Aerobic Growth of Escherichia coli Is Reduced, and ATP Synthesis Is Selectively Inhibited when Five C-terminal Residues Are Deleted from the ε Subunit of ATP Synthase

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Background: Bacterial ATP synthases are autoinhibited by subunit ε.
Results: Altering the regulatory interactions of ε increases inhibition of ATP synthesis and reduces respiratory growth of E. coli.
Conclusion: The ε subunit can have distinct regulatory interactions during ATP synthesis versus hydrolysis.
Significance: Inhibition by ε provides a bacteria-specific means to target ATP synthase for antibiotic development.

F-type ATP synthases are rotary nanomotor enzymes involved in cellular energy metabolism in eukaryotes and eubacteria. The ATP synthase from Gram-positive and -negative model bacteria can be autoinhibited by the C-terminal domain of its ε subunit (εCTD), but the importance of ε inhibition in vivo is unclear. Functional rotation is thought to be blocked by insertion of the latter half of the εCTD into the central cavity of the catalytic complex (F1). In the inhibited state of the Escherichia coli enzyme, the final segment of εCTD is deeply buried but has few specific interactions with other subunits. This region of the εCTD is variable or absent in other bacteria that exhibit strong ε-inhibition in vitro. Here, genetically deleting the last five residues of the εCTD (εΔ5) caused a greater defect in respiratory growth than did the complete absence of the εCTD. Isolated membranes with εΔ5 generated proton-motive force by respiration as effectively as with wild-type ε but showed a nearly 3-fold decrease in ATP synthesis rate. In contrast, the εΔ5 truncation did not change the intrinsic rate of ATP hydrolysis with membranes. Further, the εΔ5 subunit retained high affinity for isolated F1 but reduced the maximal inhibition of F1-ATPase by ε from >90% to ~20%. The results suggest that the εCTD has distinct regulatory interactions with F1 when rotary catalysis operates in opposite directions for the hydrolysis or synthesis of ATP.

The F-type ATP synthase is a self-contained rotary motor enzyme that is critical for efficient energy metabolism in eukaryotes and eubacteria (1–4). It is composed of a membrane-embedded F0 complex that catalyzes proton transport and an external F1 complex with three cooperative catalytic nucleotide-binding sites, and all bacterial subunits are conserved in the eukaryotic enzymes of mitochondria and chloroplasts (3, 5). So far, no complete high resolution structures have been determined for F0, but it is well accepted that the mechanism of proton transport involves rotation of a central ring of ε-subunits (ε-ring) relative to two half-channels at the interface of the c-ring with subunit a (2–4). The structural assembly of F1 (Fig. 1A) includes a hexamer of alternating α and β subunits that surround the central rotor stalk region of the asymmetric γ subunit. The lower region of γ and the N-terminal domain (εNTD) of the ε subunit form the central rotor stalk that connects with the rotary c-ring of F0, and although structural details are still lacking, a peripheral stator stalk connection is formed by F1 subunit δ and the b2 dimer of F0 (6). For ATP synthase, proton motive force (PMF) generated by the electron transport chain drives rotation of the c-ring, which is coupled with the rotation of γ within F1. Rotation of the asymmetric γ subunit helps drive conformational changes in α and β subunits that are crucial for cooperative, alternating catalysis at the three catalytic nucleotide binding sites located mainly on the β subunits.

ATP synthases can also rotate in the reverse direction by hydrolyzing ATP to generate PMF. In mitochondria, this ATPase-driven proton pumping can be blocked by a mitochondria-specific inhibitor protein called IF1 (7, 8). In contrast, many bacterial ATP synthases can be autoinhibited by the C-terminal domain (CTD) of the ε subunit (2, 9), which can inhibit both synthesis and hydrolysis of ATP (10, 11). The εCTD can transition between at least two observed states: a compact conformation (εC; Fig. 1C) that allows coupled functions (12), and an extended conformation (εX; Fig. 1A) (13) that likely corresponds to an inactive or paused state of the enzyme (14, 15). In the εC state, the two α-helices of the εCTD form a hairpin coiled coil that packs against the εNTD, as observed

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ATP Synthesis by E. coli F₀F₁ Is Inhibited by Shorter ε-Hook

FIGURE 1. Location of the ε subunit CTD and five C-terminal residues in two known conformations. A, ribbon diagram of an ε-inhibited structure of the F₁ catalytic complex from E. coli ATP synthase (13). The hexameric “head” is composed of three catalytic β subunits (shades of blue and numbered) that alternate with three α subunits (green); the frontmost α is omitted to reveal the portions of subunit γ (yellow) and of the εCTD (magenta) that are buried in the central cavity of the αβγ hexamer. The “foot” of γ and the εNTD (pink) form the rotor connection to the c-ring of F₀, B, magnified view of the εCTD segment that is buried in the central cavity of F₁. The five terminal residues of ε₁34–138, are surface-rendered, colored by element, and contact only γ (yellow) and β3 (blue). Contacts with β3 account for 78% of the buried surface area lost with the εΔ5 truncation. C, ribbon diagram of the compact conformation (ε₁) of isolated ε (16), with the εNTD oriented as in A; of the five terminal residues, ε₁34–138 (surface rendered as in B), only ε₁34 contacts the εNTD. In A and C, arrows mark the position of ε88, the point at which the εCTD is truncated for the ε88stop mutant. All proteins were rendered with Chimera (77).

