The Role of the ε Subunit in the *Escherichia coli* ATP Synthase

THE C-TERMINAL DOMAIN IS REQUIRED FOR EFFICIENT ENERGY COUPLING

Received for publication, September 12, 2005, and in revised form, October 31, 2005. Published, JBC Papers in Press, November 2, 2005, DOI 10.1074/jbc.M509986200

Daniel J. Cipriano and Stanley D. Dunn

From the Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1, Canada

The role of the C-domain of the ε subunit of ATP synthase was investigated by fusing either the 20-kDa flavodoxin (Fd) or the 5-kDa chitin binding domain (CBD) to the N termini of both full-length ε and a truncation mutant ε_{88-stop}. All mutant ε proteins were stable in cells and supported F_{1}F_{0} assembly. Cells expressing the Fd-ε or Fd-ε_{88-stop} mutants were unable to grow on acetate minimal medium, indicating their inability to carry out oxidative phosphorylation because of steric blockage of rotation. The other forms of ε supported growth on acetate. Membrane vesicles containing Fd-ε showed 23% of the wild type ATPase activity but no proton pumping, suggesting that the ATP synthase is intrinsically partially uncoupled. Vesicles containing CBD-ε were indistinguishable from the wild type in ATPase activity and proton pumping, indicating that the N-terminal fusions alone do not promote uncoupling. Fd-ε_{88-stop} caused higher rates of uncoupled ATP hydrolysis than Fd-ε, and ε_{88-stop} showed an increased rate of membrane-bound ATP hydrolysis but decreased proton pumping relative to the wild type. Both results demonstrate the role of the C-domain in coupling. Analysis of the wild type and ε_{88-stop} mutant membrane ATPase activities at concentrations of ATP from 50 μM to 8 mM showed no significant dependence of the ratio of bound/released ATPase activity on ATP concentration. These results support the hypothesis that the main function of the C-domain in the *Escherichia coli* ε subunit is to reduce uncoupled ATPase activity, rather than to regulate coupled activity.

ATP synthase is the enzyme responsible for the formation of ATP during oxidative phosphorylation. This enzyme, found on the inner membranes of mitochondria and bacteria, and on the thylakoid membranes in chloroplasts, uses the energy stored in a transmembrane proton gradient to produce ATP from the precursors ADP and P_{i}. The enzyme can be subdivided into two sectors. In *Escherichia coli*, the F_{1} sector is composed of 5 different polypeptides with a stoichiometry of α_{3}β_{3}γεσ, and houses the three catalytic nucleotide binding sites. The F_{0} sector forms a proton-specific pore and is comprised of three different integral membrane proteins, showing a stoichiometry of α_{3}β_{3}γεσ. During ATP synthesis, protons move through the F_{0} pore and drive the rotation of the c_{i0}εγ oligomer. Movement of this “rotor” drives the sequential conformational changes in α_{3}β_{3} that promote both the binding of substrates, ADP and P_{i}, and the formation and release of ATP as predicted by the binding change mechanism of Paul Boyer (1). The two δ subunits, combined with the δ subunits, form a peripheral stalk that connects the F_{1} and F_{0} sectors, preventing their rotation relative to each other. In *E. coli*, ATP synthase is reversible and under anaerobic conditions can act as an ATP-driven proton pump energizing the inner membrane to power membrane transporters and the flagellar motor. For recent reviews see Refs. 2–4.

The ε subunit, which lies at the interface of F_{1} and F_{0}, has been suggested to function within the enzyme as an inhibitor, a regulator, or a coupling factor (5–9). The high resolution structure of the isolated *E. coli* ε, solved by both x-ray crystallography (10) and NMR spectroscopy (11, 12), shows a two domain protein with an N-terminal 10-stranded β-barrel (residues 1–87, termed the N-domain) and a C-terminal helix-turn-helix domain (residues 88–138, termed the C-domain). The latter is not absolutely required for oxidative phosphorylation, or photophosphorylation in the case of the chloroplast enzyme, but is necessary for the inhibition of ATPase activity (5, 13–15). The structure of ε in the ATP synthase complex has been controversial as two different conformations of ε: the “up conformation” (16–18) and the “down conformation” (19), have been seen in crystal structures. The N-domain is located in essentially the same position in both conformations, but in the up conformation the helices of the C-domain are extended and partially wrap around γ to make contact with α_{3}β_{3}, whereas in the down conformation the two α-helices fold on themselves and lie next to the N-domain on top of the ε_{10} oligomer. It has been recently suggested (8, 9) that when the cellular concentration of ATP is low, ε adopts the up conformation and inhibits ATP hydrolysis by allowing for rotation only in the direction of synthesis. The ε subunit was suggested to adopt the down conformation when ATP is high, allowing ATP synthase to catalyze either hydrolysis or synthesis of ATP.

Previously, we have provided in vivo evidence for rotation by fusing proteins of various size to the C terminus of ε (20). In addition to stopping rotation, the larger fusion proteins also generated an uncoupled ATPase activity that was sensitive to inhibition by DCCD, implying that it required movement of the rotor. Because the full 360° rotation of the rotor is blocked by the fusion protein, any such movement must be in a partial, reciprocating fashion. We hypothesized that the C-domain of ε serves a coupling function in ATP synthase, keeping the enzyme efficient by preventing rotation in the wrong direction during ATP hydrolysis. Blocking the proper interactions of the C-domain with α_{3}β_{3} by addition of the fusion protein would block this function and allow the rotor to slip backwards. Based on these findings, we suggested that ε is involved in keeping the ATP synthase efficient by preventing uncoupled ATP hydrolysis.

