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

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The rotational mechanism of ATP synthase was investigated by fusing three proteins from Escherichia coli, the 12-kDa soluble cytochrome b562, the 20-kDa flavodoxin, and the 28-kDa flavodoxin reductase, to the C terminus of the ε subunit of the enzyme. According to the concept of rotational catalysis, because ε is part of the rotor a large domain added at this site should sterically clash with the second stalk, blocking rotation and fully inhibiting the enzyme. E. coli cells expressing the cytochrome b562 fusion in place of wild-type ε grew using acetate as the energy source, indicating their capacity for oxidative phosphorylation. Cells expressing the larger flavodoxin or flavodoxin reductase fusions failed to grow on acetate. Immunoblot analysis showed that the fusion proteins were stable in the cells and that they had no effect on enzyme assembly. These results provide initial evidence supporting rotational catalysis in vivo. In membrane vesicles, the cytochrome b562 fusion caused an increase in the apparent ATPase activity but a minor decrease in proton pumping. Vesicles bearing ATP synthase containing the larger fusion proteins showed reduced but significant levels of ATPase activity that was sensitive to inhibition by dicyclohexylcarbodi-imide (DCCD) but no proton pumping. Thus, all fusions to ε generated an uncoupled component of ATPase activity. These results imply that a function of the C terminus of ε in F1F0 is to increase the efficiency of the enzyme by specifically preventing the uncoupled hydrolysis of ATP. Given the sensitivity to DCCD, this uncoupled ATP hydrolysis may arise from rotational steps of γε in the inappropriate direction after ATP is bound at the catalytic site. It is proposed that the C-terminal domain of ε functions to ensure that rotation occurs only in the direction of ATP synthesis when ADP is bound and only in the direction of hydrolysis when ATP is bound.

ATP synthase is the enzyme responsible for the production of ATP during oxidative phosphorylation. It is found throughout all forms of life, from the membranes of bacteria to the mitochondria and chloroplasts of eukaryotes. The enzyme can be easily dissociated into two components, a membrane integral F0 component that forms a proton-permeable pore through the membrane and a peripheral F1 component that houses the three catalytic sites responsible for the synthesis or hydrolysis of ATP. The F1 portion of the Escherichia coli enzyme is composed of five subunits in the stoichiometry α3β3γε6, whereas the F0 sector is made up of three subunits with stoichiometry ab3γε10–14. The generally accepted mechanism of ATP synthase function is commonly referred to as “rotational catalysis.” In this mechanism proton translocation through F0 causes the rotation of the cγ10–14ε complex with respect to the remainder of the enzyme. During the process of oxidative phosphorylation, movement of this “rotor” is believed to drive the sequential conformational changes in αβγ3 causing binding of the substrates, ADP and P(i), and the release of the product, ATP, as predicted by Paul Boyer’s binding change mechanism. For the rotational mechanism to operate, a second, peripheral stalk composed of the two 6 β subunits and δ must link the αβγ3 hexamer of F1 with the ε subunit of F0, preventing rotation of either cγ10–14ε (for recent reviews see Refs. 1–4).

The theory of rotational catalysis received substantial support when Walker and co-workers (5) solved the high resolution structure of the αβγ3 complex from beef heart mitochondria. The structure revealed a hexamer of alternating α and β subunits surrounding a central γ subunit. The three active sites for ATP synthesis/hydrolysis are located in clefts between α and β subunits and are occupied by different nucleotides. Since then, ATP-dependent rotational motion of the γε complex in the isolated F1 sector has been demonstrated by a number of methods (6, 7), culminating in the direct observation that filaments or beads attached to either γ or ε undergo continuous rotation when ATP is added (8–10). More recently, efforts have been directed toward demonstrating that this mechanism also functions in the holoenzyme, i.e. that ATP induces rotation of γεc10–14 in ATP synthase (11–14). Rotation of filaments linked to ε was observed in these studies, but the interpretation is controversial because of concern that the F0 and F1 sectors were not fully coupled in these in vitro experimental systems. This concern was raised by the lack of sensitivity to F0-specific inhibitors (14) and the tendency of the second stalk to dissociate from the purified yeast enzyme (15). In the absence of the proper interactions of the ε oligomer with the a and b subunits, the enzyme would be uncoupled and rotation of the ε ring would be expected, regardless of whether or not it occurred during coupled activity. In another approach to rotation in F1F0, specific sites on γ or ε could be linked through disulfide bonds to any of the three β subunits, provided ATP hydrolysis was allowed to proceed (16, 17). Although consistent with rotation, this result could also be satisfied if γ and ε were to swivel in a reciprocating fashion through 240°. Such motion would allow γ and ε to sit in each of the three different positions they have.
been shown to occupy, while not actually rotating in a repeated circular fashion.

