more LDAO was required to reach near-saturation ATPase activity (Fig. 3B).

To determine the effect of ε on ATP hydrolysis activity, $TA2F_1(\varepsilon^{WT})$ was compared to $TA2F_1(\varepsilon^{\Delta C})$. At low concentrations of ATP (50 μM), $TA2F_1(\varepsilon^{WT})$ exhibited very low levels of ATP hydrolysis with a specific activity of 0.12 units $protein^{-1}$ (Fig. 4A). In contrast, $TA2F_1(\varepsilon^{\Delta C})$ exhibited levels of ATPase activity that were approximately sevenfold higher (0.85 units mg $protein^{-1}$) (Fig. 4A). At higher concentrations of ATP (2 mM), $TA2F_1(\varepsilon^{WT})$ had a rate of ATP hydrolysis activity of 1.2 units mg $protein^{-1}$ and the ATP hydrolysis activity of $TA2F_1(\varepsilon^{\Delta C})$ was 4.3 units mg $protein^{-1}$ (Fig. 4B).

FIG. 4. Effect of ε on ATPase activity of recombinant $TA2F_1$ complexes. (A and B) ATP hydrolysis by $TA2F_1(\varepsilon^{WT})$ and $TA2F_1(\varepsilon^{\Delta C})$ complexes was measured with the ATP-regenerating assay, as described in Materials and Methods, by monitoring the continuous change in absorbance at 340 nm (OD_{340}). The reaction was initiated by the addition of ATP at the times indicated by arrows. ATP concentrations were 50 μM (A) and 2 mM (B). (C and D) ATPase activity of $TA2F_1$ complexes and $TA2F_1F_o$-ATP synthase using 50 μM ATP (C) and 2 mM ATP (D). (E and F) ATPase activity of $F_1(\varepsilon^{WT})$ (□), $F_1(\varepsilon^{\Delta C})$ (△), and $F_1(\varepsilon^{6A})$ (●) with increasing amounts of ATP without LDAO (E) and with 0.05% LDAO (F).
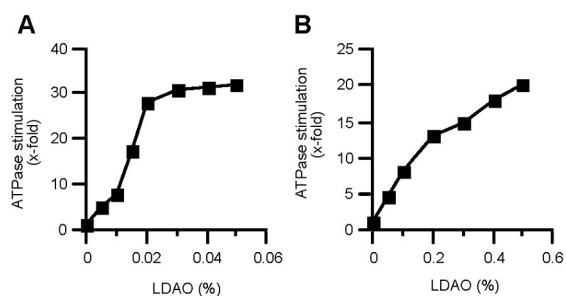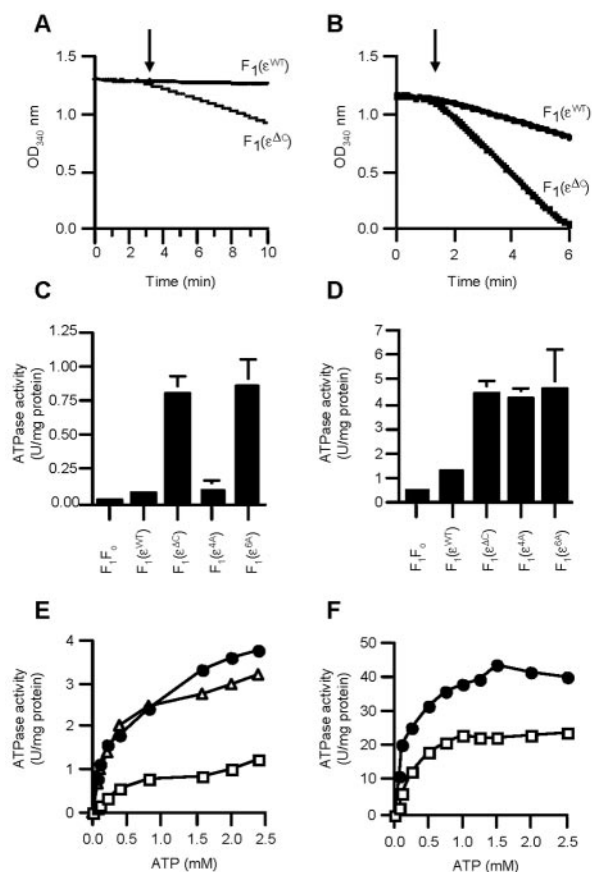

FIG. 3. Effect of LDAO on ATPase activity. ATP hydrolysis activity of purified $TA2F_1(\varepsilon^{WT})$ complex from membrane vesicles (A) and purified $TA2F_1F_o$-ATP synthase from native membranes (B) was measured with increasing amounts of LDAO at 45°C using the ATP-regenerating assay described in Materials and Methods. The reaction was initiated with 2 mM ATP. The basal ATPase activities (no LDAO addition) of $TA2F_1(\varepsilon^{WT})$ and $TA2F_1F_o$ was 0.90 units mg $protein^{-1}$ and 0.97 units mg $protein^{-1}$, respectively.