with isolated ε from two species (16, 17) and in one bacterial F₁ structure (18). In mitochondrial F₁ structures, the ε homolog appears to be locked in the εΔ conformation by a unique mitochondrial subunit (19). Thus far, an extended state of the εCTD has only been observed within the enzyme in a crystal structure of Escherichia coli F₁ (13), in which the latter half of the εCTD inserts into the central cavity of F₁ and has extensive contacts with other subunits. The second α-helix of the εΔ state (ε112–125) contacts five other subunits, with apparent H-bonds and/or salt bridges to α1, α2, β1, and γ subunits. The terminal segment of εCTD (ε₁26–138) was called the ε-hook in the εΔ state, because it bends around γ and “hooks” the CTD of another catalytic subunit, β3 (Fig. 1B). The ε-hook buries extensive surface area within F₁, but has minimal specific interactions, with perhaps one H-bond (εle₁31 amide to β3-Asp₃⁷² side chain). The final C-terminal segment of ε varies significantly in sequence and length between diverse bacterial species (9). High resolution structures have been determined for bacterial ε from two other species, Bacillus PS3 (17) and Caldalkalibacillus thermarum TA2.A1 (18); both superimpose well with the εΔ conformation of E. coli ε but are shorter at the C terminus by 4 and 3 residues, respectively. Despite this, the activity of each ATP synthase can be strongly inhibited by the shorter εCTD (10, 20, 21). Thus, we postulated that the final segment of the ε-hook might be dispensable or even destabilizing for inhibition by the εCTD. Inhibition by ε is not essential for respiratory growth of E. coli, because significant growth on a nonfermentable carbon source can be achieved with the entire εCTD genetically truncated (22, 23). Interestingly, however, an early mutagenic study with E. coli found that combined deletion of 4 C-terminal and 15 N-terminal residues disrupted growth by oxidative phosphorylation, whereas the N-terminal truncation alone did not (24). In this study, we genetically deleted just the final five amino acids of εCTD (ε₁34–138) to generate an E. coli εΔ5 mutant. Growth on a nonfermentable carbon source was reduced ~60% by εΔ5, whereas complete deletion of the εCTD (ε88stop) reduced growth by ~20%. With isolated membranes, εΔ5 reduced the ATP synthesis rate by >2.7-fold, but did not alter ε inhibition of ATPase activity. Thus, the εΔ5 truncation has distinct effects on ATP synthesis versus hydrolysis. Since a new class of effective antibacterial agents has been found to target the ATP synthase (25), our results show that regulation by the εCTD provides a bacteria-specific target for future development of antibacterials against the ATP synthase.

Experimental Procedures

Plasmids and Mutagenesis—Plasmid p3DC (26), which encodes subunits β and ε, was used as template to truncate the terminal five amino acids of εCTD via site-directed mutagenesis (27). Primer 5'-CAGCTGCGGCTTACGAGTTCGTTAATAAAGAGCGATGTAACACCGGC-3 (mutations underlined) was used to replace codons for εThr₁³⁴/Lys₁³⁵ with two ochre stop codons (bold type). DNA sequencing (Upstate Medical University core facility) was used to confirm that only the desired mutations were created. DNA encoding ε with the stop codons (εΔ5) was extracted in a Ndel-Xbal restriction fragment and used to replace the corresponding fragment of pAU1 (26) to obtain pAU1εΔ5. The Ndel-Xba1 fragment was also used to move εΔ5 into pBK88H (15), creating pBKH8εΔ5. The ε88stop truncation was also moved in a Ndel-Xba1 fragment from pBK9H (15) into pAU1. The βM209L mutation was originally a gift from A. E. Senior (28) and was transferred to pAU1 in a SacI-EagI fragment.

Phenotypic Assay for Respiratory Growth—F₀F₁, either WT or with the mutants noted (Fig. 2 and Table 1), was expressed from the atp operon on low copy plasmid pAU1. For most phenotypic growth tests, pAU1 constructs were transformed into strain LE392Δ(atpl-C) (29). Individual bacterial colonies were inoculated into 10 ml of Luria Bertani broth (LB); Lennox type, Sigma Aldrich) + ampicillin (0.1 mg/ml) and grown overnight at 37 °C, with shaking at 200 rpm in a 125-ml Erlenmeyer flask. Cells were then diluted into 10 ml of fresh LB + ampicillin to obtain an A₆₀₀ of 0.1. When growth reached A₆₀₀ ~0.8, cells were diluted 100-fold into defined minimal salts medium (30) including 1 mM MgSO₄, 0.1% (v/v) trace elements (30), 0.06%
cas-amino acids (BD Difco), 6 μM thiamine, 0.1 mg/ml ampicillin, 50 μg/ml methionine, and 30 mM succinate as the nonfermentable carbon source. Growth at 37 °C was measured with 0.4 ml of culture per well in a 48-well transparent microtiter plate with lid (catalog no. 6777102; Greiner Bio-one), with triplicate samples for each distinct pAU1 construct. Growth was monitored every 15 min by A_{600} using a plate reader (Biotek Synergy HT or TECAN Infinite F200). Plates were shaken at 88.6 rpm (TECAN) or at “slow” setting (Biotek) for 20–30 h. Some assays were repeated with the same plasmids in a distinct ATP-deletion strain, DK8 (31), so the defined growth medium included 0.3 mM isoleucine and valine and omitted methionine. The DK8 strains showed less stringent differences in growth between WT and negative controls in initial tests. For more consistent performance, defined medium for DK8 had reduced cas-amino acids (0.03%) and succinate (6 mM). Also, to reduce carryover of LB, DK8 cells from starter cultures were sedimented and resuspended with defined medium before final dilution into defined medium for the assay.