Thus, questions remain regarding the rotational mechanism in intact ATP synthase (18). Furthermore, the mechanism has never been tested in vivo. In this work we describe an approach that allows tests of rotation and function both in vivo and in vitro. During rotational catalysis, the \( \gamma \) rotor must pass the \( \beta_2 \) stator; the size of the rotor is therefore limited by the space available. Any matter added to the rotor must also rotate within this confined space, sweeping out a larger volume of revolution. One would expect that if the added material is too large, it will sterically clash with \( \beta_2 \) and rotation will be blocked. As a result, both ATP synthesis/hydrolysis and proton pumping should be strongly or completely inhibited. We selected the C terminus of \( \epsilon \) as the site to make additions, because deletion or mutation of residues in this region has only minimal effect on function (19, 20). In addition, effects on expression of ATP synthase subunits should be minor because \( \text{uncC} \), encoding \( \epsilon \), is the last gene of the \( \text{unc} \) operon. By fusing a series of proteins of increasing size to this site, we have altered the effective size of the rotor, allowing us to test rotation in vivo and to establish limits for the maximal volume of revolution. In addition, our studies indicate a novel role for the \( \epsilon \) subunit in the holoenzyme, to increase the efficiency of ATP synthase by specifically preventing the uncoupled hydrolysis of ATP.

### EXPERIMENTAL PROCEDURES

**General Methods**—Recombinant DNA techniques were performed as described by Sambrook et al. (21). All plasmid sequences produced by PCR were confirmed by DNA sequencing. SDS-PAGE was carried out using 15% separating gels according to the method of Laemmli (22) and 35% stacking gels. Protein concentrations were determined by the method of Bradford (23) for soluble proteins and the method of Lowry et al. (24) for membranes. Western blotting was performed as described previously (25) using polyvinylidene difluoride membranes and probed with antibodies that were labeled with \( ^{125}\text{I} \) by the IODO-GEN method (26). The monoclonal antibody raised against \( \alpha (\alphaII) \) (27) was a generous gift from Drs. Robert Aggerle and Rod Capaldi of the University of Oregon, and the monoclonal antibody directed against \( \beta (\beta10-61) \) was kindly provided by Drs. Gabriele Deckers-Hebestreit and Karlheinz Altendorf of Universität Osnabruk. The anti-\( \gamma \) (\( \gamma II \)) and anti-\( \epsilon \) (\( \epsilon I \)) monoclonal antibodies have been described previously (20, 25).

Inverted membrane vesicles were prepared from DK8 strains containing the various pACWU1.2-based plasmids as described by McLachlin and Dunn (29). Stripped membranes were prepared similarly, substituting 1 mM Tris-\( \text{HCl} \), pH 8, 0.5 mM EDTA, 10% glycerol in place of the membrane wash buffer, and stirring on ice for 15 min between centrifugations.

**Plasmid Construction and Mutagenesis**—The general scheme for plasmid construction was to create the C-terminal \( \epsilon \) fusions in a vector overexpressing the isolated subunit and to then move the fusions to plasmid pACWU1.2 (30) containing a cysteine-less copy of the entire \( E. \) coli \( \text{unc} \) operon expressing ATP synthase.

**Plasmid** pSD2, containing the \( 3' \) end (bp 7019–7526) of the \( \text{unc} \) operon incorporated into the \( \text{Accl} \) site of pUC8 (31), was the parent for the overexpression plasmids. Site-directed PCR mutagenesis using primer 1 (see Fig. 1 for primer sequences) and the M13 forward universal primer was used to insert an \( \text{NdeI} \) site after the 3' stop codon of the \( \text{uncC} \) gene encoding \( \epsilon \), producing an EA137H substitution in the process. The PCR product was inserted into pSD2, using the \( \text{SfiI} \) and \( \text{PstI} \) restriction sites, to produce pSD132.

DNA carrying the \( \text{E. coli} \) gene for the soluble cytochrome \( \text{b}_{562} \) (cybC, Swiss-Prot accession number P00192) was amplified by PCR using plasmid pACE110 (32) as template. The 5' primer (primer 2) contained an \( \text{NdeI} \) restriction site, the codons for a Gly-Ser addition, and then sequence encoding the N terminus of the mature cytochrome \( \text{b}_{562} \). The 3' primer (primer 3) contained the C terminus of the gene, followed by an \( \text{AvrII} \) restriction site. The PCR product was inserted into the \( \text{NdeI} \) and \( \text{AvrII} \) sites of pSD132 to produce pSD143, a plasmid containing the cytochrome \( \text{b}_{562} \) gene fused to the C terminus of \( \epsilon \) through a Gly-Ser linker.