To test this hypothesis, and to test for intrinsic uncoupling in ATP synthase, we have separated the effect of blocking rotation from the uncoupling effect of C-terminal modifications by creating a series of N-terminal fusion proteins, C-terminal truncations, and a combination

---

3 The abbreviations used are: DCCD, dicyclohexylcarbodiimide; CBD, chitin binding domain; FCCP, p-(trifluoromethoxy)phenylhydrazone; Fd, flavodoxin; PVDF, polyvinylidene difluoride.
Role of the ε Subunit in ATP Synthase

of the two. Fusion of a large 20-kDa protein to the N terminus of ε blocked rotation and inhibited ATP hydrolysis, but did not eliminate ATPase activity. The residual ATPase activity of this “rotation-blocked” enzyme was doubled if the C-domain was truncated. Truncation of the C-domain of normal ε resulted in an increased rate of ATP hydrolysis but a decreased rate of proton pumping, again indicating the generation of uncoupled activity. These results support our hypothesis that the C-domain functions to reduce uncoupled ATP hydrolysis. The mechanism for the generation of the uncoupled activity is discussed.

EXPERIMENTAL PROCEDURES

General Methods—Recombinant DNA techniques were performed as described by Sambrook et al. (21). All plasmid sequences produced by PCR and primed synthesis were confirmed by DNA sequencing. Membrane protein concentrations were determined by the method of Lowry et al. (22). Membrane preparation, assays of ATP hydrolysis, ATP-dependent proton pumping assays, and growth assays were performed as described by Cipriano et al. (20). ATPase assays performed under different concentrations of ATP were performed with the following modifications: The hydrolysis reaction was started by the addition of 0.3 ml of reaction mix containing 50 mM Tris-HCl pH 8, 2.5 mM PEP, 0.2 mg/ml pyruvate kinase, and either 8 mM ATP/4 mM MgCl₂, 4 mM ATP/2 mM MgCl₂, 0.5 mM ATP/0.25 mM MgCl₂, or 0.05 mM ATP/0.025 mM MgCl₂. Aurovertin D was prepared as described previously (23). The purified ε subunit was expressed from plasmid pES52 (24) and purified as described (20). Western blots were performed essentially as described by Cipriano et al. (20) using PVDF membranes and probed with antibodies raised against the α, β, and ε subunits. Because the N-terminal domain of ε by itself washes off of the PVDF membranes, a modified procedure for blotting the ε mutants was devised where 20 μg of membrane protein were blotted and then fixed to the membrane prior to the blocking step by soaking in transfer buffer containing 0.5% glutaraldehyde for 10 min at room temperature. The monoclonal antibody raised against α (α-II) (25) was a generous gift from Drs. Robert Aggeler and Rod Capaldi of the University of Oregon, and the monoclonal antibody raised against b (b-10–6 D1) was kindly provided by Drs. Gabriele Deckers-Hebestreit and Karlheinz Altendorf of Universität Osnabrück. The anti ε (ε-1) monoclonal antibody has been previously described (26, 27). Final plasmids containing the appropriate ε mutants in the entire unc operon encoding ATP synthase were transformed and expressed in the unc deletion strain DK8 (28).

Construction of C-terminal ε Truncations—Plasmid pDC1 (20), containing the uncC gene encoding εA137H with an AvrII site immediately following the stop codon, was the starting point for construction of the C-terminal ε deletions. Products of 3 PCR reactions using pDC1 as the template, the M13R universal primer as the 5’-primer, and primers 1, 2, or 3 (see Fig. 1A for primer sequences) as the 3’-primer, were cut with Xmal and AvrII and inserted into pDC1 cut with the same enzymes to make pDC41 (wild-type), pDC42 (ε88-stop), and pDC43 (ε108-stop), respectively. pDC41, pDC42, and pDC43 were then cut with Psyl and AvrII, and the small fragments isolated and ligated into the 8830 bp Psyl/AvrII fragment of pSD135 (20) to produce pDC44, pDC45, and pDC46, respectively. These plasmids contain all genes encoding ATP synthase with wild-type ε (pDC44), ε88-stop (pDC45), and ε108-stop (pDC46).

Construction of an N-terminal ε Fusion Cloning Vector—Plasmid pSD15 containing the 1182-bp PstI fragment of the unc operon cloned into the PstI site of pUC8 (29) was the starting point for construction of the N-terminal ε fusions. To facilitate the construction, the single BamHI restriction site and one of two AatII sites were removed. The single BamHI site in pUC8 sequence was removed by digestion with BamHI, blunting the 5’ overhangs with the Klenow fragment of DNA polymerase I and religating to produce pDC39. The AatII site in pUC8 sequence was removed by partial digestion with AatII, isolation of singly cut linear plasmid by gel purification, treatment with T4 DNA polymerase to remove the 3’ overhangs, and ligation of the blunt ends. Transformants for which the AatII site within pUC8 vector sequence had been removed were screened by restriction mapping to identify pDC62. Two partially overlapping oligonucleotides (primers 4 and 5) were annealed and made double-stranded by treatment with Klenow fragment of DNA polymerase, digested with AatII, and inserted into the single AatII site in pDC62 to make pDC40. To facilitate future cloning steps the AvrII site just after the stop codon of the ε gene was moved into pDC40. The 465-bp BsaBI/ HindIII fragment of pDC41 was inserted into pDC40 using those sites to produce pDC47. The sequence of pDC47 corresponding to the N terminus of ε is shown in detail in Fig. 1A.