To identify the residues in the C-terminal domain of the ε subunit important in this process, we changed the cluster of basic residues at positions Arg[116], His[117], Lys[118], and Arg[119], as well as Arg[123] and Arg[127], to alanine residues (Fig. 1). In a mutant in which four of the basic residues (viz., Arg[116], His[117], Lys[118], and Arg[119]) were changed to alanines [i.e., TA2F$_1$(ε[4A])], ATP hydrolysis activity was still blocked at 50 μM ATP, but at 2 mM the ATPase activity was similar to TA2F$_1$(ε[ΔC]) (Fig. 4C and D). The TA2F$_1$(ε[6A]) mutant in which all six basic residues were changed to alanines exhibited levels of ATP hydrolysis activity that were equal to that of TA2F1(ε[ΔC]) at both low and high concentrations of ATP (Fig. 4C and D). The kinetics of ATP hydrolysis for TA2F$_1$(ε[WT]), TA2F$_1$(ε[ΔC]), and TA2F$_1$(ε[6A]) were determined (Fig. 4E). With increasing concentrations of ATP, TA2F$_1$(ε[WT]) hydrolyzed ATP at a rate that was almost proportional to the external ATP concentration, and the kinetic analyses using the Lineweaver-Burk equation indicated that the apparent K_m and V_{max} were 0.52 mM and 1.27 units mg protein^{-1}, respectively. In contrast, TA2F$_1$(ε[ΔC]) and TA2F$_1$(ε[6A]) showed high rates of ATP hydrolysis at low ATP concentrations (Fig. 4E). For TA2F$_1$(ε[ΔC]), the apparent K_m and V_{max} were 0.20 mM and 3.03 units mg protein^{-1}, respectively. Similar values were obtained for TA2F$_1$(ε[6A]) (i.e., the K_m and V_{max} were 0.17 mM and 3.11 units mg protein^{-1}, respectively). Based on the observation that TA2F$_1$(ε[6A]) behaved like TA2F$_1$(ε[ΔC]), we decided to use TA2F$_1$(ε[6A]) for further characterization studies. When the rates of ATP hydrolysis for TA2F$_1$(ε[WT]) and TA2F$_1$(ε[6A]) were measured at increasing concentrations of ATP in the presence of 0.05% LDAO, typical saturation kinetics were observed (Fig. 4F). The V_{max} of TA2F$_1$(ε[6A]) was significantly higher (twofold) than that of TA2F$_1$(ε[WT]) (Fig. 4F), but the affinity constants were in similar ranges (i.e., between 0.2 to 0.3 mM ATP).

The above results demonstrate that the C-terminal arm of the ε subunit inhibits ATP hydrolysis activity of TA2F$_1$, and the most likely mechanism for this inhibition is a strong electrostatic interaction between the cluster of basic residues in the second α-helix of ε and another subunit in the F$_1$ complex. The other subunit in this interaction remains to be identified, but, on the basis of previous studies, the DELSEED region of the β subunit seems a likely possibility (12). Studies with the chloroplast ATP synthase have shown that the C-terminal domain of the ε subunit is a regulator of ATP hydrolysis activity both in CF$_1$ and CF$_1$F$_o$, and most of this inhibitory activity can be attributed to the last 45 C-terminal amino acids (38, 39). While the ε subunit is a regulator of ATP hydrolysis activity, ATP synthesis experiments revealed that the C-terminal domain of the ε subunit is not required for photophosphorylation (39).

Lowry and Frasch (33) have highlighted that other subunits of the soluble E. coli F$_1$-ATP synthase (ECF$_1$) play a role in regulating ATP hydrolysis activity, namely, the salt bridges between the residues βD372 and γK9. We noted some variation in the apparent K_m values for ATP of the TA2F$_1$(ε[WT]) and TA2F$_1$(ε[6A]) complexes, suggesting a potential role for ε in modulating the K_m of the β subunits for ATP. ε has been proposed to act as a sensor of intracellular ATP in vivo for some bacterial species (26). This could be mediated through an effect whereby ε converts the enzyme from a form with a high affinity for binding ATP to one where the affinity is greatly

reduced. Under physiological conditions within the cell, ATP hydrolysis could simply be impaired by the lowered affinity of the enzyme for ATP. ATP hydrolysis would therefore be observed only if the intracellular ATP concentration increased to very high levels. In strain TA2.A1, the intracellular ATP content of exponential-phase cells is in the range 0.5 to 3.5 mM and this drops to below 0.1 mM in stationary-phase cells (41). A role for ε in modulating the affinity of the β subunits for Mg-nucleotide complexes has been reported for ECF$_1$ (56). In ECF$_1$, ε alters the binding affinity of high-affinity catalytic nucleotide binding site 1 for MgADP and MgATP. The affinity for MgADP and MgATP is 10-fold higher in an ε-replete ECF$_1$ compared to a ε-depleted form of the enzyme (56). The authors suggest that a potential explanation for this difference could be the reduced off rate for the Mg-nucleotide complex in the presence of ε and hypothesize that the ε subunit decelerates a conformational change step that is necessary to release ligands from the high-affinity binding site.