To facilitate future cloning procedures, the single \( \text{BamHI} \) site on pSD132 derived from pUC8 sequence was then removed by digestion with \( \text{BamHI} \) and the subsequent filling of the overhangs with the Klenow fragment of DNA polymerase I to produce plasmid pSD146. An \( \text{NdeI} \) site on pSD146 derived from the pUC8 sequence was removed by the partial digestion with \( \text{NdeI} \) and similar filling procedure to produce pDC1.

PCRs using primers 4 and 5 and primers 6 and 7 were used to amplify DNA fragments carrying the \( \text{E. coli} \) genes encoding flavodoxin (\( \text{flfdA} \), Swiss-Prot accession number P23243) and flavodoxin reductase (\( \text{fnr} \), Swiss-Prot accession number P28861), respectively. Chromosomal DNA from strain K12a was used as template. The primers were designed in a fashion similar to that mentioned above for the cytochrome \( \text{b}_{562} \) fusion but included a \( \text{BamHI} \) site encoding the Gly-Ser linker. Insertion of the PCR products into pDC1 using \( \text{NdeI} \) and \( \text{AvrII} \) yielded plasmids pDC2 and pDC3 that encode \( \epsilon \) fused to flavodoxin and flavodoxin reductase, respectively.

The same set of fusion proteins were then constructed with a 20-amino acid linker between the fused proteins. The linker sequence was designed such that the translated peptide would likely form an a-helix. First a PCR using primers 8 and 9 was used to insert a \( \text{BamHI} \) site encoding the Gly-Ser linker in pSD146, producing pDC4. Two partially overlapping oligonucleotides (primers 9 and 10) were annealed and made double-stranded by treatment with the Klenow fragment of DNA polymerase I and dNTPs. This synthetic double-stranded DNA contained an \( \text{NdeI} \) site followed by sequence encoding GSAEEAAAKAAAE- AARAAEEAAGS where the final GS was encoded by a \( \epsilon \) site. The resulting double-stranded DNA was cut with \( \text{NdeI} \) and \( \text{BamHI} \) and inserted into pDC6, pDC2, and pDC3 using the same restriction sites to produce plasmids pDC10, pDC7, and pDC8.

To facilitate the cloning of the \( \epsilon \) fusion proteins into the \( \text{unc} \) operon, the 238-bp \( \text{SfiI} / \text{PstI} \) fragment of pSD132 was moved into pACWU1.2 using the same sites to produce plasmid pSD135. This plasmid contains the entire \( \text{unc} \) operon with the EA137H mutation encoding the \( \text{NdeI} \) site and the \( \text{AvrII} \) site after the stop codon of the \( \text{uncC} \) gene. Plasmids pSD143, pDC2, pDC3, pDC10, pDC7, and pDC8 were digested with \( \text{SfiI} \) and \( \text{AvrII} \), and the small fragments were inserted into pSD135 using the same enzyme sites to produce plasmids pSD144, pDC4, pDC5, pDC11, pDC12, and pDC13, respectively. These plasmids contain the \( \text{unc} \) operon with the respective fusions to the \( \text{uncC} \) gene.

To produce a plasmid for use as a negative control, pACWU1.2 was digested with \( \text{PvuII} \), and the large fragment ligated on itself to form plasmid pDC14. This plasmid has a deletion of almost the entire \( \text{unc} \) operon, with remnants of the 5' end of \( \text{uncA} \) fused out of frame to the 3' end of \( \text{uncC} \). This plasmid produces none of the subunits of ATP synthase and was used to confer ampicillin resistance to control strains DK5 (\( \Delta \text{ufu} \)) and DK7 (\( \Delta \epsilon \)) (33).

### Table: Primers used in plasmid construction and mutagenesis.

| Primer Sequence | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-----------------|---|---|---|---|---|---|---|---|---|---|
|                 | 5'-GGCCGGTATCGGGCAGCTCCCGGGTTCCT-3' | 5'-GGCCATATGGGTCTACAGTATTTTGCATGGTG-3' | 5'-GGCCCTATAGGGTCAAGCCACCATATGATA-3' | 5'-GACCTCCATATGGGTCACCATGCTGATAAC-3' | 5'-GACCTGCCATGGTTAGCACTGAGATGTC-3' | 5'-GACCTCCATAGGGTCAAGCCACCATATGATAA-3' | 5'-GACCTGGTATCAGGGTCAAGCCACCATATGATA-3' | 5'-GACCTCCATAGGGTCAAGCCACCATATGATAA-3' | 5'-GACCTCCATAGGGTCAAGCCACCATATGATAA-3' | 5'-GACCTGGTATCAGGGTCAAGCCACCATATGATAA-3' |