Construction of N-terminal ε Fusions—PCR reactions using either primers 6 and 7 or else primers 8 and 9 were used to amplify DNA fragments carrying the CBD from the Bacillus circulans chitinase A1 (chia1, Swiss-Prot P20533) and the E. coli flavodoxin (Fd, Swiss-Prot P23243) using plasmid pTYB1 (New England Biolabs) and E. coli chromosomal DNA as templates, respectively. Insertion of the PCR products into pDC47 using KpnI and BamHI yielded plasmids pDC48 and pDC49, which contain the nuclear-CBD-ε and flavodoxin-ε (Fd-ε) fusions, respectively. These mutants were then moved into the unc operon on plasmid pSD135 using the EagI and AvrII sites to produce pDC52 (CBD-ε) and pDC53 (Fd-ε).

Construction of N-terminal Fusion/C-terminal Deletion ε Double Mutants—The deletion mutant ε88-stop was fused with the N-terminal fusions as follows: The 312-bp HindIII/BsaBI fragment of pDC42 was moved into pDC48 and pDC49, which had been cut with the same enzymes to produce pDC50 (CBD-ε88-stop) and pDC51 (flavodoxin-ε88-stop), respectively. These mutants were then moved into the unc operon on plasmid pSD135 using the EagI and AvrII sites to produce pDC54 (CBD-ε88-stop) and pDC55 (flavodoxin-ε88-stop).

RESULTS

Construction of N-terminal ε Fusions and C-terminal ε Truncations—To investigate the effect of physically blocking rotation in ATP synthase and the role of the C-domain in energy coupling, a series of N-terminal fusion proteins were constructed both in the presence and absence of the C-domain. The strategy was: 1) to block rotation of the rotor with the N-terminal fusions while leaving the C terminus intact, allowing us to ask how tightly ATP hydrolysis is coupled to rotation; 2) to remove the C-domain from this rotation-blocked ATP synthase to study the uncoupling effect of a C-terminal truncation in the absence of rotation, and 3) to test the uncoupling effect of C-terminal ε truncations in rotation-allowed ATP synthase. Based on previous results with the small C-terminal ε fusion protein (20), the removal of the ε C-domain should result in increased rates of DCCD-sensitive ATP hydrolysis that are uncoupled from proton pumping.

The N-terminal fusions were designed to avoid or minimize effects on the translation of the mRNA, the folding of the ε polypeptide, or the assembly of ATP synthase (Fig. 1, A and B). Because translation efficiency depends on nucleotide sequences both upstream and downstream of the start codon, no changes were made in either the upstream region or the sequence encoding the first 10 amino acid residues of ε. A double-stranded oligonucleotide prepared from primers 4 and 5 (Fig.
The $\epsilon$ subunit of ATP synthase functions in the proton-motive force driving ATP synthesis. The $\epsilon$ subunit is involved in the coupling of ATP synthesis to the proton gradient across the mitochondrial inner membrane. The $\epsilon$ subunit is composed of two domains: the N-terminal domain and the C-terminal domain. The C-terminal domain interacts with the $\delta$ and $\gamma$ subunits, while the N-terminal domain interacts with the $\alpha$ and $\beta$ subunits. The N-terminal domain is composed of a series of $\alpha$-helical segments and flexible Gly-Ser hinges, which provide flexibility between the two fused proteins. Following the linker is the sequence of the N-terminal region of the $\epsilon$ subunit, starting with residue Ala-1. PCR reactions encoding CBD and flavodoxin sequence were cloned into the KpnI and BamHI sites. Two potential concerns when constructing work of this nature is that the fusion protein could be undergoing cleavage. To address these concerns membrane vesicles were prepared and Western blots performed probing with antibodies raised against the $\alpha$, $\epsilon$, and $\delta$ subunits (Fig. 3). There was a low but detectable level of $\epsilon$ in membranes prepared from the $\Delta\epsilon$ strain likely attributed to a small amount of $F_1$ that was trapped in the pellet during the preparation. Membranes prepared from strains carrying the various $\epsilon$ deletions and fusions all had a relatively normal amount of $\alpha$ in the membranes indicating that the $\epsilon$ mutants do not interfere with the proper assembly of $F_1$.

Western Blot Analysis of Expressed $\epsilon$ Mutants—Two potential concerns when conducting work of this nature is that the fusion protein could be undergoing cleavage. To address these concerns membrane vesicles were prepared and Western blots performed probing with antibodies raised against the $\alpha$, $\epsilon$, and $\delta$ subunits (Fig. 3). There was a low but detectable level of $\epsilon$ in membranes prepared from the $\Delta\epsilon$ strain likely attributed to a small amount of $F_1$ that was trapped in the pellet during the preparation. Membranes prepared from strains carrying the various $\epsilon$ deletions and fusions all had a relatively normal amount of $\alpha$ in the membranes indicating that the $\epsilon$ mutants do not interfere with the proper assembly of $F_1$.