Proteolytic digestion of TA2F$_1$ complexes. The above results indicate that alanine substitutions in the C-terminal arm of the ε subunit prevent inhibition of ATPase activity in TA2F$_1$. To determine if these alanine substitutions induced a change in the conformation of the C-terminal arm, we studied the effect of protease treatment on TA2F$_1$, TA2F$_1$(ε[6A]), and TA2F$_1$F$_o$ (Fig. 5). When TA2F$_1$F$_o$ was treated either with chymotrypsin or trypsin, low ATP hydrolysis activities were observed (i.e., 0.32 and 0.25 units mg protein^{-1}, respectively), indicating that the enzyme was still blocked in ATP hydrolysis activity. SDS-PAGE revealed some proteolytic digestion products in both the chymotrypsin- and trypsin-treated TA2F$_1$F$_o$ enzyme when compared to the untreated control; however, the ε subunit was unaffected (Fig. 5A). Extensive proteolytic digestion was noted in TA2F$_1$(ε[WT]), but no proteolysis of the ε subunit was discernible (Fig. 5B, lanes 2 and 3), and the ATPase activity of the protease-digested TA2F$_1$(ε[WT]) remained low (i.e., 0.58 to 0.9 units mg protein^{-1}). In contrast, the ε subunit in TA2F$_1$(ε[6A]) was completely degraded by trypsin (Fig. 5C, lane 3) but not by chymotrypsin (Fig. 5C, lane 2). In neither case did protease treatment further activate ATP hydrolysis in TA2F$_1$(ε[6A]) (2.8 to 2.92 units mg protein^{-1}) compared with the untreated control (3.8 units mg protein^{-1}). These data imply that the conformation of the ε subunit in both TA2F$_1$ and TA2F$_1$F$_o$ is protected by proteolytic enzymes compared to TA2F$_1$(ε[6A]), where ε is degraded. Since no further activation of ATP hydrolysis in TA2F$_1$(ε[6A]) was observed, despite extensive proteolytic digestion, the latent ATPase activity of the enzyme seems to be controlled by the ε subunit, at least in terms of protein-protein interactions. The conformation of ε in strain TA2F$_1$ would appear to be different to that in ECF$_1$ since, under similar experimental conditions, trypsin was shown to activate ATPase activity by the removal of ε from the ECF$_1$ complex (32, 49). High-resolution structural data have indicated that the ε subunit can exist in two conformations with different arrangements of the two α-helices. In the "up-state form" the two α-helices extend into the catalytic head of the F$_1$. In the "down-state" or retracted form the helices lie in a plane parallel to the oligomeric c ring (10, 43, 52). A third conformation, where the α-helices are fully extended, has been suggested by cross-linking studies (51). Biochemical data have accumulated that indicate that the ε subunit adopts the extended form in the
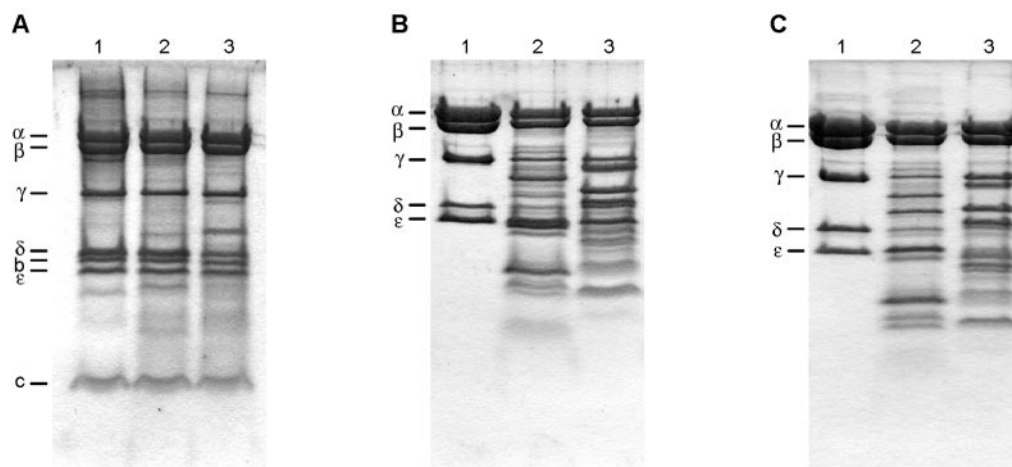
FIG. 5. Effect of protease treatment on recombinant $TA2F_1$ complexes and $TA2F_1F_o$-ATP synthase. $TA2F_1F_o$ (A), $TA2F_1(\varepsilon^{WT})$ (B), and $TA2F_1(\varepsilon^{6A})$ (C) complexes were treated with chymotrypsin (lanes 2) and trypsin (lanes 3) as described in Materials and Methods, and samples containing 50 μg of protein were electrophoresed on a 13% SDS-polyacrylamide gel. Lanes 1 contained the untreated subunit complexes.

absence of added nucleotide or in the presence of ADP, in which ATPase activity is inhibited, and that ATP counteracts ADP by favoring the retracted form through the binding of ATP to the catalytic β subunit, which is a noninhibitory form (19, 51). The blockage in ATP hydrolysis activity of the $TA2F_1F_o$-ATP synthase, even under conditions of increasing ATP, suggests that under physiological conditions the enzyme never works in the ATPase direction, and thus it is tempting to speculate that the ε subunit α-helices are fully extended, resulting in ATP hydrolysis inhibition and protection from trypsin.

Johnson and McCarty (23) have provided evidence that the chloroplast ATP synthase undergoes a conformational shift during its activation by the $\Delta\mu_{H^+}$ in light conditions, which specifically induces a change in the conformation of the C-terminal domain of the ε subunit from a protected to a solvent-exposed state. Studies on the orientation of the ε subunit within CF_1 demonstrate that it shares a common structure and positioning within the ATP synthase to that of the *E. coli* ε subunit (4, 22).