**Fig. 1. Primers used in plasmid construction and mutagenesis.** Restriction sites are indicated as follows: \( \text{NdeI} \), underscore; \( \text{SfiI} \), double underscore; \( \text{AvrII} \), overscore; and \( \text{BamHI} \), double overscore. Italicized sequence on primers 9 and 10 indicates the complementary regions used for primer extensions.
Protein Purification—\(\varepsilon\) and \(\epsilon\) fusion proteins were expressed from derivatives of pUC8 in strain JM103. Cells were grown in 2X YT media, induced with isopropylthiogalactoside at an \(A_{600}\) of ~0.8, and harvested when cultures reached stationary phase. Cell pellets were washed once in 50 mM Tris-HCl, pH 8, 10 mM MgCl₂, and suspended in a volume of the buffer equivalent to 10 times their packed wet weight. Phenylmethylsulfonyl fluoride was added to 1 mM, and cells were broken by passage through a French pressure cell at 20,000 pounds/square inch. The cell lysates were then subjected to centrifugation at 38,000 rpm for 1.5 h in a Beckman Ti-45 rotor. Supernatant solutions were fractionated by precipitation with ammonium sulfate and by ion exchange and gel filtration chromatography with the modifications listed below. Unless otherwise stated all ion exchange chromatography steps were performed in 50 mM Tris-HCl, 1 mM EDTA at the specified pH and gel filtration chromatography in 50 mM Tris-HCl, pH 8, 1 mM EDTA. Between each step the proteins were dialedyzed into the appropriate buffer for the next step. SDS-PAGE was used to analyze all fractions, and purified products were stored at ~80 °C.

Red \(\epsilon\) (fused to cytochrome \(b_{562}\)) was precipitated between 60 and 70% saturated ammonium sulfate, and then subjected to chromatography on DEAE-Sepharose at pH 8. The protein was eluted with a linear gradient of 0–500 mM NaCl. Appropriate fractions were pooled and loaded onto a column of Sephacryl G-75. Pooled fractions were then loaded onto DEAE-Sepharose at pH 6.4 and eluted with a linear gradient of 0–500 mM NaCl.

Orange \(\epsilon\) (fused to flavodoxin) was precipitated with 50% saturated ammonium sulfate. The resuspended pellet was loaded onto a DEAE-Sepharose column at pH 8 and eluted with a linear gradient of 100–600 mM NaCl. Pooled fractions were subjected to gel filtration on a Sephacryl S-200 column, pooled again, loaded onto a DEAE-Sepharose column at pH 6.4, and eluted with a linear gradient of 100–600 mM NaCl.

Yellow \(\epsilon\) (fused to flavodoxin reductase) was precipitated between 45 and 50% saturated ammonium sulfate, loaded onto a DEAE-Sepharose column at pH 8, and eluted with a linear 0–600 mM NaCl gradient. Pooled fractions were dialyzed into 30 mM sodium acetate, pH 5, loaded onto a carboxymethyl-Sepharose column, and eluted with a 0–600 mM NaCl gradient. Fractions were then concentrated and run on a Sephacryl S-200 column.

E-Red \(\epsilon\) (fused to cytochrome \(b_{562}\) through a 20-residue linker) was precipitated between 40 and 60% saturated ammonium sulfate. It was then subjected to DEAE-Sepharose chromatography, first at pH 8 and then at pH 6.4. In both cases the protein was eluted from the column with a linear gradient of 100–600 mM NaCl. Pooled fractions were then subjected to DEAE-Sepharose chromatography, first at pH 8 and then at pH 6.4. Fractions were then concentrated and run on a Sephacryl S-200 column.

E-Orange \(\epsilon\) (fused to flavodoxin through a 20-residue linker) was precipitated with 40–55% saturated ammonium sulfate, applied to a DEAE-Sepharose column, and eluted with a linear 0–600 mM NaCl gradient. The resulting fractions were pooled and subjected to gel filtration on a Sephacryl S-200 column.

E-Yellow \(\epsilon\) (fused to flavodoxin reductase through a 20-residue linker) was precipitated with 50% saturated ammonium sulfate. The resuspended pellet was applied to a DEAE-Sepharose column at pH 8 and eluted with a linear gradient of 0–600 mM NaCl. The pooled fractions were adjusted to pH 6.4, loaded onto another DEAE-Sepharose column, and chromatographed at pH 6.4 with a linear gradient of 0–500 mM NaCl. Pooled fractions were then applied to a Sephacryl S-200 column for gel filtration chromatography.

Plasmid Construction and Protein Purification—To investigate subunit rotation in ATP synthase in vivo, a number of proteins of increasing mass were fused to the C terminus of \(\epsilon\). The strategy used was to make each \(\epsilon\) fusion construct in a pUC8-based plasmid where the individual proteins could be expressed for purification and then to move each fusion to the unc operon on plasmid pACWU1.2 (30) so that the mutant FlF₀ could be expressed in an unc-deficient strain, such as DK8 (Jal fgy68 abc) (33) for analysis of function in vivo.

