Alanine-scanning Mutagenesis of the $\epsilon$ Subunit of the F$_{1}$-F$_{0}$ ATP Synthase from Escherichia coli Reveals Two Classes of Mutants*

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The abbreviations used are: ACMA, 9-amino,6-chloro,2-methoxy-

Alanine-scanning mutagenesis was applied to the $\epsilon$ subunit of the F$_{1}$-F$_{0}$ ATP synthase from E. coli. Nineteen amino acid residues were changed to alanine, either singly or in pairs, between residues 10 and 93. All mutants, when expressed in the $\epsilon$ deletion strain XH1, were able to grow on succinate minimal medium. Membranes were prepared from all mutants and assayed for ATP-driven proton translocation, ATP hydrolysis $\pm$ lauryldiethylenelamine oxide, and sensitivity of ATPase activity to N,N'-dicyclohexylcarbodiimide (DCCD). Most of the mutants fell into 2 distinct classes. The first group had inhibited ATPase activity, with near normal levels of membrane-bound F$_{1}$, but decreased sensitivity to DCCD. The second group had stimulated ATPase activity, with a reduced level of membrane-bound F$_{1}$, but normal sensitivity to DCCD. Membranes from all mutants were further characterized by immunoblotting using 2 monoclonal antibodies. A model for the secondary structure of $\epsilon$ and its role in the function of the ATP synthase has been developed. Some residues are important for the binding of $\epsilon$ to F$_{1}$ and therefore for inhibition. Other residues, from Glu-59 through Glu-70, are important for the release of inhibition by $\epsilon$ that is part of the normal enzyme cycle.

The F$_{1}$-F$_{0}$ ATP synthase from Escherichia coli (for reviews, see Refs. 1-3) is composed of eight different types of polypeptide subunits which are coded by eight genes found in a single operon called unc (or atp). The enzyme can be physically separated into a membrane-bound portion called F$_{0}$ and a water-soluble portion called F$_{1}$. F$_{0}$ consists of five types of subunits: $\alpha$ (uncA), $\beta$ (uncD), $\gamma$ (uncG), $\delta$ (uncH), and $\epsilon$ (uncE) in a stoichiometry of 3:3:1:1:1. F$_{0}$ consists of three types of subunits: a (uncB), b (uncF), and c (uncE) in a stoichiometry of 1:2:10 ± 1 (4).

In the intact F$_{1}$-F$_{0}$ complex, the net synthesis of ATP at sites in F$_{0}$ is in response to an electrochemical proton gradient across the membrane, which drives proton movement through F$_{0}$. This proton movement is coupled to net synthesis of ATP at sites in F$_{1}$, probably via a series of conformational changes. Isolated F$_{1}$ catalyzes the hydrolysis of ATP, but not its synthesis. The molecular events linking the movement of protons through the membrane-bound portion of the ATPase to the synthesis of ATP at distant sites, remain essentially unknown. Recently, x-ray crystallographic studies of F$_{1}$ from bovine mi-

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The following symbols indicate 50% mixtures of bases at a designated position: M = A + C, R = A + G, S = G + C, Y = C + T.

Fig. 1. A, the gene for the \( \epsilon \) subunit, unc\( ^2 \). The darkest regions of the gene indicate segments that have been replaced by synthetic DNA containing additional restriction sites (27). Sites used in this study have been indicated. B, deoxyoligonucleotides used for cassette mutagenesis. For each pair of oligonucleotides, the two restriction sites used for ligation are indicated at the appropriate ends. The residues that are mutagenized are indicated above the oligonucleotides, and the corresponding new codons are underlined. The following symbols indicate 50% mixtures of bases at a designated position: M = A + C, R = A + G, S = G + C, Y = C + T.

tained from Molecular Probes. LDAO and DCCD were obtained from Fluka. Immunoblotting reagents were obtained from Bio-Rad, and anti-\( \epsilon \) monoclonal antibodies, \( \epsilon \)I and \( \epsilon \)II, were a generous gift of Dr. Roderick Capaldi, University of Oregon (23).