**Isolation of Inverted Membrane Vesicles**—Strain LE392Δ (atpI-C) containing pAU1 (WT or δ mutants) was inoculated from individual colonies into 10 ml of LB + ampicillin (0.1 mg/ml) and grown overnight in a 125-ml Erlenmeyer flask at 37 °C with shaking (200 rpm). Cells were then diluted to 2 liters of defined minimal salts medium (30) to obtain an A_{600} of ~0.05. Additions were as noted earlier except that 30 mM glucose and 1% glycerol were the carbon sources. The cells were grown at 37 °C with constant aeration and were harvested during logarithmic growth phase. Inverted membrane vesicles (membranes) were prepared as described before (32), but with a final exchange into 50 mM MOPS-Tris, 10% (v/v) glycerol, 5 mM magnesium acetate, pH 7.5.

**Expression and Purification of Proteins**—WT-F0F1 was expressed, and F1 was released from membranes, purified, and depleted of subunits δ and ε as before (13, 15). For expression of biotinylated ε as an MBP-Bap-ε fusion protein, pBK8 (WT or δ5) was transformed into strain DH5α (33). Biotinylated ε (WT or δ5) was expressed and purified as before (15). Concentrations and purity of proteins were determined by a modified Lowry assay (34) and SDS-PAGE (35).

**Detection of F_{0}F_{1} Content in Membrane Vesicles by Immunoblotting**—Membrane samples (at least two amounts each) and known amounts of purified F1 were subjected to SDS-PAGE (35) on precast 4–20% gradient polyacrylamide gels (Bio-Rad) at 200 V for 33 min. Proteins were then transferred to a polyvinylidene difluoride membrane (Invitrogen) in a Bio-Rad Mini Trans-Blot cell at 200 mA for 1 h using 1× electrophoresis buffer (35) + 10% (v/v) methanol. The blot was blocked with TBST (10 mM Tris-Cl, 150 mM NaCl, pH 8, 0.05% Tween 20) + 5% (w/v) nonfat dried milk and then washed three times for 5 min each with TBST (0.3 M NaCl total). The blot was then incubated for 1 h with the primary rabbit anti-β antibody (1:200) in TBST + BSA (10 mg/ml); this anti-β antibody (antibody AS05-85; Agrisera) was previously tested for this purpose with *E. coli* membranes (36). The blot was washed three times as above, followed by 1 h with a fluorescent goat anti-rabbit secondary antibody (antibody 35553; Thermo Scientific), 1:1000 dilution in TBST + BSA. After three final washes as above, the blot was air-dried, and fluorescence was detected on a Typhoon 9410 imager (GE Healthcare Life Sciences) with a 532-nm laser and 526-nm short pass filter. Signals for β from known amounts of F1 provided a linear response range that was used to quantify the amount of β in different membrane samples.

**ATP Hydrolysis**—ATP hydrolysis rates were measured at 30 °C with a coupled enzymes assay (37) as described (15). Assays with membranes contained 5 mM magnesium acetate and 2 mM ATP, 5 mM KCN to inhibit NADH oxidation by the electron transport chain and 5 μM FCCP as uncoupler to prevent generation of PMF. Assays to measure ε inhibition of isolated F1(δε) included preincubation of F1(δε) with ε, 2 mM ATP, and 0.1 mM EDTA, and the values for K_{1} and maximal inhibition with Bap-εδ5 were determined as before for WT-ε and ε88stop (15). Assays of NADH oxidation by the electron transport chain were done with the same conditions but without ATP or coupling enzymes, and ≤ KCN; oxidation rates were >88% inhibited by KCN, confirming that most NADH oxidation was through the electron transport chain in all membranes.

**ATP Synthesis**—Assays of ATP synthesis by membranes were modified from (38). The membranes were diluted to 0.105 mg/ml final in 1910 μl of synthesis reaction buffer (50 mM MOPS-Tris, pH 7.5 + 10 mM magnesium acetate) in a 1 × 1-cm cuvette. Aeration was achieved throughout the assay by stirring with a cylindrical magnetic stirrer with cross-cut channels. Reactions were done at ambient temperature (±22 °C). Membranes were allowed to equilibrate for 2 min after dilution into the cuvette. NADH (50 μl of 0.1 M stock) was added to 2.5 mM final concentration, and after 1 min to establish PMF, ATP synthesis was started by adding 40 μl of ADP/Pi mixture to obtain 1 μM ADP and 3 mM P_{i} final (total assay volume, 2.0 ml). Over 4 min, 100 μl of reaction was withdrawn at 1-min intervals and added to 400 μl of ice-cold stop solution (1% TCA, 2 mM EDTA) with vortexing, and the quenched samples were kept on ice. For each membrane sample tested, a control time course was done with 10 μM FCCP present to prevent PMF formation; this corrected for (i) minimal ATP synthesis from ADP by contaminating pyruvate kinase and (ii) residual ATP in the assay (primarily from the ADP stock). For each quenched sample in duplicate, 10 μl was added to 390 μl of ice-cold luciferase assay buffer (0.1 M Tris acetate, 2 mM EDTA, pH 7.5). Samples of ATP standards were treated with stop solution and diluted as above to provide a linear response over 0.25–12 pmol in the final measurement. Samples could be frozen at this point, if needed. For each neutralized sample, 100 μl was transferred to a well of a white, opaque 96-well microtiter plate (catalog no. 236108; Nunc), which was then equilibrated to ambient temperature. The plate was placed in a Synergy HT microplate reader (Biotek) equipped with autoinjectors. For each sequential sample well, 50 μl of luciferase reagent (ATP bioluminescence assay kit CLS II; Roche Diagnostics) was injected, and luminescence was measured for 10 s (top path, no emission filter; integration, 1 s; gain, 135). The rates of synthesis for control samples (+ FCCP) were minimal and were subtracted from rates with energized membranes to obtain ATP synthesis rates caused by the ATP synthase. All membranes assayed showed linear rates of ATP syn-
growth required oxidative phosphorylation (41). As shown in Fig. 2 and Table 1, growth on succinate was negligible for cells expressing F0F1 with a control mutation, βM209L, which allows assembly of normal levels of ATP synthase on the membrane but renders it essentially inactive (28, 42). Deletion of the entire εCTD (ε88stop) reduced respiratory growth yield only ~20%, with little effect on growth rate. This is consistent with another group’s study in which ε88stop allowed respiratory growth on acetate and caused a minimal decrease in growth yield on limiting glucose (22). In contrast, deleting only five C-terminal amino acids from ε (εΔ5) reduced growth yield and growth rate (Table 1) by ~60%. Growth assays were repeated with the same plasmids expressed in a distinct Δatp-operon host strain, DK8. Initially, the DK8 strains showed less robust differences in phenotypic growth, possibly because of greater Cε-dicarboxylate transporter activity (43). For subsequent assays, succinate concentration was reduced 5-fold, and results were similar to the effects of mutations seen in Table 1 and Fig. 2 (DK8 growth yields relative to WT: ε88stop, 95%; εΔ5, 46%; βM209L, 9%). The greater phenotypic defect of εΔ5 was not simply due to poor expression or assembly of F0F1 because εΔ5 membranes showed higher F0F1 content than ε88stop (Table 1). Thus, the entire εCTD can be removed with minimal effects, but the small εΔ5 truncation perturbs the regulatory interactions of εCTD so that the capacity for in vivo oxidative phosphorylation is significantly degraded.