RESULTS

The \(\epsilon\) fusion proteins that were constructed are illustrated in Fig. 2. To facilitate the cloning process, an NdeI restriction site was introduced in the DNA encoding the last two amino acids of \(\epsilon\). This resulted in an Ala to His substitution in the penultimate residue (eA137H). Proteins were chosen based on size and the presence of a chromophore to assist purification and characterization. The chosen proteins were the soluble cytochrome \(b_{562}\) (12 kDa), flavodoxin (20 kDa), and flavodoxin reductase (27 kDa) all from \(E. coli\). The \(\epsilon\) fusion proteins were purified and found to retain the characteristic color of their chromophore. Based on these colors the cytochrome \(b_{562}\), flavodoxin, and flavodoxin reductase \(\epsilon\) fusion proteins were given.

\[\text{FIG. 2. A schematic representation of the } \epsilon \text{ fusion proteins created for this study.} \]

\[\text{The fused protein in the Red } \epsilon \text{ mutants is cytochrome } b_{562} \text{ (12 kDa); in the Orange } \epsilon \text{ mutants, flavodoxin (20 kDa); and in the Yellow } \epsilon \text{ mutants, flavodoxin reductase (27 kDa). The constructs were given common names based on the color of the purified proteins. Similar constructs were made with a 20-residue linker and named E-Red } \epsilon, \text{ E-Orange } \epsilon, \text{ and E-Yellow } \epsilon. \text{ All protein sequences, with the exception of the synthetic linker, were derived from } E. coli. \text{ The black regions represent sequence of the } \epsilon \text{ subunit; the dark gray regions represent a Gly-Ser hinge; the white regions represent a synthetic 20-residue linker; and the light gray regions represent sequence of the fused protein domains. Sizes shown are the molecular weight of the entire fusion construct.} \]
common names of Red ε, Orange ε, and Yellow ε, respectively. The same set of fusion proteins was constructed with a 20-residue linker between the two domains. The linker sequence GSAAEAAAKAAEAAARAAEAAAGS was designed to favor a helical conformation. These fusion proteins were purified and named E-Red ε, E-Orange ε, and E-Yellow ε, where E indicates extended.

**Growth Characteristics of C-terminal ε fusion Strains**—To address the ability of these mutant ε subunits to provide normal ε function *in vivo*, plasmid-borne F₁F₀ containing the different mutants was expressed in the *unc* deletion strain DK8. Results from these studies are summarized in Table I. Strains bearing the Red ε and E-Red ε mutants grew on plates containing acetate as the sole carbon and energy source, producing colonies of the same size as the wild-type, but strains carrying the larger fusion proteins did not. Growth on acetate is a good test for the function of ATP synthase as cells depend solely on oxidative phosphorylation to produce ATP. Another commonly used test for the function of ATP synthase as cells depend solely on oxidative phosphorylation to produce ATP. Another commonly used test for the function of ATP synthase as cells depend solely on oxidative phosphorylation to produce ATP.

**TABLE I**

| Plasmid/strain | ATP synthase type | Growth on acetate | Growth yield | Doubling time (min) |
|----------------|-------------------|------------------|--------------|---------------------|
| pDC14/DK7      | Δε                | –                | 39           | 70                  |
| pDC14/DK8      | Δεβγδεabc         | –                | 59           | 60                  |
| pACWU1.2/DK8   | Wild type        | +                | 100          | 53                  |
| pSD135/DK8     | εα137H           | +                | 104          | 53                  |
| pSD144/DK8     | Red ε            | +                | 85           | 55                  |
| pDC4/DK5       | Orange ε         | –                | 50           | 64                  |
| pDC5/DK5       | Yellow ε         | –                | 49           | 65                  |
| pDC11/DK8      | E-Red ε          | +                | 80           | 57                  |
| pDC12/DK8      | E-Orange ε       | –                | 55           | 64                  |
| pDC13/DK8      | E-Yellow ε       | –                | 54           | 72                  |

*All cells were grown at 37°C using M9 minimal media and the appropriate carbon source. Acetate growth tests were performed using agar plates containing 0.2% sodium acetate. Growth was assessed after incubation for 3 days. Growth yields were obtained by measuring the *A*₅₆₀ of stationary phase liquid cultures containing 0.04% glucose and expressed as a percentage of the wild type. Doubling times were calculated from plots of the *A*₅₆₀ of liquid cultures containing 0.2% glucose as a function of time.*