Proton pumping of reconstituted $TA2F_1$ complexes with F_1-stripped membranes. To determine what effect the alanine substitutions in ε had on the entire $TA2F_1F_o$-ATP synthase, purified $TA2F_1(\varepsilon^{WT})$ and $TA2F_1(\varepsilon^{6A})$ were reconstituted with F_1-stripped native membranes of *Bacillus* sp. strain TA2.A1. Native membranes exhibited approximately 4.4 units mg protein^{-1} of ATP hydrolysis activity in the presence of 0.5% LDAO. After F_1 was stripped off the membranes, only 13% of the original LDAO-stimulated ATPase activity could be detected, indicating that the procedure removed up to 87% of the F_1 moiety (data not shown).

After reconstitution of $TA2F_1(\varepsilon^{WT})$ to F_o membranes, proteins were visualized by SDS-PAGE (Fig. 6). Even when stripped membranes were loaded in excess onto the SDS-PAGE gel, no F_1 subunits were visible (Fig. 6, lane 3). These are clearly visible in native membranes that are overloaded on the same gel (Fig. 6, lane 5). When $TA2F_1(\varepsilon^{WT})$ was reconstituted with F_1-stripped F_o membranes [i.e., $TA2F_oF_1(\varepsilon^{WT})$], all

F_1 subunits are clearly visible, indicating that reconstitution of F_1 to F_o had indeed taken place (Fig. 6, lane 2).

When proton transport was measured by the quenching of ACMA fluorescence in native membranes, only very low levels of proton pumping (fluorescence quenching) could be detected (Fig. 7A), which is consistent with the latent ATPase activity of this enzyme in strain TA2.A1. The residual ATP-dependent proton pumping in native membranes was dissipated by the addition of the protonophore CCCP (Fig. 7A). Importantly, no proton pumping could be detected in the stripped membranes (Fig. 7B). When proton pumping measurements were performed with reconstituted $TA2F_oF_1(\varepsilon^{WT})$, ACMA fluorescence was quenched to some extent by the addition of ATP, and this was dissipated by CCCP addition (Fig. 7C). Identical experiments were performed with $TA2F_oF_1(\varepsilon^{6A})$ and
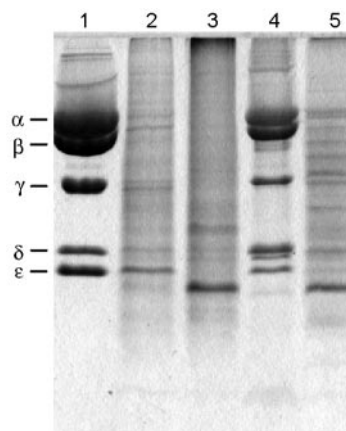

FIG. 6. Analysis of reconstituted $TA2F_1(\varepsilon^{WT})$ complex with F_1-stripped F_o membranes by 13% SDS-polyacrylamide gel electrophoresis. Lane 1, 50 μg of purified $F_1(\varepsilon^{WT})$ complex; lane 2, 50 μg of reconstituted $TA2F_oF_1(\varepsilon^{WT})$ membranes; lane 3, 75 μg of F_1-stripped membranes; lane 4, 50 μg of purified $TA2F_1F_o$-ATP synthase; lane 5, 50 μg of native membranes.
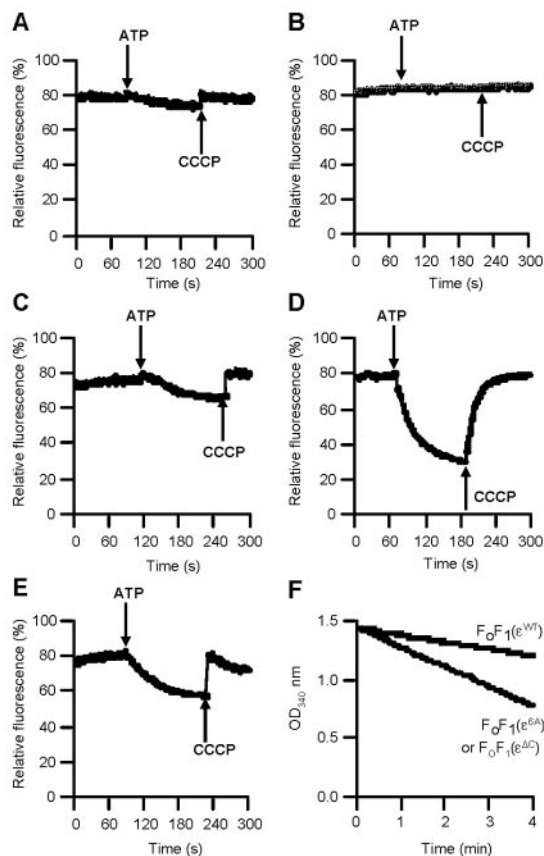
FIG. 7. ATP-driven proton translocation and ATPase activity of reconstituted $TA2F_1$ complexes with F_1-stripped F_o membranes. ATP-dependent proton translocation was determined at 45°C by the quenching of ACMA as described in Materials and Methods. The reaction was initiated by adding 1.7 mM ATP and terminated with 1.5 μM CCCP at the times indicated by arrows. (A) Native membranes; (B) F_1-stripped membranes; (C) reconstituted $F_oF_1(\varepsilon^{WT})$ membranes; (D) reconstituted $F_oF_1(\varepsilon^{6A})$ membranes; (E) reconstituted $F_oF_1(\varepsilon^{\Delta C})$ membranes. (F) ATPase activity of reconstituted $F_oF_1(\varepsilon^{WT})$, $F_oF_1(\varepsilon^{6A})$, and $F_oF_1(\varepsilon^{\Delta C})$ membranes assayed at 45°C with the ATP-regenerating assay and the addition of 2 mM ATP.