Bacterial Strains—Strain XL1-Blue (reA1, endA1, gyrA96, thi, hsdR17(rK \( ^{-} \), m\( ^{J} \)), supE44, relA1, lac- (Iac), (P', proAB, lac') Z18M15, Tn10(Tet\( ^{R} \))) was obtained from Stratagene and was used routinely for subclonings and mutagenesis. Strain XH1 (unc\( ^2 \), bgl\( ^{-} \), thi\( ^{-} \), relE44, lacI\( ^{-} \)), (F\( ^{-} \) rec\( ^{-} \) end\( ^{-} \) pro\( ^{-} \) bgl\( ^{-} \) thi\( ^{-} \) lac\( ^{-} \) MI5, M15) was used to characterize mutations in unc\( ^2 \). It contains a deletion from \( \lambda \) to 271 in unc\( ^2 \) and produces no detectable \( \epsilon \) subunit. It is complemented by plasmid pXH302S, which contains a synthetic version of unc\( ^2 \). Cultures were grown at 37°C, and cell density was monitored by optical density at 600 nm using a Milton Roy 1001 spectrophotometer. Rich medium was Luria Broth supplemented with 0.2% glucose, and minimal medium was salts supplemented with succinate (0.2%) or with glucose, as indicated (25). Media were supplemented with ampicillin (100 mg/liter) or tetracycline (12.5 mg/liter) as appropriate.

Construction of Mutations—Plasmid pXH302S was used to construct the mutations (shown in Fig. 1) using the cassette mutagenesis technique, as described previously (26, 27). Plasmids were subjected to sequential digestion by each of the two indicated restriction enzymes, followed by ligation with annealed oligonucleotides. Following mutagenesis, plasmids were isolated from transformants (28) and screened by various restriction enzyme digestions to detect the presence of mutations. Subsequently, the DNA sequences of plasmids thought to bear the mutations were analyzed using \( \epsilon I \) dATP as described before (26) or by a silver staining technique (Promega) according to the manufacturer's instructions. As indicated in Fig. 1, S10A and F16A were constructed simultaneously in the same plasmid. These mutations were later isolated using a unique SacI restriction site which lies between the two mutations. Most of the other mutations were constructed using degenerate oligonucleotides, which produced mixtures of wild type and mutant residues that were identified after sequence analysis. One mutation, E90A, was never isolated. Mutations were characterized following transformation of the strain XH1.

Preparation of Cell Fractions and Assays—Fractionation of cells and isolation of membranes and stripped membranes were carried out as described (29). Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as standard. ATP hydrolysis assays were carried out as described (9) with 50 mM Tris acetate, pH 7.5, 3 mM ATP, 5 mM MgCl\( _2 \), 5 mM KCN, 2 mM phoshoenolpyruvate, 0.25 mM NADH, pyruvate kinase (6 units), and lactate dehydrogenase (8 units) at 37°C using a Beckman DU70 spectrophotometer. For release of inhibition by \( \epsilon \), LDAO was added to the assay medium to a final concentration of 1% (9). Inhibition of ATP hydrolysis by DCCD was performed as described previously (26). Fluorescence quenching assays were performed using 400 \( \mu \)mol/mg membrane protein in a solution of 50 mM Mops, pH 7.3, 10 mM MgCl\( _2 \), 1 mM ACMA, and either 0.5 mM NADH or 0.1 mM ATP, as appropriate. The excitation wavelength was 410 nm and the emission wavelength was 490 nm. Fluorescence quenching was abolished by addition of 1 \( \mu \)M carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone.

Immunoblotting—Samples of membranes (75 \( \mu \)g of protein) were electrophoresed and immunoblotted as described previously (30), using anti-\( \epsilon \) monoclonal antibodies and goat, anti-mouse IgG-alkaline phosphatase conjugate.

RESULTS

Mutations were constructed as outlined in Fig. 1. Amino acid residues in \( \epsilon \) were converted to alanine to maximize the possibility that the effects of mutagenesis would be confined to local changes. Residues were selected for mutagenesis based on the
Eighteen mutants, with either single or double alanine substitutions in $\epsilon$, were analyzed for growth properties following transformation of the $\epsilon$ deletion strain X1H1. The mutants that were constructed and their growth properties are shown in Table I. Only 2 mutants, S10A and F16A, grew poorly on minimal glucose medium, as compared to wild type. The mutants with normal growth on succinate were T77A, R85A, D81A/R85A, T82A/R85A, R51A, K54A, R93A, F16A, E70A, T77A, and R93A. The mutants with properties that were intermediate between the other two classes and were more similar to wild type were E70A, E29A, R51A, K54A, and R93A. This group also tended to have lower levels of bound $F_1$, ATPase, averaging 61% of wild type, and to have normal sensitivity to DCCD, averaging 60% (95% of the wild type level). Six additional mutants, E21A, Q24A, E29A, R51A, K54A, and R93A, had properties that were intermediate between the other two classes and were more similar to wild type.