**Western Blot Analysis of Expressed ε Mutants**—Two potential concerns in interpreting results obtained through this approach are that the added domain might sterically interfere with the assembly of the enzyme or be subject to proteolytic removal *in vivo*. To address these concerns, membranes were prepared from cells and subjected to Western blot analysis, probing with antibodies raised against the α, γ, ε, and b subunits of ATP synthase (Fig. 3). There were low but detectable levels of α and γ in the Δε strain, indicating the presence of a small amount of F₁ in these membrane preparations (Fig. 3, panels A and B). The presence of these bands was likely due to soluble F₁ that was trapped in the pellet during the preparation of the membranes. Strains containing wild-type ε or each of the ε fusion proteins showed similar levels of α and γ in the membrane preparations, indicating that the assembly of the enzyme was normal in all cases. Blots probed with monoclonal antibodies directed against α (panel A), γ (panel B), ε (panel C), and b (panel D).

**FIG. 3.** Western blots of membranes prepared from cells containing F₁F₀ bearing various C-terminal ε fusions. Membrane samples containing 2 μg of membrane protein were analyzed by SDS-PAGE followed by Western blotting. Blots were probed with 125I-labeled antibodies directed against α (panel A), γ (panel B), ε (panel C), and b (panel D).

**Analysis of ATPase Activity of Membrane-bound F₁F₀**—To take a closer look at the effect of the ε fusions on the function of F₁F₀, the membranes described above were assayed for ATPase activity as described under “Experimental Procedures” (Table II). Besides determining the ATPase activity under conditions where F₁ remains associated with F₀, the sensitivity of this activity to the F₀-specific inhibitor, DCCD, was also assayed the ATPase activity under conditions where F₁ would be released from the membrane. Under these dilute assay conditions, the ε subunit dissociates from the F₁ complex, so the measured ATPase activity provides a good measure of the total F₁ present, without influence from potential inhibition by ε. Therefore the ratio of membrane-bound to released activity represents the relative activities of the various membrane-bound ATPases, independent of their concentrations in the membranes.

As expected, the Δεβγδεabc deletion control showed no ATPase activity. The Δε strain showed a small amount of ATP hydrolysis activity that was insensitive to DCCD inhibition, confirming that the trace amounts of α and γ detected in the Western blots of membranes prepared from this strain resulted...
is coupled to proton translocation, the ability of F₁F₀ to establish a proton gradient (curve c) was almost complete failure to establish a proton gradient (curve d). Quenching by the wild-type and fusion mutants showed a modestly decreased quenching response (curves b), compared with the wild-type despite their elevated ATPase activity (Table II). The Orange ε, E-Orange ε, and E-Yellow ε mutants showed an almost complete failure to establish a proton gradient (curves d) implying that their residual membrane-bound ATPase activity was functionally uncoupled. The Yellow ε mutant showed a low but detectable level of proton pumping (curve f), but this could be attributed to the trace levels of proteolysis of the ε fusion in the membrane preparations, as was detected in Western blot analysis (Fig. 3, panel C). In all cases the established proton gradients were quickly dissipated by addition of the uncoupler carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone.

To ensure that the F₀ portion of ATP synthase is unaffected (Fig. 4, panel B), quenching by membranes reconstituted with wild-type F₁ showed an increase in absolute membrane-bound ATPase activities that, although reduced, retained substantial sensitivity to inhibition by DCCD. These results imply that this ATPase activity that, although reduced, retained substantial sensitivity to inhibition by DCCD.
membranes prepared from any of the ε mutants and the wild-type (curves f) suggesting that the F₄₁ portion of ATP synthase assembled properly. The Δε control showed a decreased quenching response (curve g), likely due to cellular degradation of the unprotected and protruding subunit in the membrane during normal cell growth. As expected the Δεβγδεabc mutant showed no quenching response (curve h).

The ability to maintain a proton gradient in response to NADH was also tested on intact membranes containing the various F₁, F₄₁ mutants. There was no distinguishable difference between the wild-type and any of the mutant membranes (data not shown), suggesting that the fusions did not render the membranes leaky to protons.

**The Effect of the Fusions on the Ability of ε to Inhibit F₁-ATPase**—Because the C-terminal α-helix of ε has been implicated in the inhibition of ATPase activity of soluble F₁-ATPase (39), it was important to assess the effect of the ε fusions on inhibition. The consequence of adding 100 nM of each ε mutant on the activity of ε-depleted F₁-ATPase is shown in Fig. 5, panel A. Given the affinity of ε for F₁-ATPase under these conditions (35), virtually all of the ATPase molecules will be converted to the ε-bound, low activity form of the enzyme. As expected addition of wild-type ε or εA137H brought the level of ATP hydrolysis activity down to 25–35% of the activity of the ε-depleted enzyme. Fusing protein mass to the C terminus of ε essentially abolished its ability to inhibit soluble F₁-ATPase, as in all cases the residual ATPase activity ranged from 84 to 98%. To ensure that this lack in inhibition activity is not due to a failure of the mutant ε subunits to bind to F₁, the ability of the Red, Orange, and Yellow ε mutants to reverse inhibition by wild-type ε was measured (Fig. 5, panel B). If these mutants bind to F₁, but cannot inhibit its activity, they should be able to compete with wild-type ε and reverse inhibition. The three different fusions tested in this manner show decreased binding affinities for F₁ but were still able to bind, and at high concentrations were able to compete with wild-type ε and prevent inhibition. These results imply that the fused domains prevent the proper interactions of the C-terminal domain of ε with F₁ while still allowing ε to associate with F₁.