The $\epsilon_{88-stop}$ construct was washed off the PVDF membranes using our standard blotting procedure (32). To correct for this the blot was fixed prior to the blocking step by soaking in transfer buffer containing 0.5% glutaraldehyde for 10 min. This allowed us to detect the $\epsilon_{88-stop}$ construct, although it could not be quantified. With this limitation, there was no detectable degradation of the $\epsilon$ fusion proteins (even when blots were overexposed) indicating that the fusion proteins were stable (Fig. 3C).
Role of the ε Subunit in ATP Synthase

Analysis of ATPase Activity of Membrane-bound F1F0—To further analyze the effect of the N-terminal fusions and C-terminal truncations, the membranes described above were assayed for ATPase activity (Table 2). As noted previously with this expression system (20), there is significant variability in the amount of F1F0 present in different membranes. This was true not only for preparations from cells with different plasmids but also for different preparations from the same plasmid/strain. For this reason, membranes were assayed for the ATPase activity of both membrane-bound F1F0 and for the activity of F1 after dissociation from F0 and ε, the latter providing a measure of the amount of ATP synthase present in the membranes. The membrane-bound ATPase activities were then normalized to the activity of soluble F1 released from these preparations, and expressed as the fraction of units bound per unit released, a unitless ratio that we call normalized activity. This treatment of the data gave reproducible results independent of the level of F1F0 in different preparations from the same strain. Previously we have shown that membrane-bound F1F0 typically shows a normalized activity of ~0.4 (20). Here the membrane-bound wild-type ATP synthase showed a normalized activity of 0.44 and was 85% inhibited by treatment with the F1-specific inhibitor, DCCD (Table 2). It should be noted that in all cases DCCD treatment showed less than 12% inhibition of the released activity (data not shown), indicating the specific labeling of F0 and not F1. The fusion of the 5-kDa CBD to the N terminus of ε had no effect on either the normalized hydrolysis activity (0.42), or the DCCD sensitivity (83%), of the membrane-bound enzyme implying that the N-terminal modification itself had no effect on expression of ε or assembly of the ATP synthase.

Fusion of the 20-kDa flavodoxin protein to the N terminus of ε had a substantial effect on the normalized ATPase activity, decreasing it to 0.10. This represents 23% of the normalized activity of the wild-type enzyme. Whereas still significant, this is the lowest observed activity for rotation-blocked ATP synthase. The activity was 47% inhibited by DCCD treatment, suggesting that it required partial movement of the rotor. Furthermore, this activity was sensitive to the F1-specific inhibitor aurovertin D (Table 3) showing that it originated from ATP synthase or F1-ATPase. To ensure that the F1 was not dissociating from F0 during the hydrolysis reaction, an excess of wild-type ε was added to the assay. The ε subunit is a natural inhibitor of F1-ATPase (33), and 100 nM ε has been shown to inhibit free F1 in our ATP hydrolysis reaction (20). The addition of 100 nM wild-type ε had no effect on the activity of mem-

![FIGURE 3. Effect of ε mutation on F0F1 assembly and ε stability. Samples of membrane protein (2 μg) were subjected to SDS-PAGE analysis followed by Western blotting to PVD membranes. Blots were probed with [35S]-labeled antibodies raised against α (A), b (B), and ε (C). Anti-ε blots failed to show ε38-kDa, unless 20 μg of protein were blotted and fixed to the membrane by soaking in transfer buffer with 0.5% glutaraldehyde before the blocking step.](image)

**TABLE 2**
ATPase activity of membrane vesicles

Membranes were suspended to 2 mg/ml in 50 mM Tris-HCl, pH 8, 5 mM MgCl2, 300 mM KCl in the presence or absence of 50 μM DCCD, and incubated for 15 min at room temperature. Membranes were then diluted into ATPase assays to test for coupled activity as described under “Experimental Procedures.” Data shown are the average of triplicate assays ± S.D. 1 unit of activity is defined as 1 μmol of product formed per min.

| ATP synthase type | Membrane-bound ATPase | Released ATPase activity | Normalized activity |
|-------------------|-----------------------|--------------------------|---------------------|
| Activity<sup>a</sup> | % | units/mg | N/A<sup>b</sup> | units/mg |
| ΔNΔβΔεabc | 0.00 ± 0.02 | N/A<sup>c</sup> | 0.00 ± 0.05 | N/A |
| Δε | 0.03 ± 0.01 | N/A | 0.05 ± 0.01 | N/A |
| Wild-type ε | 0.80 ± 0.00 | 85 | 1.83 ± 0.04 | 0.44 ± 0.01 |
| Flavodoxin-ε | 0.15 ± 0.01 | 47 | 1.48 ± 0.00 | 0.10 ± 0.01 |
| Flavodoxin-ε<sub>38-kDa-stop</sub> | 0.41 ± 0.01 | 54 | 1.95 ± 0.04 | 0.21 ± 0.01 |
| CBD-ε | 0.78 ± 0.02 | 83 | 1.87 ± 0.02 | 0.42 ± 0.01 |
| CBD-ε<sub>38-kDa-stop</sub> | 1.24 ± 0.03 | 66 | 2.17 ± 0.07 | 0.57 ± 0.02 |
| ε<sub>38-kDa-stop</sub> | 1.20 ± 0.01 | 83 | 2.53 ± 0.06 | 0.45 ± 0.01 |

<sup>a</sup> Activity values were corrected for background activity of 0.05 units/mg seen in the ΔNΔβΔεabc control strain.