**Effects of εCTD Truncations on in Vitro Functions of Membrane-bound ATP Synthase**—To further examine why εΔ5 is more deleterious than ε88stop in vivo, membranes were isolated and tested for effects of εCTD truncations on activities of F0F1 in vitro. Membrane ATP hydrolysis was measured with excess uncoupler present in all conditions, to ensure that activity was not inhibited by “back pressure” from PMF. Nearly all ATPase activity of WT and mutant membranes was likely due to F0F1 because sodium azide, a catalytic site inhibitor, reduced ATPase ≈98%. In direct comparison, εΔ5 and ε88stop membranes showed 63 and 60% ATPase activity versus WT (Table 2). However, when results were normalized for the F0F1 content in membranes, intrinsic ATPase activity was 2.6-fold higher in the absence of the εCTD (ε88stop). This is consistent with prior demonstration that WT membrane ATPase activity doubled when ε inhibition was disrupted (12, 44). In contrast, the εΔ5 truncation did not significantly alter the intrinsic ATPase activity of F0F1 in membranes. This is also supported by the effects of LDAO, a detergent that is known to activate ATPase of E. coli F0F1 and F1 mostly by disrupting ε inhibition (45, 46). LDAO activated ATPase activity to the same extent for WT and εΔ5 membranes but less for ε88stop membranes, which were already activated by the absence of the εCTD (Table 1). These results suggest that the loss of ε’s 5 C-terminal residues does not significantly alter the inherent energetic balance between active and ε-inhibited forms of F0F1 in membranes.

As suggested previously for ε88stop (22), it is possible that partial functional uncoupling of F1 from F0 contributes to the in vivo phenotypic defect of εΔ5. One result of this could be that some ATPase activity is not thermodynamically linked to PMF; under respiratory conditions that would drive net ATP synthesis through well coupled F0F1, unregulated ATP hydrolysis

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**TABLE 1**

| Strain       | Growth yield | Growth rate | Relative F0F1 content<sup>a</sup> |
|--------------|--------------|-------------|----------------------------------|
| WT           | 100 ± 9 (5)  | 0.2 ± 0.01 (5) | 1                                |
| ε88stop      | 78 ± 13 (4)  | 0.14 ± 0.03 (4) | 0.23 ± 0.006 (2)                 |
| εΔ5          | 38 ± 4 (6)   | 0.08 ± 0.01 (6) | 0.54 ± 0.001 (2)                 |
| βM209L       | 4 ± 2 (3)    | NS<sup>b</sup>  | ND<sup>c</sup>                     |

<sup>a</sup> Amounts of F0F1 in membrane were quantified by anti-β antibody (Experimental Procedures). The values were normalized relative to WT.

<sup>b</sup> NS, not significant.

<sup>c</sup> ND, not determined, but previously measured as equivalent to WT (28).

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**Proton Pumping**—Proton pumping activity of membranes was measured by monitoring fluorescence quenching of ACMA, which reflects ΔpH (39). Membranes were diluted to 0.1 mg/ml in assay buffer (20 mM MOPS-Tris, pH 7.5, 50 mM KCl, 5 mM magnesium acetate) + 1 μM ACMA and equilibrated for ~9 min, and the assay was started by adding NADH or ATP to drive proton pumping. Total assay volume was 2 ml in a 1 × 1-cm fluorescence cuvette, and aeration was maintained by stirring, as in the ATP synthesis assays. Assays were done at 30 °C on a Fluoromax-4 or Fluorolog-3 (Horiba Scientific) with excitation/emission wavelengths (nm) of 430/560, excitation/ emission slits of 5/4 nm, gratings set at 1200, integration time of 0.5 s, and interval time of 7.5 s. For WT and each ε mutant, at least two separate preparations of membranes from different cell growths were tested.

**BioLayer Interferometry (BLI)**—Biolayer interferometry was used to study interactions between isolated F1 (εδε) and biotinylated ε variants. Experiments were done in an Octet RED system (Pall ForteBio) as described (15, 40).