$TA2F_oF_1(\varepsilon^{\Delta C})$ membranes (Fig. 7D and E). Under these conditions, proton pumping was faster than with $TA2F_oF_1(\varepsilon^{WT})$ and the quenching of ACMA fluorescence was again reversed by CCCP. While $TA2F_oF_1(\varepsilon^{\Delta C})$ membranes showed a greater level of proton pumping when compared to $TA2F_oF_1(\varepsilon^{WT})$, the level was lower than that of $TA2F_oF_1(\varepsilon^{6A})$ despite both mutant forms having similar ATP hydrolysis activities. ATP-dependent proton pumping of $TA2F_oF_1(\varepsilon^{WT})$, $TA2F_oF_1(\varepsilon^{6A})$, and $TA2F_oF_1(\varepsilon^{\Delta C})$ was sensitive to DCCD (N,N'-dicyclohexyl-carbodiimide) (data not shown). ATPase activity was determined in $TA2F_oF_1(\varepsilon^{WT})$, $TA2F_oF_1(\varepsilon^{6A})$, and $TA2F_oF_1(\varepsilon^{\Delta C})$, and the ATP hydrolysis rates at high ATP concentration (i.e., 2 mM) (Fig. 7F) agree with those observed in the corresponding $TA2F_1$ complexes (Fig. 4D).

Cipriano et al. (5) have put forward a model that proposes that the ε subunit plays an important role in keeping the ATP synthase efficient by preventing uncoupled ATP hydrolysis. This is further supported by single-molecule experiments with F_1-ATPase from the thermophilic *Bacillus* sp. strain PS3 which demonstrate unequivocally that the ε subunit is essential for

coupling catalysis with mechanical rotation in the ATP synthesis direction (44). Dunn and coworkers showed that if the *E. coli* ATP synthase is blocked in rotation by the fusion of a 20-kDa protein to the N terminus of ε, ATPase activity is not eliminated (6). Moreover, the residual ATPase activity of this enzyme is doubled if the C-terminal domain is truncated. In the wild-type enzyme, truncation of the C-terminal domain resulted in an increased rate of ATP hydrolysis activity but a decreased rate of proton pumping, indicating uncoupled activity (6). Rondelez et al. (44) report that ε is dispensable for ATP hydrolysis activity using $\alpha_3\beta_3\gamma$ subcomplexes where the mechanochemical coupling efficiency was three ATPs hydrolyzed per turn either in the presence or absence of ε. However, these studies did not measure proton-pumping activity, and therefore it is not known whether ATP hydrolysis was coupled to proton translocation. Our data suggest that an activation of ATPase activity in $TA2F_1(\varepsilon^{6A})$ does correlate with increased proton pumping. However, in $TA2F_1(\varepsilon^{\Delta C})$, we observed a 50% decrease in proton pumping, suggesting that, in this form, ATP hydrolysis was partially uncoupled from proton-pumping activity.

We have previously demonstrated that the F_1F_o-ATP synthase of the thermoalkaliphile *Bacillus* sp. strain TA2.A1 is blocked in ATP hydrolysis activity, and this is intrinsic to the F_1 moiety (7). The results of the present study demonstrate that the ε subunit plays an important role in blocking ATP hydrolysis activity of both $TA2F_1$ and $TA2F_1F_o$. This inhibition of ATPase activity was attributed to a number of basic residues located in the C-terminal arm of the ε subunit (viz., Arg[116], His[117], Lys[118], Arg[119], Arg[123], and Arg[127]). As shown in Fig. 1, various members of the genus *Bacillus* (alkaliphilic and non-alkaliphilic) show a higher proportion of basic residues in the C-terminal arm of the ε subunit when compared to *E. coli*. We hypothesize that this serves an important regulatory function when cells become either limited for oxygen (decrease in $\Delta\mu_{H^+}$) or grow at high pH (low $\Delta\mu_{H^+}$ due to an inverted pH gradient). Notably, both conditions are prevalent in the lifestyle of alkaliphilic bacilli. Under these conditions, there is a need to overcome the thermodynamic problem of uncontrolled ATPase activity, and kinetically these bacteria have solved this problem by blocking ATP hydrolysis activity (7, 14, 15, 17). We have recently performed side-by-side experiments with inverted membrane vesicles of all *Bacillus* species listed in Fig. 1 and report that they all show various degrees of latent ATPase activity, but importantly all were able to synthesize ATP at high rates (B. Seale, S. Keis, and G. M. Cook, unpublished data). For alkaliphilic bacteria, the blockage in the ATP hydrolysis direction, but not in the ATP synthesis (proton import) direction, is further magnified by the requirement for intracellular pH homeostasis, both in the retention and acquisition of protons to prevent intracellular alkalinization. The role of the ATP synthase in alkali adaptation has been reported for *E. coli*, where the enzyme has been shown to be up-regulated at pH 8.7 as a mechanism of proton import (34). Conversely, systems involved in proton export were down-regulated in *E. coli* at high pH (e.g., cytochrome *o* and NADH dehydrogenases I and II).