<sup>b</sup> Membrane-bound activities were normalized to the activity released from membranes and expressed as the fraction of units bound per unit released.

<sup>c</sup> N/A, not applicable.

**TABLE 3**
Effect of Aurovertin D and excess ε on the ATPase activity of membrane vesicles

Membranes were diluted into ATPase assays as described under “Experimental Procedures” with the addition of the inhibitors to give the indicated amount during hydrolysis conditions. Data shown are the average of triplicate assays ± S.D. 1 unit of activity is defined as 1 μmol product formed per min.

| Inhibitor | Wild type | Fd-ε |
|-----------|-----------|------|
| Membrane-bound ATPase activity | Released ATPase activity | Membrane-bound ATPase activity | Released ATPase activity |
| units/mg | units/mg | units/mg | units/mg |
| None | 1.52 ± 0.05 | 2.93 ± 0.05 | 0.41 ± 0.02 | 3.15 ± 0.09 |
| 1 μM Aurovertin D | 0.35 ± 0.01 | 1.22 ± 0.01 | 0.13 ± 0.02 | 1.26 ± 0.06 |
| 10 μM Aurovertin D | 0.09 ± 0.01 | 0.53 ± 0.00 | 0.06 ± 0.01 | 0.56 ± 0.02 |
| 100 nM ε | 1.53 ± 0.03 | 0.93 ± 0.04 | 0.42 ± 0.00 | 1.00 ± 0.04 |

<sup>a</sup> Numbers in parentheses represent the percent residual activity of the inhibited forms of the enzyme when compared to the uninhibited enzyme.
brane-bound ATP synthase carrying either wild-type ε or Fd-ε, but it did inhibit the released activity by 68% (Table 3). These results show that the ATPase activity of membranes from the Fd-ε mutant was caused by intact, membrane-bound ATP synthase, rather than a contaminating phosphatase or dissociated F1-ATPase.

The normalized activity of F1/F0 carrying the Fd-ε88-stop construct was increased to 0.21, double that seen with Fd-ε. This activity was 54% inhibited by DCCD. The normalized activities with CBD-ε88-stop and ε88-stop were 0.57 and 0.58, representing a 30% increase in the ATP hydrolysis activity compared with CBD-ε or wild-type ε, respectively. Membranes carrying these constructs showed 66 and 73% DCCD sensitivity, respectively. Interestingly, truncation of only the final helix from normal ε (ε108-stop) had no significant effect on ATP hydrolysis, yielding a normalized activity of 0.45 that was 83% sensitive to DCCD.

**ATP-dependent Proton Translocation in Membrane Vesicles**—To investigate whether the ATPase activity of ε mutants is coupled to proton translocation, the ability of F1/F0 to establish a proton gradient was tested by measuring the ATP-dependent quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence (Fig. 4). Membranes bearing wild-type F1/F0 (A, curve c) showed very strong quenching upon addition of ATP, whereas the Δε (A, curve a) and Δαβεabc (A, curve b) membranes showed no quenching response. The CBD-ε fusion resulted in a level of proton pumping (A, curve e) similar to the wild type. The Fd-ε (A, curve d) and Fd-ε88-stop (B, curve f) membranes showed no quenching, consistent with blocked rotation. Both the CBD-ε88-stop (B, curve g) and ε88-stop (B, curve h) constructs showed a reduced quenching response despite the elevated hydrolysis activity. The ε88-stop (B, curve i) construct also showed a reduced level of quenching even though this mutation had no effect on normalized membrane-bound ATPase activity. In all cases the addition of the uncoupler carbonyl cyanide p-<sub>(trifluoromethoxy)phenyl</sub>hydradrazine (FCCP) quickly dissipated established proton gradients.

**Effect of ATP Concentration on ATP Hydrolysis**—If ε regulates ATP synthase by inhibiting ATP hydrolysis at low ATP concentrations as has been suggested (9), then the rate of hydrolysis by F1/F0 should be lower, compared with the activity measured after release of F1, at low ATP concentrations than at high ATP concentrations. If the regulation involves the C-domain of ε then we should see this effect only with the wild-type enzyme and not with F1/F0 bearing ε88-stop, which should only show the elevated, or stimulated ATP hydrolysis rates. To test this we conducted ATP hydrolysis measurements (Fig. 5) on membranes carrying both the wild type (open bars) and ε88-stop mutant (filled bars) in the presence of various concentrations of ATP. Neither type of membrane showed any substantial change in the normalized rate of ATP hydrolysis over a range of 50 μM to 8 mM ATP. At all concentrations, the membrane-bound ε88-stop mutant was ~40% more active than the wild type. These data show that high concentrations of ATP do not specifically activate hydrolysis by the membrane-bound form of the E. coli enzyme.