**Results**

**Effects of εCTD Truncations on Aerobic Growth**—To observe whether εCTD truncations affect in vivo function of the ATP synthase, bacteria expressing WT or mutant forms of F0F1 were grown with a nonfermentable carbon source, succinate, so that some ATPase activity is not thermodynamically linked to PMF; under respiratory conditions that would drive net ATP synthesis through well coupled F0F1, unregulated ATP hydrolysis...
**TABLE 2**  
Effects of eCTD truncations on the *in vitro* activities of membranes

|     | ATP hydrolysis | Proton-pumping assays† |
|-----|---------------|------------------------|
| ε   | Specific activity‡ | Relative‡ | Stimulation by LDAO* | Inhibition by DCCD‡ | ATP synthesis‡ | Driven by NADH | Driven by ATP |
| WT  | 5.2 ± 0.3 (7)    | 1.0        | 1.9 ± 0.3 (7)        | 77 ± 2 (5)         | 105 ± 7 (8)   | 71 ± 2.8 (7)   | 64 ± 2.6 (8)  |
| εΔ5 | 3.3 ± 0.3 (9)    | 1.1        | 2.1 ± 0.1 (9)        | 73 ± 2 (5)         | 38 ± 2 (5)    | 77 ± 3.7 (3)   | 60 ± 5.4 (4)  |
| ε88stop | 3.1 ± 0.6 (3)  | 2.6        | 1.4 ± 0.04 (3)       | 81 ± 3 (3)         | 100 ± 8 (4)   | 59 ± 1.7 (4)‡  | 53 ± 6.5 (4)‡  |

* The units are μmol/min/mg of membrane protein. Each experiment included duplicate assays for each sample in each condition tested.
* ATPase normalized to levels of catalytic β subunit in membranes, relative to WT.
* Ratio of activity ± 0.5% LDAO in assay.
† Inhibition (%) after preincubation of membranes with DCCD (0.1 mM, 30 min, 4 °C); εΔ5 value is not significantly less than others (unpaired t tests, p > 0.11).
‡ The units are nmol ATP/min/mg of membrane protein.
§ Maximal quenching (%) of ACMA fluorescence after addition of NADH (0.5 mM) or ATP (1 mM).
¶ Significantly different from WT or ε88stop result (unpaired t tests, p < 0.026).
∫ Not significantly different from WT or εΔ5 result (unpaired t tests, p = 0.18).

![Image](image_url)

**FIGURE 3. Respiratory generation of proton motive force by membranes.** Proton pumping was measured by quenching of fluorescence of the dye ACMA (see "Experimental Procedures"). A, respiration was initiated by addition of NADH to 0.5 mM. Dashed lines represent proton pumping by untreated ε88stop (blue), εΔ5 (red), and WT (black) membranes. Solid lines represent proton pumping by the same membranes after treatment with DCCD to block possible proton leakage through F0. Once the NADH was depleted, the relaxation of ACMA fluorescence quenching reflects all intrinsic membrane transport processes that contributed to collapse of the ΔpH. B, ATP was added to 1 mM to initiate proton pumping by F0F1, and after 150 s, FCCP was added to 5 μM to collapse the PMF. Table 2 summarizes statistical results from multiple experiments for both NADH- and ATP-driven pumping.

would create a futile cycle that reduces the efficiency of cellular energy conversion. To test for this, ATP hydrolysis was measured after treating membranes with DCCD, a covalent modifier of the c-ring that blocks proton transport through F0. For well coupled F0F1 complexes, blocking proton transport with DCCD also inhibits ATP hydrolysis (47). As shown in Table 2, the ε88stop truncation did not significantly alter the sensitivity of membrane ATPase to DCCD, although the original study by Cipriano and Dunn (22) showed slightly reduced DCCD inhibition for ε88stop membranes. The εΔ5 truncation resulted in a small and insignificant decrease in inhibition by DCCD (Table 2).

Membranes were also tested for possible effects of eCTD truncations on ATP synthesis. As shown in Table 2, εΔ5 membranes showed a >2.7-fold lower rate for ATP synthesis. This was not due to reduced PMF, because NADH-driven respiration generated similar ΔpH gradients for WT and εΔ5 membranes (Fig. 3 and Table 2). The lower ATP synthesis rate was also not due to the ~50% lower F0F1 content in εΔ5 membranes because ε88stop membranes had even lower F0F1 content (Table 1) but had ATP synthesis rates similar to that of WT pAU1 membranes (Table 2). This is consistent with prior studies showing that F0F1 content of haploid membranes exceeds that necessary for ATP synthesis rates *in vivo* (48) and *in vitro* (49). In fact, because εΔ5 membranes had more F0F1 than ε88stop or WT haploid membranes, their 2.7-fold lower synthesis rate probably reflects an even greater intrinsic inhibition of ATP synthesis by the εΔ5 subunit. Control assays were also included to test whether reduced ATP synthesis by εΔ5 membranes was due in part to any uncoupled ATPase activity. The ATP synthesis rates of εΔ5 and WT membranes were not significantly altered by the presence of 10 μM AMPPNP, which inhibits ATPase but not ATP synthesis (50). Together, these results indicate that the εΔ5 truncation directly increases ε inhibition of ATP synthesis by F0F1.