ATP hydrolysis by membrane vesicles from these mutants was measured under three sets of conditions, and the results are shown in Table I. The standard assay was the coupled enzyme assay at pH 7.5. This assay was repeated in the presence of 1% LDAO, which is known to release the inhibition of $F_1$ ATPase activity by $\epsilon$ (9, 31). The second assay reveals two properties of the mutants. First, it yields the level of uninhibited ATPase activity of the membranes, which reflects the total amount of $F_1$ bound. Second, when compared with the first assay it shows the fold stimulation upon LDAO addition, which reflects the fold inhibition by $\epsilon$. Finally, a third assay was performed after preincubation with DCCD, a specific inhibitor of proton translocation by $F_0$. Decreased sensitivity of ATPase activity to DCCD reflects ATP hydrolysis that is not obligatorily coupled to proton translocation.

Results of the standard ATP hydrolysis assays revealed that membranes from S10A and F16A had undetectable levels of membrane-bound ATPase. Membranes from the other mutants ranged from 51% to 132% of the wild type levels of ATPase activity, suggesting 2 classes of mutants. This was confirmed by assays in the presence of LDAO, or after preincubation with DCCD. Six mutants showed stimulation of ATP hydrolysis by LDAO of less than 2.4-fold, compared to the wild type value of 3.1-fold. These mutants were T77A, R85A, D81A/R85A, T82A/R85A, T43A, and K46A. This group also tended to have lower levels of bound $F_1$, ATPase, averaging 61% of wild type, and to have normal sensitivity to DCCD, averaging 60% (95% of the wild type level). A second class consisted of E70A, S65A, E59A/E60A, K54A/E60A, E29A, R51A, and E31A. These mutants showed higher than normal stimulation of ATPase activity by LDAO, with values ranging from 3.5- to 4.6-fold. These mutants showed a more normal level of membrane-bound $F_1$, ATPase, averaging 85% of the wild type level. However, the membrane-bound ATPase activity was less sensitive to DCCD, averaging only 48% (76% of the wild type level). Six additional mutants, E21A, Q24A, E29A, R51A, K54A, and R93A, had properties that were intermediate between the other two classes and were more similar to the wild type.

Membranes from all mutants were subjected to immunoblotting using two previously described monoclonal antibodies to $\epsilon$ (23). Both monoclonal antibodies failed to recognize F16A, and S10A was recognized only weakly. The only significant difference between the two antibodies was that E1 failed to recognize E21A and Q24A.
Fig. 2. PHD prediction of secondary structure of ε. The lines labeled AA contain the amino acid sequence of ε, with the corresponding numbers above. PHD indicates the profile network prediction, where E indicates β-strand, H indicates α-helix, and blank spaces indicate a loop prediction. Rel indicates the reliability index of prediction, on a scale from 0 to 1 (most reliable). Sub indicates a subset of the predictions for which the expected accuracy will be >82%. In this line, loop predictions are denoted by L. Where Rel < 0.5, no prediction is made, and these are indicated by dots. Underlined residues are the hydrophobic residues in the predicted β-strands. Other sequences used in the prediction are Vibrio alginolyticus, Bacillus PS3, Bacillus megaterium, Bacillus firmus, Rhodosporillum rubrum, Synechocystis PCC 6803, and Synechococcus PCC 6301.

α-helix were predicted at the carboxyl terminus (79–138), consisting of 5, 16, and 21 residues. Two other regions were weakly predicted to be β-strands: residues 15–16 and 32–34. Each of the 8 predicted β-strands shown in Fig. 2 has 2 hydrophobic residues separated by a single residue. These residues are underlined. This trend exists in each of the eight ε sequences and is consistent with amphipathic β-strands, such as those which would form a β-barrel or β-sandwich. The only exception is the second predicted β-strand, in which the 2 hydrophobic residues are adjacent. Further analysis of 21 bacterial ε sequences retrieved by BLAST analysis (33) revealed that this pattern of hydrophobic residues was present in each sequence (not shown).

**DISCUSSION**

The ε subunit has a central role in energy transduction by the E. coli F₁-F₀ ATP synthase. The goal of the present work was to identify mutants that alter ε binding to other subunits, presumably γ (11), would reduce both the binding of F₁ to F₀ in membranes and also the inhibition of ATPase activity by ε. These residues extend from the eighth predicted β-strand to the α-helical region. This region has been implicated previously in the inhibitory role of ε by analysis of deletions from the carboxyl terminus (16). It has also been shown that an ε monoclonal antibody has an epitope that is buried in F₁, and part of that epitope is between residues 78 and 85 (18). Two other mutants analyzed in this study have similar properties: T43A and K46A. These residues lie in a stretch of 15 amino acids between the fourth and fifth predicted β-strands. It is possible that these residues make up part of the epitope for ε-mAb-1, described previously (18).

A second group of mutants has an opposite effect on the inhibitory properties of ε. This group also contains residues from two distinct regions: residue 31 and residues 59–70. With these mutants a relatively high level of F₁ is bound to membranes, averaging 85% of wild type, but the ATPase activity is highly inhibited. Upon addition of LDAA, the activity is stimulated up to 4.6-fold. It appears that these mutants, E31A, E59A, E60A/K54A, E59A/E60A, and E70A, form stable F₁–F₀ complexes, but that the F₁ is locked in an abnormally inhibited state.