**DISCUSSION**

In our previous study (20), we showed that a relatively small 12-kDa protein could be added to the C terminus of ε, and cells would still carry out oxidative phosphorylation, although fusion of larger proteins were not tolerated. Because the larger fusions would be expected to physically block rotation of the γε rotor past the peripheral stock of the enzyme, these results provided support for <i>in vivo</i> operation of the rotational mechanism. Unexpectedly, all ε fusions resulted in generation of an uncoupled ATP hydrolysis activity. Despite being uncoupled from proton translocation, this activity was partially sensitive to the F0-specific inhibitor DCCD, which prevents movement of ε subunits past ab<sub>2</sub> (34). As full rotation was blocked by the large fusion proteins, the uncoupled hydrolysis was proposed to occur by reciprocating rotational movements. Because the fusions might be expected to prevent the C-domain from assuming its normal position or conformation, we inferred that during ATP hydrolysis the C-domain of ε functions to ensure tight coupling. Definitive interpretation of the results, however, was complicated because both steric and uncoupling effects were caused by the same modification, the C-terminal fusion. In the current study we have separated these effects by: 1) fusing the 20-kDa flavodoxin to the N terminus of ε to block rotation with the C terminus kept intact; 2) removing the C-domain to generate an uncoupled activity in this rotation-blocked construct; and 3) removing the C-domain from normal ε to see the uncoupling effect on rotation-allowed ATP synthase.

**ATP Synthase Is Intrinsically Uncoupled**—This approach has allowed us to investigate how tightly ATP hydrolysis is coupled to rotation,
Role of the $\epsilon$ Subunit in ATP Synthase

because blocking rotation in a fully coupled enzyme should completely inhibit hydrolysis. Other attempts to block rotation using disulfide bond formation have been hard to interpret, either because of effects of the introduced cysteines (34) or incomplete disulfide formation (35). We have avoided these problems by genetically fusing a large protein to the N terminus of $\epsilon$ to stop rotation. Cells expressing ATP synthase containing Fd-$\epsilon$ were unable to carry out oxidative phosphorylation indicating a blockage of rotation. This rotation-blocked ATP synthase showed a substantial level of ATP hydrolysis, 23% of the normalized activity of the wild-type enzyme (Table 2), but extremely low proton pumping (Fig. 4). The ATPase activity was specific to ATP synthase as shown by Aurovertin D inhibition (Table 3) and was sensitive to movement of the rotor as shown by DCCD inhibition (Table 2). Furthermore, the activity arose neither from dissociation of F$_1$ from F$_0$ since it was not inhibited by purified wild-type $\epsilon$ (Table 3), nor from degradation of the fusion protein as shown by Western blotting (Fig. 3). These results imply that the ATP synthase is intrinsically partially uncoupled, even when $\epsilon$ is fully functional. One alternative explanation for the uncoupled activity we observed is that the N-terminal modification itself caused the partial uncoupling, but we think this unlikely since the N-terminal CBD-$\epsilon$ fusion had no effect on ATP hydrolysis or proton pumping. It may be possible that the level of uncoupled activity is lower in the wild type enzyme than that observed in the rotation-blocked enzyme, because blocking rotation could directly enhance the uncoupling. However, the existence of uncoupled activity in the wild-type ATP synthase is also supported by the observation that blocking rotation with the F$_0$-specific inhibitor, DCCD, never completely inhibits ATP hydrolysis.

In a mechanism as complex as that of ATP synthase, modest failure of many steps might result in partial uncoupling. An intrinsic partial uncoupling observed in the absence of ADP and P$_i$ has recently been reported for the ATP synthase from _Rhodobacter capsulatus_ (36). Other forms of uncoupling caused by failure of linking rotation to proton movement through F$_0$ have also been reported (37–42).

_The C-Domain of $\epsilon$ Promotes Efficient Energy Coupling_—Removal of the C-domain from the rotation-blocked Fd-$\epsilon$ construct to produce the Fd-$\epsilon_{88\text{-stop}}$ resulted in a 2-fold stimulation of the ATPase activity. This elevated ATP hydrolysis was completely uncoupled from proton transport, yet DCCD-sensitive, implying a reciprocating rotational movement. Truncation of the C-domain in forms of $\epsilon$ that can undergo normal rotation ($\epsilon_{88\text{-stop}}$ and CBD-$\epsilon_{88\text{-stop}}$) resulted in increased ATPase activity but less proton pumping, again indicating generation of an uncoupled ATPase activity. In retrospect, increased ATPase activities and lowered proton pumping resulting from C-terminal deletions (5, 15), or other C-domain impairment (8, 43), may be discerned in previously published work, though these effects were difficult to recognize in the absence of the rotation-blocking fusions. The results presented here utilizing the fusion approach do reveal that removal of the C-domain of $\epsilon$ causes an increased uncoupled ATPase activity, confirming the postulated role of the C-domain of $\epsilon$ in maintaining coupling of ATP hydrolysis to proton movements.

Whereas fusions to $\epsilon_{88\text{-stop}}$ were not constructed, it is notable that this lesser truncation resulted in decreased proton pumping (Fig. 4), but no significant change in ATPase activity (Table 2), indicative of partial uncoupling. This opens the possibility that the activation of ATPase activity accompanying C-domain deletion, and the uncoupling effect that we have demonstrated, may arise through different mechanisms.