Another test for possible coupling defects between F1 and F0 is to monitor the kinetics of proton pumping by isolated, inverted membranes. Altered coupling between F1 and F0 might allow uncontrolled, passive flux of protons, which would decrease the capacity to generate PMF by respiration or by ATPase-driven proton pumping (51). As shown in Table 2 and Fig. 3A for NADH-driven proton pumping, εΔ5 membranes generated similar or better PMF than did WT, but ε88stop membranes generated partially reduced PMF. To test for F0-specific proton leaks, membranes were treated with DCCD before addition of NADH. DCCD had a similar effect on NADH-driven proton pumping for WT and εΔ5 membranes (Fig. 3), so εΔ5 did not cause any increased leak through F0. Fig. 3 also shows that the lower PMF achieved with ε88stop membranes (Table 2) was not due to greater proton leaks, because (i) upon depletion of NADH, the gradient collapsed with a time course similar to WT and εΔ5 membranes, and (ii) DCCD had a minimal effect on proton pumping by ε88stop membranes. The reduced PMF generated with ε88stop membranes was not
due to gross changes in the capacity of the electron transport chain, because two preparations of e88stop membranes showed NADH oxidation rates at least as fast as with WT membranes (0.8 and 0.7 μmol/min/mg protein, respectively). In any case, the current proton pumping results show that the eCTD truncations do not cause any increased proton leak in the membrane preparations. Proton pumping was also tested when PMF was generated by ATP hydrolysis via F0,F1, and results were similar for WT, eΔ5, and e88stop membranes (Table 2 and Fig. 3B). Cipriano and Dunn (22) noted a more significant defect in ATPase-driven proton pumping for eΔ5 (15). Cipriano and Dunn (22) noted a more significant defect in ATPase-driven proton pumping for eΔ5 (15). Cipriano and Dunn (22) noted a more significant defect in ATPase-driven proton pumping for eΔ5 (15). Cipriano and Dunn (22) noted a more significant defect in ATPase-driven proton pumping for eΔ5 (15). 

*Effects of eΔ5 Truncation on Interactions of eCTD with Isolated F1*—In vitro, the catalytic complex of ATP synthases can be released from the membrane as a soluble F1, ATPase. Isolated bacterial F1 is strongly inhibited by e but, upon dilution, e can dissociate, activating the enzyme (2, 9). Previously, BLI kinetic assays of protein-protein interactions showed that the conformation of the eCTD controls dissociation of e from E. coli F1 (15), and e probably does not dissociate at all when it adopts the inhibitory extended conformation (e2), with part of the eCTD buried in the central cavity of F1 (13, 15). Here, BLI was used to test whether the eΔ5 truncation changes the interactions of eCTD with F1. With F1 bound to immobilized eΔ5 in buffer alone, 87% of F1/eΔ5 complexes dissociated very slowly in buffer only (Fig. 4, trace 1, and Table 3). This is similar to the behavior of F1/WT-e (15), but the difference in their slow dissociation rates is near the limit of sensitivity for BLI in these conditions. Although WT-e on F1 is strongly biased toward the tightly bound inhibitory state, transition in and out of that state is dynamic (14, 15); addition of excess ATP in the BLI dissociation step (with EDTA present to prevent hydrolysis) rapidly shifts F1/WT-e complexes to dissociate ~80-fold faster, as if the eCTD were completely absent (Fig. 4, traces 3 and 6). ATP/EDTA in the dissociation step produced faster, essentially monophasic dissociation of F1/eΔ5 complexes (Fig. 4, trace 2) with no noticeable lag, but with a rate ~6-fold slower than for F1/WT-e (Table 3). As seen before (15), when F1/WT-e was bound in the presence of ATP/EDTA, subsequent exposure to Mg2+ allowed hydrolysis and rapid switching to the e-inhibited state at the catalytic dwell, and post-hydrolysis conditions (MgADP/Pi) in the dissociation step stabilized e in the tightly bound form (Fig. 4, trace 4). F1/eΔ5 complexes also showed rapid reversal to a tightly bound state on switching from ATP/EDTA during F1/eΔ5 association to MgADP/Pi in the dissociation phase (Fig. 4, trace 5). Overall, these results indicate that although the eCTD can still undergo dynamic transitions between different conformations, the absence of five terminal residues from the eCTD significantly stabilizes a tightly bound state of eΔ5 on F1 relative to the dissociable state.

The ATPase activity of isolated E. coli F1 is inhibited >90% by bound WT-e (9, 15). Because eΔ5 showed a bias toward tight binding, we investigated whether this correlates with greater inhibition. An N-terminal Bap tag on e does not affect its inhibition of isolated F1 (15). As shown in Fig. 5, the Kd of 0.7 nm for Bap-eΔ5 is similar to the Kd for WT-e (0.5 nm) but does not reflect the increased stability of the tightly bound state as indicated by the BLI assays of F1/eΔ5 binding. Furthermore, maximal inhibition by eΔ5 was only ~20%. This is similar to the ~24% maximal inhibition by e88stop under the same conditions, although the Kd for e88stop is nearly 20-fold weaker because of the complete absence of the eCTD (15). This surprising finding indicates that the shorter eCTD of eΔ5 still con-

### TABLE 3

| Conditions for association | Conditions for dissociation | Amplitude 1 | koff 1 | Amplitude 2 | koff 2 |
|---------------------------|-----------------------------|-------------|--------|-------------|--------|
| Buffer                     | Buffer                      | e88stop (trace 6) | 96% | 3.0 × 10^{-3} | 13% | <10^{-1} |
| Buffer                     | Buffer                      | eΔ5 (trace 1)    | 94% | 6.3 × 10^{-4} | 6% | 9.5 × 10^{-4} |
| Buffer                     | ATP-EDTA                    | WT-e (trace 2)   | 94% | 4.0 × 10^{-3} | 5% | <10^{-3} |
| ATP-EDTA                  | MgADP-Pi                    | WT-e (trace 4)   | <100% | <10^{-6} |
| ATP-EDTA                  | MgADP-Pi                    | eΔ5 (trace 5)    | ~100% | <10^{-6} |