The precise molecular mechanism whereby the enzyme can block rotation in one direction but rotates in the ATP synthesis direction remains unknown but clearly involves the ε subunit

and other protein partners. Notwithstanding this important role, the ε subunit alone is not sufficient for ATP synthase function at high pH. Wang and coworkers have identified the presence of amino acid motifs that are unique to the c and a subunits of alkaliphilic bacilli (55). Studies by this group with the F_1F_o-ATP synthase from the facultative alkaliphile *B. pseudofirmus* OF4 have demonstrated that these alkaliphilic motifs are critical for growth on nonfermentable carbon sources and ATP synthesis at pH 10.5 but not pH 7.5 (55).

If the ATP synthase of strain TA2.A1 is specifically adapted to work only in the ATP synthesis direction, then one could speculate that the C-terminal arm of ε is obligatory for growth under oxidative phosphorylation conditions at high pH. To our knowledge, the role of ε on the energetics of growth of *E. coli* on nonfermentable carbon sources has not been assessed, and therefore the physiological role of ε in vivo remains unknown. Future research will focus on developing a genetic system to make in vivo mutations in the ε subunit of strain TA2.A1 and assess the effect on growth at high pH.

ACKNOWLEDGMENTS

REFERENCES

1. **Abrahams, J. P., A. G. W. Leslie, R. Lutter, and J. E. Walker.** 1994. Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria. Nature **370:**621–628.
2. **Boyer, P. D.** 1997. The ATP synthase—a splendid molecular machine. Annu. Rev. Biochem. **66:**717–749.
3. **Brusilow, W. S. A., M. A. Scarpetta, C. A. Hawthorne, and W. P. Clark.** 1989. Organization and sequence of the genes coding for the proton-translocating ATPase of *Bacillus megaterium*. J. Biol. Chem. **264:**1528–1533.
4. **Capaldi, R. A., and B. Schulenberg.** 2000. The ε subunit of bacterial and chloroplast F_1F_o ATPases. Structure, arrangement, and role of the ε subunit in energy coupling within the complex. Biochim. Biophys. Acta **1458:**263–269.
5. **Cipriano, D. J., Y. Bi, and S. D. Dunn.** 2002. Genetic fusions of globular proteins to the ε subunit of the *Escherichia coli* ATP synthase: implications for *in vivo* rotational catalysis and ε subunit function. J. Biol. Chem. **277:**16782–16790.
6. **Cipriano, D. J., and S. D. Dunn.** 2006. The role of the ε subunit in *Escherichia coli* ATP synthase: the C-terminal domain is required for efficient energy coupling. J. Biol. Chem. **281:**501–507.
7. **Cook, G. M., S. Keis, H. W. Morgan, C. von Ballmoos, U. Matthey, G. Kaim, and P. Dimroth.** 2003. Purification and biochemical characterization of the F_1F_o-ATP synthase from thermoalkaliphilic *Bacillus* sp. strain TA2.A1. J. Bacteriol. **185:**4442–4449.
8. **Digel, J. G., N. D. Moore, and R. E. McCarty.** 1998. Influence of divalent cations on nucleotide exchange and ATPase activity of chloroplast coupling factor 1. Biochemistry **37:**17209–17215.
9. **Dimroth, P., and G. M. Cook.** 2004. Bacterial Na^+- or H^+-coupled ATP synthases operating at low electrochemical potential. Adv. Microb. Physiol. **49:**175–218.
10. **Gibbons, C., M. G. Montgomery, A. G. W. Leslie, and J. E. Walker.** 2000. The structure of the central stalk in bovine F_1-ATPase at 2.4 Å resolution. Nat. Struct. Biol. **7:**1055–1061.
11. **Hanahan, D., J. Jessee, and F. R. Bloom.** 1991. Plasmid transformation of *Escherichia coli* and other bacteria. Methods Enzymol. **204:**63–113.
12. **Hara, K. Y., Y. Kato-Yamada, Y. Kikuchi, T. Hisabori, and M. Yoshida.** 2001. The role of the βDELSEED motif of F_1-ATPase: propagation of the inhibitory effect of the ε subunit. J. Biol. Chem. **276:**23969–23973.
13. **Harris, D. A., P. John, and G. K. Radda.** 1977. Tightly bound nucleotides of the energy-transducing ATPase, and their role in oxidative phosphorylation. I. The *Paracoccus denitrificans* system. Biochim. Biophys. Acta **459:**546–559.
14. **Hicks, D. B., D. M. Cohen, and T. A. Krulwich.** 1994. Reconstitution of energy-linked activities of the solubilized F_1F_o ATP synthase from *Bacillus subtilis*. J. Bacteriol. **176:**4192–4195.
15. **Hicks, D. B., and T. A. Krulwich.** 1990. Purification and reconstitution of the F_1F_o-ATP synthase from alkaliphilic *Bacillus firmus* OF4. Evidence that the enzyme translocates H^+ but not Na^+. J. Biol. Chem. **265:**20547–20554.
16. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
17. **Hoffmann, A., and P. Dimroth.** 1990. The ATPase of *Bacillus alcalophilus*. Purification and properties of the enzyme. Eur. J. Biochem. **194:**423–430.