Residue Glu-31 is located in the fourth predicted β-strand and has been mutated previously (27). This residue has also been identified as the site of partial suppressors of the ε subunit mutant Q42E (34). In the present study, E31A was found to reduce the sensitivity of F₁ ATPase to DCCD (76% of wild type). Mutations at the nearby residue His-38 were shown (27) to reduce the sensitivity of F₁ ATPase to DCCD. This evidence tends to support either of the possibilities offered (34), that this region of ε interacts directly with the polar loop of c or that it interacts with “stalk” subunits, which interact with c. A reduced sensitivity to DCCD by membrane-bound F₁ ATPase is also seen with most of the other members of this group, especially S65A and E70A (70% and 62% of wild type, respectively). Residue 65 is predicted to lie in a loop between the sixth and seventh β-strands, and residue 70 is part of the predicted seventh β-strand. The double mutant E59A/E60A also has reduced sensitivity to DCCD, and these residues are found in a loop between the fifth and sixth predicted β-strands.

The two monoclonal antibodies have been characterized previously with respect to binding of ε in F₁ and F₁-F₀, εL3 (5A3-C11), and εII (13A7-E9) (23). Our results reveal a single difference between these two antibodies: εL does not recognize the mutants E21A and Q24A. Since it was determined previously (23) that εL does not recognize ε in F₁–F₀ complexes, but that εII does weakly, it is likely that residues 21 and 24 of ε are shielded by the binding of F₁ to F₀.

The rate of the present study contribute to the growing picture of the role of ε in the structure and function of the F₁–F₀ ATP synthase. Previous work by others has indicated a two-domain structure for ε (16), separated by a “hinge” region near residues 80–85 (18). Conformational changes in ε have been observed via differences in protease sensitivity and in response to ligands at nucleotide binding sites (19). DCCD has been shown to alter the nucleotide dependence of tryptophan cleavage (35). Movement of ε relative to other F₁ subunits has been observed via specific cross-linking reagents, also in response to ligands at nucleotide binding sites (6, 12, 13). The effects of several mutations at the strictly conserved residue His-38 have also been studied. H38C has been shown to be accessible to Ellman’s reagent, and the modified ε remains inhibitory (36). Two other mutants H38I and H38R were found to have de-
creased sensitivity to DCCD, but ε seemed to be highly inhibitory (27).

A schematic model for the secondary structure of ε is shown in Fig. 3, based on the PHD secondary structure predictions, hydrophobicity considerations, and the results of our mutagenesis. The model is oriented such that the top would be near the α and β subunits in F₁, and the bottom would be near the ε subunits of F₀. The model includes 8 antiparallel β-strands, 3 α-helical segments, and one significant region of undesignated secondary structure. It is proposed that 6 β-strands are organized into an anti-parallel β-barrel, and that 2 additional β-strands form a β-hairpin. Three of the β-strands in the barrel are proposed to interact with γ; primarily strands 2 and 8, and to a small extent strand 1. The other 3 β-strands in the barrel, 5, 6, and 7, are proposed to form a relatively exposed face that interacts with one or both of the α-helical segments. The β-hairpin, formed by β-strands 3 and 4, is proposed to be close to F₂ subunits. A region of undesignated secondary structure, found between β-strands 4 and 5, and the short α-helix following β-strand 8 are both proposed to have interactions with γ. The sites of alanine mutations are also indicated in Fig. 3.

In view of the secondary structure model and the previously described conformational changes of ε, the following interpretation of these mutations is offered: residues Ser-10, Phe-16, Thr-43, Lys-46, Thr-77, Asp-81, Thr-82, and Arg-85 are proposed to be important for the binding of ε to other subunits, primarily γ. Failure to bind properly, due to mutations at these residues, leads to decreased inhibition by ε, and to some loss of F₁ from membranes. Specifically, the conformation of ε that stabilizes bound products at the active site is affected, but the functional coupling of F₁ and F₀ is not affected. Residues Glu-31, Lys-54, Glu-59, Glu-60, Ser-65, and Glu-70 are proposed to be important for the release of inhibition that is part of the normal conformational cycle of ε. Mutations at these residues lead to a highly inhibited form of membrane-bound F₁, and ATPase activity is partially decoupled from proton translocation. These residues might interact with carboxyl-terminal residues of ε, or, in the case of Glu-31, with ε subunits (34). One prediction is that some of these mutants might be resistant to protolysis by trypsin, independent of nucleotide (19, 35).