**DISCUSSION**

**In Vivo Evidence Supporting the Rotational Mechanism of ATP Synthase**—It is believed that the ATP synthesis/hydrolysis function of the peripheral F₁₁ sector is coupled to the movement of protons through the F₄₁ sector by the rotation of the γε₁₀–₁₄ complex relative to the remainder of the enzyme. Because the α₁₂β₁ hexamer of F₁ and the α₁ subunit of F₄₁ are linked by the peripheral second stalk, the volume of revolution that may be swept out by the rotor is limited. By fusing unrelated proteins to the C terminus of ε, we have increased that volume of revolution to determine whether oxidative phosphorylation and other energy coupling functions of ATP synthase would be inhibited as predicted.

Initially, although it might have been expected that fusion of the 12-kDa cytochrome b would interfere with movement of the rotor, we found that these mutants retained function, as observed both in vivo through growth on acetate and in vitro through ATP-dependent proton pumping activity. This prompted us to construct the larger ε fusion proteins, which were nonfunctional by both criteria. These nonfunctional complexes appear to have assembled properly and not to have been subject to significant degradation, because subunits of the expected sizes were observed in membrane samples analyzed by Western blotting, and stripped membranes reconstituted with wild-type F₁ exhibited normal levels of ATP-dependent proton pumping.

These results provide evidence supporting rotational catalysis by ATP synthase in vivo and may be interpreted in terms of conformational changes and unoccupied space in the region around the rotor. The cytochrome b molecule forms a four-helix bundle with dimensions of 23 × 24 × 48 Å (40), whereas flavodoxin (24 × 37 × 48 Å) (41) and flavodoxin reductase (30 × 35 × 55 Å) (42) have somewhat larger cross-sectional areas based on their crystal structures. Thus, it appears that the rotational path inside the second stalk provides between 24 and 37 Å of clearance. In addition, limits are placed on any obligatory conformational changes linked to catalysis, such as vertical movement of α₁₁β₁ toward the ε oligomer.

Previous studies of rotation in ATP synthase have been carried out in vitro, using dilute buffers, sometimes in the presence of detergents. Protoplasts are extremely different from these buffers. Biological membranes are packed tightly with proteins, and the high concentrations of both proteins and
nucleic acids in the cytoplasm give rise to the phenomenon called macromolecular crowding (43). The paucity of space leads to altered viscosity, activity coefficients, diffusion rates, and binding equilibria. Although it might be expected that molecular motions would be affected, our results support the rotational mechanism of ATP synthase in this natural environment.

Location of the C-terminal ε fusion Site in ATP Synthase—When these studies were begun, the C terminus of ε was selected as the site for the fusions because it was known that modification of this region does not disrupt oxidative phosphorylation (19, 20), but exact knowledge of the position of the C terminus was unavailable. High resolution structures of isolated ε reveal a two-domain protein, with an N-terminal 10-stranded β-sandwich and a C-terminal helix-loop-helix hairpin (44, 45). Whether or not the two domains remain closely associated in ATP synthase is controversial, because new crystal structures have revealed two different conformations of the C-terminal domain. In the yeast F1ε10 structure of Stock et al. (15), the δ subunit (the mitochondrial homolog of bacterial ε) assumes a conformation similar to that seen in the isolated E. coli subunit. Both domains are located near the polar loops of the membrane-integral ε subunits, and the C-terminal helix points in a direction tangential to that ring. This could be called the “down” conformation because of the proximity of the C-terminal domain to the membrane; a similar orientation was seen in the recent beef mitochondrial F1δ structure by Gibbons et al. (46). This position would be expected to accommodate assembly of enzyme containing the ε fusions, but given the evidence that the membrane-spanning domains of the b subunits are adjacent to the c ring (47), it seems unlikely that the C-terminal domain and the fused protein could remain in this down position without blocking rotation past bδ. In the E. coli γεε structure of Rodgers and Wilce (48), where the N- and C-terminal helices of γ have been removed, the C terminus of ε is located far from the membrane in a position that would be adjacent to the lower surfaces of α and/or β (the “up” conformation). A similar conformation of ε is implied in a low resolution E. coli F1ε structure (49, 50). This position is in accordance with cross-linking studies (51, 52). The globular domains fused to ε would probably block assembly of the enzyme if this up conformation was obligatory. Thus, in the fusion proteins the C-terminal helix of ε is unlikely to adopt either of these conformations. Instead, it is probably mobile, providing flexibility between the essential N-terminal domain of ε and the fused domain.