FIGURE 4. Effects of eΔ5 on dissociation of F1/e complexes. Only the dissociation phase is shown for representative BLI assays. In previous steps, similar amounts of biotinylated e (Bap-e, WT, e88stop, or eΔ5) were immobilized on streptavidin-coated BLI sensors, and then sensors were incubated 15 min with excess F1-IgG to form F1/e complexes (100% BLI signal, nm: trace 1, 0.5; traces 2–5, done in parallel, 0.39 ± 0.02; trace 6, 0.6). Buffer conditions for association/dissociation phases: trace 1 (eΔ5, cyan) and trace 6 (e88stop, brown), buffer/buffer; trace 2 (eΔ5, blue) and trace 3 (WT-e, magenta), buffer/ATP-EDTA (1 mM each); trace 4 (WT-e, red) and trace 5 (eΔ5, green), 1 mM ATP-EDTA/2 mM Mg2+, 1 mM ADP, 1 mM Pi). Black lines are nonlinear regression fits (GraphPad Prism) for two phases of exponential decay; fitting results are summarized in Table 3. Note that data for trace 6 are reproduced from Ref. 15.
ATP Synthesis by E. coli F_0F_1 Is Inhibited by Shorter ε-Hook

FIGURE 5. Inhibition of F_1-ATPase by WT and truncated forms of the ε subunit. Results for WT-ε (○) and ε88stop (▼) are reproduced from Ref. 15. Assays for varying concentrations of εΔ5 subunit (●) were measured with 0.75 nM F_1(εΔ5). The specific activity of F_1(εΔ5) alone was 60.9 μmol/mg. For each data set, the curve shown is from a nonlinear regression fit to a quadratic equation described in Ref. 15. For εΔ5, regression indicated maximal inhibition of F_1 (εΔ5) = 20% (95% confidence interval, 19–22%), and K_inh = 0.68 nM (95% confidence interval, 0.33–1.02 nM); R^2 = 0.972 (GraphPad Prism). In parallel with εΔ5 assays, control assays confirmed that 100 nM WT-ε inhibited F_1(εΔ5) > 85%.

Contributions to tight binding to F_1, but that the five terminal residues of ε are critical for strong inhibition of F_1-ATPase activity. However, εΔ5 does inhibit ATP synthesis and hydrolysis by F_0F_1 on membranes (Table 2), suggesting that F_0-F_1 interactions are important for the εΔ5 subunit to achieve inhibition of ATP hydrolysis.

Discussion

Earlier studies with F_0F_1 of E. coli (52) and B. PS3 (53) suggested that the extended εCTD inhibits ATPase but not ATP synthesis, based on disulfide cross-links to trap the εCTD in extended states. However, it is not clear that those γ–ε cross-links occurred in native conformations of the enzyme. For example, the E. coli cross-linking sites (γ99, ε118) were based on a structure of an isolated complex of truncated γ with ε (54), but are 28 Å apart (Ca–Ca) in the structure determined for ε-inhibited F_1 (13). Subsequent studies showed that deleting the εCTD increased the ATP synthesis rate 3-fold for B. PS3 F_0F_1 (10) and activated ATP synthesis more than it activated ATPase for E. coli F_0F_1 (11). Thus, it is clear that the εCTD can inhibit both ATP hydrolytic and synthetic directions of rotary catalysis in bacterial ATP synthases. Here, in vitro results for F_0F_1 containing the εΔ5 subunit further show that altering interactions of the εCTD with F_1 can preferentially increase inhibition of ATP synthesis (Table 2).

Prior studies with membrane-bound E. coli F_0F_1 (12, 44) indicate that, on average, ~50% of F_0F_1 complexes are in an ε-inhibited state. Current results with ε88stop membranes support this, because the intrinsic ATPase activity is 2.6-fold greater in the absence of the εCTD (relative ATP hydrolysis in Table 2). ATP synthesis results with ε88stop membranes also likely reflect a greater fraction of active F_0F_1 complexes without the εCTD: compared with WT, ε88stop membranes showed about the same synthesis rates (Table 2), although they contained ~4-fold less F_0F_1 (Table 1) and generated lower PMF by NADH oxidation (Fig. 3). In the presence of MgADP/PI, PMF activates F_0F_1 in E. coli membranes (55), probably because of release from the ε-inhibited state (13, 15). Thus, without the inhibitory εCTD, ε88stop membranes in this study likely contained a higher fraction of active F_0F_1 complexes and so achieved high ATP synthesis rates even with a reduced PMF. In contrast, εΔ5 membranes showed ATP synthesis rates nearly 3-fold less than those for WT or ε88stop (Table 2), even though F_0F_1 content was ~2-fold greater in εΔ5 than in ε88stop membranes (Table 1). The low synthesis rate was not due to uncoupling, because εΔ5 membranes generated a greater NADH-driven pH gradient than did WT and showed no greater F_0-specific proton leak (Fig. 3). On the other hand, εΔ5 membranes showed intrinsic ATPase rates, activation by LDAO, and ATPase-driven proton pumping that were very similar to the values obtained with WT membranes (Table 2). Thus, the εΔ5 truncation specifically increased ε inhibition of ATP synthesis without increasing inhibition of ATP hydrolysis or uncoupling ATPase from proton pumping. Further, without interactions with F_0, εΔ5 subunit bound isolated F_1 with high affinity (Fig. 4) but inhibited F_1-ATPase minimally, as seen with the εNTD alone (Fig. 5). This suggests that contacts of the ε-hook with the CTD of subunit β3 (Fig. 1B) are important for inhibition of F_1-ATPase.