Fig. 6 shows a model adapted from Ref. 44 to explain the mechanism of generation of the uncoupled ATPase activity in ATP synthase bearing the Fd-$\epsilon_{88\text{-stop}}$ mutant. During steps 1 and 2, the enzyme hydrolyzes ATP, and the rotor turns in a clockwise direction. During step 3 the fusion protein comes into direct contact with the $b_2$ stator, physically blocking rotation. We postulate that the C-domain of $\epsilon$ normally inhibits step 4 in which the rotor turns backwards (i.e. the direction of synthesis) without ADP and P$_i$ bound at the relevant catalytic site. We cannot specify whether the step occurs while the site is still empty or after it has bound ATP. Step 4 differs from the reversal of step 2 in that the latter would only occur with bound ADP and P$_i$ and would result in ATP synthase. However, with the C-domain missing in the Fd-$\epsilon_{88\text{-stop}}$ construct, or compromised by C-terminal fusions, the rotor can turn backwards via step 4 without concomitant ATP synthase, and then step 2 can be repeated. Together steps 2 and 4 constitute a futile cycle of uncoupled activity. It is envisioned that in ATP synthase carrying the C-terminal $\epsilon$ truncations, or a small C-terminal fusion that can undergo rotation, this backwards step 4 can occur at any given point in the catalytic cycle, and would likely be driven by protonmotive force. Whereas the relationship between the uncoupling seen with the C-terminal $\epsilon$ truncations and the uncoupling that can be remedied by ADP+P$_i$ (36) is uncertain, it is notable that occupation of the empty catalytic site in Fig. 6C by ADP and P$_i$ might be expected to prevent uncoupling by the postulated mechanism, because reverse rotation under these conditions would result in the reversal of the coupled step 2 and the synthesis of ATP. It is the occurrence of step 4, rotation in the direction of ATP synthesis without synthesis actually occurring, that is the key component leading to uncoupling in our model. The _in vivo_ occurrence of step 4 will result in the inward flow of protons through F$_0$ without concomitant synthesis of ATP.

_Function of the $\epsilon$ Subunit in ATP Synthase_—Two different conformations have been reported for $\epsilon$ in complex with other subunits of ATP synthase (16–19). Studies of the propensities to form disulfide bonds suggested that both conformations can exist in the enzyme (8, 43) and suggest that $\epsilon$ can preferentially adopt the different conformations depending on the nucleotides or protonmotive force present (9). A model for the regulation of ATP hydrolysis in the thermophilic _Bacillus_ PS3 TF$_1$F$_{10}$ has been proposed such that if the ATP concentration is low, $\epsilon$ adopts the up conformation and permits only ATP synthesis, while if the ATP concentration is high, the down conformation permitting both synthesis and hydrolysis is preferred (9). If this were the case for the
E. coli ε, then the wild-type enzyme should have a higher normalized ATPase activity at high concentrations of ATP than at low concentrations, and this difference should disappear with the C-terminal ε truncation. We have shown here that the normalized activity did not change significantly in the range of 50 μM to 8 mM ATP, which includes physiologically relevant levels, and that the activation attributed to the truncation of the C-domain of ε was independent of ATP concentration (Fig. 5). These results do not support a role for ε as an ATP-dependent regulator of ATP hydrolysis in E. coli.

A coupling role for ε has been well established in the chloroplast and mitochondrial enzymes. Assembly of these enzymes is not ε-dependent, but forms lacking ε are uncoupled (6, 7, 45). Establishing ε as a coupling factor in E. coli has been more difficult because ε is required for F1-F0 assembly in this system. Through the use of fusions and partial deletions, this coupling role has been demonstrated and a possible mechanism for the uncoupled activity has been developed (20, 44). Besides explaining our own results, the mechanism we propose (44) is also supported by the recent studies of Rondelez et al. (46). These authors have shown that mechanically driven rotation in F1 is more effective in ATP synthesis with ε-replete F1 than with ε-depleted enzyme, supporting our hypothesis that ε inhibits rotation in the direction of synthesis without synthesis actually occurring.

Acknowledgments—We thank Dr. Robert Nakamoto of the University of Virginia for the gift of plasmid pACWU1.2, Drs. Gabriele Deckers-Hebestreit and Karlheinz Altendorf of Universität Osnabrück for providing the anti-b monoclonal antibody, and Drs. Robert Aggeler and Rod Capaldi of the University of Western Ontario for useful discussion and critical reading of the manuscript. Some of the experiments described were carried out at the UWO Biomolecular Interactions and Conformations Facility, which is supported by a Multi-User Maintenance and Equipment Grant from the Canadian Institutes of Health Research.