18. **Hoffmann, A., and P. Dimroth.** 1991. The ATPase of *Bacillus alcalophilus*. Reconstitution of energy-transducing functions. Eur. J. Biochem. **196:**493–497.
19. **Iino, R., T. Murakami, S. Iizuka, Y. Kato-Yamada, T. Suzuki, and M. Yoshida.** 2005. Real-time monitoring of conformational dynamics of the ε subunit in F_1-ATPase. J. Biol. Chem. **280:**40130–40134.
20. **Ivey, D. M., and T. A. Krulwich.** 1991. Organization and nucleotide sequence of the *atp* genes encoding the ATP synthase from alkaliphilic *Bacillus firmus* OF4. Mol. Gen. Genet. **229:**292–300.
21. **Jackson, P. J., and D. A. Harris.** 1988. The mitochondrial ATP synthase inhibitor protein binds near the C-terminus of the F_1 β-subunit. FEBS Lett. **229:**224–228.
22. **Johnson, E. A., Y. Evron, and R. E. McCarty.** 2001. Resonance energy transfer between tryptophan 57 in the ε subunit and pyrene maleimide labeled γ subunit of the chloroplast ATP synthase. Biochemistry **40:**1804–1811.
23. **Johnson, E. A., and R. E. McCarty.** 2002. The carboxyl terminus of the ε subunit of the chloroplast ATP synthase is exposed during illumination. Biochemistry **41:**2446–2451.
24. **Kato, Y., T. Matsui, N. Tanaka, E. Muneyuki, T. Hisabori, and M. Yoshida.** 1997. Thermophilic F_1-ATPase is activated without dissociation of an endogenous inhibitor, ε subunit. J. Biol. Chem. **272:**24906–24912.
25. **Kato-Yamada, Y., D. Bald, M. Koike, K. Motohashi, T. Hisabori, and M. Yoshida.** 1999. ε subunit, an endogenous inhibitor of bacterial F_1-ATPase, also inhibits F_1F_o-ATPase. J. Biol. Chem. **274:**33991–33994.
26. **Kato-Yamada, Y., and M. Yoshida.** 2003. Isolated ε subunit of thermophilic F_1-ATPase binds ATP. J. Biol. Chem. **278:**36013–36016.
27. **Keis, S., G. Kaim, P. Dimroth, and G. M. Cook.** 2004. Cloning and molecular characterization of the *atp* operon encoding for the F_1F_o-ATP synthase from a thermoalkaliphilic *Bacillus* sp. strain TA2.A1. Biochim. Biophys. Acta **1676:**112–117.
28. **Klionsky, D. J., W. S. A. Brusilow, and R. D. Simoni.** 1984. In vivo evidence for the role of the ε subunit as an inhibitor of the proton-translocating ATPase of *Escherichia coli*. J. Bacteriol. **160:**1055–1060.
29. **Kuki, M., T. Noumi, M. Maeda, A. Amemura, and M. Futai.** 1988. Functional domains of ε subunit of *Escherichia coli* H^+-ATPase (F_0F_1). J. Biol. Chem. **263:**17437–17442.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
31. **Laget, P. P., and J. B. Smith.** 1979. Inhibitory properties of endogenous subunit ε in the *Escherichia coli* F_1 ATPase. Arch. Biochem. Biophys. **197:**83–89.
32. **Lötscher, H.-R., C. deJong, and R. A. Capaldi.** 1994. Interconversion of high and low adenosinetriphosphatase activity forms of *Escherichia coli* F_1 by the detergent lauryldimethylamine oxide. Biochemistry **23:**4140–4143.
33. **Lowry, D. S., and W. D. Frasch.** 2005. Interactions between βD372 and γ subunit N-terminus residues γK9 and γS12 are important to catalytic activity catalyzed by *Escherichia coli* F_1F_o-ATP synthase. Biochemistry **44:**7275–7281.
34. **Maurer, L. M., E. Yohannes, S. S. Bondurant, M. Radmacher, and J. L. Slonczewski.** 2005. pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. J. Bacteriol. **187:**304–319.
35. **Nakagawa, H., S. H. Lee, V. K. Kalra, and A. F. Brodie.** 1977. Trypsin-induced changes in the orientation of latent ATPase in protoplast ghosts from *Mycobacterium phlei*. J. Biol. Chem. **252:**8229–8234.
36. **Nakamoto, R. K.** 1996. Mechanisms of active transport in the F_oF_1 ATP synthase. J. Membr. Biol. **151:**101–111.
37. **Nalin, C. M., and R. E. McCarty.** 1984. Role of a disulfide bond in the γ subunit in activation of the ATPase of chloroplast coupling factor 1. J. Biol. Chem. **259:**7275–7280.
38. **Nowak, K. F., and R. E. McCarty.** 2004. Regulatory role of the C-terminus of the ε subunit from the chloroplast ATP synthase. Biochemistry **43:**3273–3279.
39. **Nowak, K. F., V. Tabidze, and R. E. McCarty.** 2002. The C-terminal domain of the ε subunit of the chloroplast ATP synthase is not required for ATP synthesis. Biochemistry **41:**15130–15134.
40. **Ohta, S., M. Yohda, M. Ishizuka, H. Hirata, T. Hamamoto, Y. Otawara-Hamamoto, K. Matsuda, and Y. Kagawa.** 1988. Sequence and over-expression of subunits of adenosine triphosphate synthase in thermophilic bacterium PS3. Biochim. Biophys. Acta **933:**141–155.
41. **Olsson, K., S. Keis, H. W. Morgan, P. Dimroth, and G. M. Cook.** 2003.