Different effects on ATP synthesis versus hydrolysis modes have been noted for other inhibitors (reviewed in Ref. 50). For example, azide or AMPPNP inhibit ATP hydrolysis but not ATP synthesis, whereas some fluorescent analogs of ADP inhibit ATP synthesis more than hydrolysis. However, what mechanisms might explain how the εΔ5 truncation selectively increases inhibition of ATP synthesis? Thus far, only one ε-inhibited state has been observed structurally (13). If one assumes that the observed ε_X state is responsible for inhibition of both synthesis and hydrolysis, then the εΔ5 truncation could preferentially increase the energy barrier for activation from the ε_X state during rotation in the direction of ATP synthesis. Control of ε conformation by rotational direction has been proposed before (56). Such directional asymmetry has been demonstrated for an ADP-inhibited state that pauses the enzyme at a specific rotary angle: in single-molecule studies with B. PS3 F_1, magnetically driven torque reactivated the enzyme after 40° of forced rotation in the direction of hydrolysis but not after 120° in the direction of ATP synthesis (57). For regulation by the εCTD, an alternative is the bidirectional ratchet model, in which the εCTD has distinct regulatory interactions with F_1 during opposite directions of rotary catalysis (6, 58). With this model, the εΔ5 truncation could preferentially enhance the stability of the inhibitory state that forms primarily during ATP synthesis mode. Our present results on interactions of the εΔ5 subunit with isolated F_1 seem more consistent with this second model: kinetic assays for F_1/ε interactions (Fig. 4) indicate that the tightly bound state of F_1/εΔ5 reverses to a dissociable state more slowly than for F_1/WT-ε, but the tightly bound state of εΔ5 causes minimal inhibition of F_1-ATPase activity (Fig. 5).

The existence of a distinct F_1/εCTD interaction state is also consistent with our recent collaborations to study conformational changes of the εCTD by single-molecule FRET with probes on γ and on helix-1 of the εCTD. Initial studies with isolated F_1 (59, 60) showed bimodal distribution of FRET efficiencies that correlate with the ε_c and ε_x states, and nucleotides shifted the balance between the two FRET states in agreement.
with our bulk assays of F/ε interactions (15). Subsequent studies with FRET-labeled F0F1-liposomes revealed a trimodal distribution of FRET efficiencies in the presence of MgATP that cannot be explained by the two known orientations of helix-1 of ε (13, 16). Thus, it seems likely that the ε-CTD can form distinct interactions with F during opposite directions of rotary catalysis and that ε5 preferentially stabilizes or promotes formation of the tightly bound state that inhibits the ATP synthesis direction.

For the direction of ATP hydrolysis, single-molecule rotation assays (14, 61, 62) and our recent enzymological study (15) show that inhibition by the ε-CTD initiates at the catalytic dwell angle after the hydrolytic step. In contrast, the only available structure of ε-inhibited F appears to be paused after further 40° rotation to an angle near the next dwell for ATP binding (13). Some rotational data could suggest dynamic oscillation between these two angles during a long inhibitory pause (Ref. 14 and Fig. 3A), so perhaps these represent two positions of the ε-CTD that have distinct regulatory effects during opposite directions of rotary catalysis. In detail, ε5 might also cause some type of mechanical slip between F and F only during rotation in the ATP synthesis direction, but further tests are needed to explore these possibilities.

Correlation of the ε5 Phenotypic Growth Defect with Inhibited ATP Synthesis—Reduced ATP synthesis rate was the only significant functional defect identified in vitro with ε5 membranes, and this is likely the primary reason that cells expressing ε5 grew poorly by oxidative phosphorylation. With the entire ε-CTD absent, cells showed better phenotypic growth, and rates of in vitro ATP synthesis were normal, even though ε88stop membranes contained less F1F1. An earlier study reported that deletion of 10 C-terminal residues from E. coli ε also allowed normal growth yield on succinate (23), indicating that in vivo ATP synthesis is more effective than with ε5. Together, these results suggest that residues between ε128–133 are important for inhibition of ATP synthesis.

It should be noted that the pAU1 construct used here expresses the entire atp operon, and ε5 membranes contained ~4-fold greater F/F than in haploid membranes. Even haploid expression of E. coli F/F is not rate-limiting for ATP synthesis in vitro (48), so the low rate measured for in vitro ATP synthesis by ε5 membranes probably represents a greater intrinsic inhibition by ε5. Thus, ε5 should cause an even larger defect in phenotypic growth in a strain expressing lower, haploid levels of F/F, and we are currently reengineering our expression system to test for this.

Summary—Overall, our results are consistent with the idea that the ε-CTD may be fine-tuned in different bacterial species to regulate ATP synthesis and hydrolysis functions according to the distinct metabolic/environmental demands of each species (2, 9). We showed that a minor truncation of the ε-hook selectively increased inhibition of ATP synthesis and reduced the capacity for cell growth on a nonfermentable carbon source. ATP synthases from two Gram-positive species appear to be missing the last 3–4 residues of the ε hook (17, 18) but still show strong inhibition of ATPase by ε (20, 21). This could suggest that inhibitory behavior in different species involves co-evolution of one or more subunits that interact with the ε-CTD. This correlates with results of recent computational studies of co-evolution in protein complexes, which used interactions of γ and ε as a test case (63, 64). There are also indications that ε inhibition also occurs in the enzyme of several species of Mycobacterium (65, 66), and the CTD of most mycobacterial ε subunits is ~17 residues shorter than that of E. coli, although different possible alignments make it uncertain how much of the hook and/or helix-2 are absent (67, 68). Mycobacterial ATP synthase is the target of a new class of antibiotics, the diarylquinolines, and the lead drug, bedaquiline, has been approved for treatment of multidrug-resistant tuberculosis (25, 69). Modified diarylquinolines have been developed to attack other Gram-positive pathogens including Staphylococcus aureus but so far, these show significant inhibition of mitochondrial ATP synthase (70). Bacterial ATP synthase function is also essential or important for the viability or virulence of Gram-negative pathogens (71–74). Thus, it will be important to explore how ε inhibits ATP synthases in a range of bacterial pathogens. Results of the current study support the concept that ε inhibition can provide a bacteria-specific means to target the ATP synthase for development of future antibiotics.

Author Contributions—N. B. S. performed all experiments shown. T. M. D. prepared Fig. 1. N. B. S. and T. M. D. conceived the study, wrote the paper, analyzed and reviewed all results, and approved the final version of the manuscript.

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