Implications of the Membrane-bound ATPase Activity of the ε Fusions for the Normal Function of ε—The inability of all of the ε fusions to inhibit the ATPase activity of soluble F1ε is consistent with much evidence linking the inhibitory function to the C-terminal domain and its interaction with β and/or α (19, 53). This domain is dispensable for ATP synthesis (19) but must fulfill some important function, and a role in inhibition or regulation of the enzyme has been suggested (54–56). Proteolytic cleavage of the C-terminal part of ε in ATP synthase activates ATP hydrolysis without causing release of F1ε from F0 (54). In addition, linking the C-terminal and N-terminal domains through an engineered disulfide gives enhanced ATPase activity (55). These effects have been interpreted as relief from inhibition by ε. The purpose of inhibiting the ATP synthase of a facultative bacterium like E. coli has been unclear, however, because the function of the enzyme is to allow equilibration of the protonotive force and the phosphorylation potential with energy flowing in either direction depending on physiological conditions.

Our results may provide an explanation for this discrepancy. Consistent with the proposed role of the C terminus of ε in inhibition of the holoenzyme, the membrane-bound ATPase activity of the Red ε fusions was higher, relative to the released ATPase activity, than the wild-type or εA137H mutants. However, the excess ATPase activity of the Red forms was not coupled, because lower levels of ATP-dependent proton pumping were observed. Thus an uncoupled ATPase activity was generated by the fusions. The very substantial levels of ATPase activity of the membrane-bound ATP synthases containing the Orange or Yellow ε support this concept. Because these enzymes were unable to synthesize ATP in vivo or to couple ATP hydrolysis to proton pumping in vitro, all of the ATPase activity is uncoupled. It is notable that if the ATPase activities of the membrane-bound enzymes are expressed as percentages of the values seen after release (Table II), the values for the Red ε enzymes are essentially the sums of those of the wild-type enzyme, which should have only coupled activity, and the Orange or Yellow ε enzymes, which have only the uncoupled activity. This implies that the coupled and uncoupled activities are not just different but independent and more-or-less additive.

The growth properties of the ε fusion strains provide in vivo evidence supporting the argument above. The yield of cells containing Red ε, intermediate between wild-type and the ATPase deletion, is consistent with the existence of both coupled and uncoupled components of ATP hydrolysis. Our results thus imply that one of the normal functions of ε in membrane-bound ATP synthase is to inhibit an uncoupled ATP hydrolysis reaction and that this function is blocked in the fusions because of steric effects of the added domain.

Proposed Mechanism of Uncoupled ATP Hydrolysis—Because recent results (47) indicate that DCCD blocks the movement of the ring of ε subunits past abδ, and the uncoupled activity was substantially DCCD-sensitive, rotation is required for the uncoupled hydrolysis. The full 360° rotation of γεε is blocked by the larger fusions, but these mutant enzymes could be undergoing partial rotation, with the rotor reciprocating 120° between two sites, or even the 240° that would allow occupation of all three resting sites. In this model, each 120° rotation could be coupled with protons moving in the appropriate direction through F0, but the protons passing in opposite directions during the steps back-and-forth would cancel each other out, so net proton pumping would not be observed. For this mechanism to occur in our hydrolysis assays, the rotor must sometimes turn in the direction that would normally drive ATP synthesis while the site that has just released ADP and phosphate is either still empty or else after it has bound ATP. The latter possibility is favored because the assays were carried out at relatively high ATP concentrations.

Function of ε in ATP Synthase—If the hydrolysis is occurring as envisaged, normal ε could block it by preventing rotation in the wrong direction when ATP has been bound to the active site. In support of this idea, the ability of ε to sense occupancy at the catalytic site has been illustrated by both proteolysis and cross-linking studies which showed that the conformation of ε subunit in ATP synthase differs depending on whether MgATP or MgADP + Pi is present (54, 57). More recently, Tsunoda et al. (56) found that formation of an engineered disulfide bridge between γ and ε, designed to lock the C terminus of ε in the up conformation described above, led to an enzyme capable of ATP synthesis but defective in coupled ATP hydrolysis. In the same study, disulfide bridges introduced between the C-terminal domain of ε and the polar loop of c to lock ε in the down conformation had little effect on ATP synthase but caused a 50% increase in ATPase activity. Based on these results, it was suggested that ε functions as a ratchet; through interactions
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