Bioenergetic properties of the thermoalkaliphilic *Bacillus* sp. strain TA2.A1. J. Bacteriol. **185:**461–465.

42. **Richter, M. L., W. J. Patrie, and R. E. McCarty.** 1984. Preparation of the ε subunit and ε subunit-deficient chloroplast coupling factor 1 in reconstitutively active forms. J. Biol. Chem. **259:**7371–7373.

43. **Rodgers, A. J. W., and M. C. J. Wilce.** 2000. Structure of the γ-ε complex of ATP synthase. Nat. Struct. Biol. **7:**1051–1054.

44. **Rondelez, Y., G. Tresset, T. Nakashima, Y. Kato-Yamada, H. Fujita, S. Takeuchi, and H. Noji.** 2005. Highly coupled ATP synthesis by F_1-ATPase single molecules. Nature **433:**773–777.

45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

46. **Santana, M., M. S. Ionescu, A. Vertes, R. Longin, F. Kunst, A. Danchin, and P. Glaser.** 1994. *Bacillus subtilis* F_0F_1 ATPase: DNA sequence of the *atp* operon and characterization of *atp* mutants. J. Bacteriol. **176:**6802–6811.

47. **Senior, A. E.** 1988. ATP synthesis by oxidative phosphorylation. Physiol. Rev. **68:**177–231.

48. **Smith, J. B., and P. C. Sternweis.** 1977. Purification of membrane attachment and inhibitory subunits of the proton translocating adenosine triphosphatase from *Escherichia coli*. Biochemistry **16:**306–311.

49. **Smith, J. B., and P. C. Sternweis.** 1982. Subunit specific antisera to the *Escherichia coli* ATP synthase: effects on ATPase activity, energy transduction, and enzyme assembly. Arch. Biochem. Biophys. **217:**376–387.

50. **Stocker, A., S. Keis, G. M. Cook, and P. Dimroth.** 2005. Purification, crystallization, and properties of F_1-ATPase complexes from the thermoalkaliphilic *Bacillus* sp. strain TA2.A1. J. Struct. Biol. **152:**140–145.

51. **Suzuki, T., T. Murakami, R. Iino, J. Suzuki, S. Ono, Y. Shirakihara, and M. Yoshida.** 2003. F_0F_1-ATPase/synthase is geared to the synthesis mode by conformational rearrangement of ε subunit in response to proton motive force and ADP/ATP balance. J. Biol. Chem. **278:**46840–46846.

52. **Tsunoda, S. P., A. J. W. Rodgers, R. Aggeler, M. C. J. Wilce, M. Yoshida, and R. A. Capaldi.** 2001. Large conformational changes of the ε subunit in the bacterial F_1F_0 ATP synthase provide a ratchet action to regulate this rotary motor enzyme. Proc. Natl. Acad. Sci. USA **98:**6560–6564.

53. **Uhlin, U., G. B. Cox, and J. M. Guss.** 1997. Crystal structure of the ε subunit of the proton-translocating ATP synthase from *Escherichia coli*. Structure **5:**1219–1230.

54. **Walker, J. E., M. Saraste, and N. J. Gay.** 1984. The *unc* operon: nucleotide sequence, regulation and structure of ATP-synthase. Biochim. Biophys. Acta **768:**164–200.

55. **Wang, Z., D. B. Hicks, A. A. Guffanti, K. Baldwin, and T. A. Krulwich.** 2004. Replacement of amino acid sequence features of *a*- and *c*-subunits of ATP synthases of alkaliphilic *Bacillus* with the *Bacillus* consensus sequence results in defective oxidative phosphorylation and non-fermentative growth at pH 10.5. J. Biol. Chem. **279:**26546–26554.

56. **Weber, J., S. D. Dunn, and A. E. Senior.** 1999. Effect of the ε-subunit on nucleotide binding to *Escherichia coli* F_1-ATPase catalytic sites. J. Biol. Chem. **274:**19124–19128.

57. **Weber, J., and A. E. Senior.** 1997. Catalytic mechanism of F_1-ATPase. Biochim. Biophys. Acta **1319:**19–58.

58. **Wilkens, S., and R. A. Capaldi.** 1998. Solution structure of the ε subunit of the F_1-ATPase from *Escherichia coli* and interactions of this subunit with β subunits in the complex. J. Biol. Chem. **273:**26645–26651.

59. **Wilkens, S., F. W. Dahlquist, L. P. McIntosh, L. W. Donaldson, and R. A. Capaldi.** 1995. Structural features of the ε subunit of the *Escherichia coli* ATP synthase determined by NMR spectroscopy. Nat. Struct. Biol. **2:**961–967.

60. **Xiong, H., D. Zhang, and S. B. Vik.** 1998. Subunit ε of the *Escherichia coli* ATP synthase: novel insights into structure and function by analysis of thirteen mutant forms. Biochemistry **37:**16423–16429.

61. **Yoshida, M., E. Muneyuki, and T. Hisabori.** 2001. ATP synthase—a marvelous rotary engine of the cell. Nat. Rev. Mol. Cell Biol. **2:**669–677.

62. **Zharova, T. V., and A. D. Vinogradov.** 2004. Energy-dependent transformation of F_0F_1-ATPase in *Paracoccus denitrificans* plasma membranes. J. Biol. Chem. **279:**12319–12324.