REFERENCES

1. Boyer, P. D. (1997) Annu. Rev. Biochem. 66, 717–749
2. Walker, J. E. (guest ed) (2000) Biochim. Biophys. Acta 1458, 221–510
3. Peterson, P. L. (ed) (2000) J. Bioenerg. Biomembr. 32, 325–540
4. Fusi, M. Wada, Y., and Kaplan, J.H. (ed) (2004) Handbook of ATPsases 237–336 Wiley-VCH, Weinheim Germany
5. Kato-Yamada, Y., Bald, D., Koike, M., Motohashi, K., Hisabori, T., and Yoshida, M. (1999) J. Biol. Chem. 274, 33991–33994
6. Xiao, Y., Metzl, M., and Mueller, D. M. (2000) J. Biol. Chem. 275, 6963–6968
7. Duvicz-Cabot, S., Caron, M., Giraud, M. F., Velours, J., and di Rago, J. P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 13235–13240
8. Tsunoda, S. P., Rodgers, A. J., Aggeler, P., Wilce, M. C., Yoshida, M., and Capaldi, R. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6560–6564
9. Suzuki, T., Murakami, T., Iino, R., Suzuki, J., Ono, S., Shirakihara, Y., and Yoshida, M. (2003) J. Biol. Chem. 278, 46840–46846
10. Uhlil, U., Cox, G. B., and Guis, J. M. (1997) Structure 5, 1219–1230
11. Wilkens, S., Dahlquist, W. F., McIntosh, L. P., Donaldson, L. W., and Capaldi, R. A. (1995) Nat. Struct. Biol. 2, 961–967
12. Wilkens, S., and Capaldi, R. A. (1998) J. Biol. Chem. 273, 26645–26651
13. Nowak, K. F., and McCarty, R. E. (2004) Biochemistry 43, 3273–3279
14. Kuki, M., Nouni, T., Maeda, M., Amemura, A., and Futai, M. (1988) J. Biol. Chem. 263, 17437–17442
15. Xiong, H., Zhang, D., and Yik, S. B. (1998) Biochemistry 37, 16423–16429
16. Rodgers, A. J., and Wilce, M. C. (2000) Nat. Struct. Biol. 7, 1051–1054
17. Hausrath, A. C., Capaldi, R. A., and Matthews, B. W. (2001) J. Biol. Chem. 276, 47227–47232
18. Hausrath, A. C., Gruber, G., Matthews, B. W., and Capaldi, R. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13697–13702
19. Gilbons, C., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2000) Nat. Struct. Biol. 7, 1055–1061
20. Cipriano, D. J., Bi, Y., and Dunn, S. D. (2002) J. Biol. Chem. 277, 16782–16790
21. Sambrook, J., Frisch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Lowrey, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
23. Dunn, S. D., Zadorozny, V. D., Tozer, R. G., and Ortiz, L. E. (1987) Biochemistry 26, 4488–4493
24. Sako, E., and Dunn, S. D. (1993) Arch. Biochem. Biophys. 302, 272–278
25. Aggeler, R., Capaldi, R. A., Dunn, S., and Gogol, E. P. (1992) Arch. Biochem. Biophys. 296, 685–690
26. Sako, E., and Dunn, S. D. (1993) Arch. Biochem. Biophys. 302, 279–284
27. Dunn, S. D., Tozer, R. G., Antczak, D. F., and Heppel, L. A. (1985) J. Biol. Chem. 260, 10418–10425
28. Klionsky, D. J., Brusilow, W. S., and Simoni, R. D. (1984) J. Bacteriol. 160, 1055–1060
29. Vieira, J., and Messing, J. (1982) Gene (Amst.) 19, 259–268
30. Dunn, S. D. (1982) J. Biol. Chem. 257, 7354–7359
31. Kuo, P. H., Ketchum, C. J., and Nakamoto, R. K. (1998) FEBS Lett. 426, 217–220
32. Dunn, S. D. (1986) Anal. Biochem. 157, 144–153
33. Sternweis, P. C., and Smith, J. B. (1980) Biochemistry 19, 526–531
34. Jones, P. C., Hermolin, J., Jiang, W., and Fillingame, R. H. (2000) J. Biol. Chem. 275, 31340–31346
35. Suzuki, T., Suzuki, J., Mitome, N., Ueno, H., and Yoshida, M. (2000) J. Biol. Chem. 275, 37902–37906
36. Turina, P., Giovannini, D., Gubellini, F., and Melandri, B. A. (2004) Biochemistry 43, 11126–11134
37. McCarty, R. E., Fuhrman, J. S., and Tsuchiya, Y. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 2522–2526
38. Evron, Y., and Avron, M. (1990) Biochim. Biophys. Acta 1019, 115–120
39. Groth, G., and Junge, W. (1993) Biochemistry 32, 8103–8111
40. Gräber, P., Burmeister, M., and Hortsch, M. (1981) FEBS Lett. 136, 25–31
41. Feniouk, B. A., Cherepanov, D. A., Junge, W., and Mulldjianian, A. Y. (1999) FEBS Lett. 445, 409–414
42. Feniouk, B. A., Mulkidjianian, A. Y., and Junge, W. (2005) Biochim. Biophys. Acta 1706, 184–194
43. Schulenberg, B., and Capaldi, R. A. (1999) J. Biol. Chem. 274, 28351–28355
44. Dunn, S. D., Cipriano, D. J., and Del Rizzo, P. A. (2004) in Handbook of ATPsases (Futai, M., Wada, Y., and Kaplan, J. H., ed), pp. 311–338, Wiley-VCH, Weinheim
45. Richter, M. L., Patrie, W. J., and McCarty, R. E. (1984) J. Biol. Chem. 259, 7371–7373
46. Rondelez, Y., Tresset, G., Nakashima, Y., Kato-Yamada, Y., Fujita, H., Takeuchi, S., and Noji, H. (2005) Nature 433, 773–777
47. Leslie, A. G., and Walker, J. E. (2000) Philos. Trans. R. Soc. Lond. B. Biol. Sci. 355, 465–471

Role of the ε Subunit in ATP Synthase