Subunit ϵ of the *Escherichia coli* ATP Synthase: Novel Insights into Structure and Function by Analysis of Thirteen Mutant Forms[†]

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ABSTRACT: Structural models of subunit ϵ of the ATP synthase from *Escherichia coli* have been determined recently by NMR [Wilkens et al. (1995) *Nat. Struct. Biol.* 2, 961−967] and by X-ray crystallography [Uhlin et al. (1997) *Structure* 5, 1219−1230], revealing a two-domain protein. In this study, six new ϵ mutants were constructed and analyzed: Y63A, D81A, T82A, and three truncated mutants, tr80(S), tr94-(LAS), and tr117(AS). Seven mutants constructed previously were also analyzed: E31A, E59A, S65A, E70A, T77A, R58A, and D81A/R85A. Subunits were purified by isoelectric focusing from extracts of cells that overproduced these 13 mutants. F_1 was prepared lacking subunit ϵ by immobilized-Ni affinity chromatography. Three mutants, E70A, S65A, and E31A, showed somewhat higher affinities and extents of inhibition than the wild type. Three mutants, T82A, R85A, and tr94(LAS), showed both lower affinities and extents of inhibition, over the concentration range tested. Two showed no inhibition, D81A and tr80(S). The others, T77A, Y63A, E59A, and tr117(AS), showed lower affinities than wild type, but the extents of inhibition were nearly normal. Results indicate that the C-terminal domain of subunit ϵ contributes to inhibition of ATP hydrolysis, but it is not necessary for ATP-driven proton translocation. Interactions with subunit γ are likely to involve a surface containing residues S65, E70, T77, D81, and T82, while residues R85 and Y63 are likely to be important in the conformation of subunit ϵ.

The F_1F_o ATP synthase from *Escherichia coli* is composed of eight different subunits, and is typical of the ATP synthases found in mitochondria, chloroplasts, and many other bacteria [for recent reviews, see (*1*−*4*)]. It consists of two complexes: a membrane-bound portion called F_o and a water-soluble portion called F_1. F_1 consists of five types of subunits: α, β, γ, δ, and ϵ in a stoichiometry of 3:3:1:1:1. F_o consists of three types of subunits: *a*, *b*, and *c* in a stoichiometry of 1:2:(9−12) (*5*). F_1 contains the nucleotide-binding subunits involved in catalysis, and F_o conducts protons across the membrane. The crystallization of F_1 from bovine mitochondria (*6*) has led to a high-resolution structure of the $\alpha_3\beta_3$ hexamer, plus parts of γ in the central core. Electron cryomicroscopic images have also contributed to an understanding of subunit arrangement (*7*, *8*) and of the movement of ϵ and γ subunits in F_1 (*9*). Direct visualization of rotation of fluorescently labeled actin filaments covalently attached to γ (*10*) during ATP hydrolysis has confirmed the hypothesis of rotation of γ relative to $\alpha_3\beta_3$ (*11*), which was previously supported by other studies involving engineered disulfide cross-linking of β to γ (*12*) and fluorescence spectroscopy (*13*). The three-dimensional structures of two other F_1 subunits from *E. coli* have been determined: δ (*14*) and ϵ (*15*, *16*). Subunit ϵ binds to γ through its N-terminal domain at the "base" of F_1 (*17*, *18*), and interacts with subunits α and β through its C-terminal domain (*19*−*21*). The two-domain structure of ϵ is shown in Figure 1. Subunit ϵ has been cross-linked to subunit *c* (*22*) via mutants ϵ (E31C) and *c* (Q42C), and it can protect a subset of the 9−12 subunits *c* from labeling by a maleimide reagent (*23*).

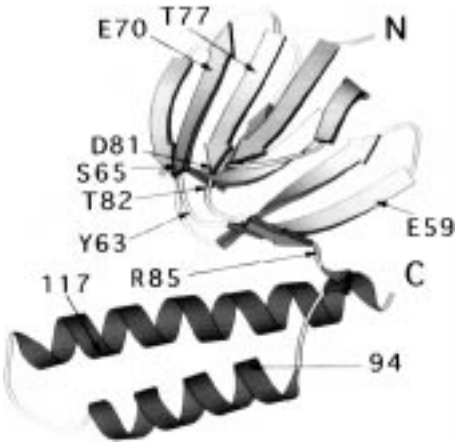

FIGURE 1: The two-domain structure of subunit ϵ. The N-terminal domain is a 10-stranded β-sandwich, and the C-terminal domain is a pair of antiparallel α-helices. Key residues for this study are indicated by arrows. This figure was generated from Protein Databank File 1aqt (*15*).

In the *E. coli* enzyme, ϵ is required for the binding of F_1 to F_o (*24*), and is also an inhibitor of ATP hydrolysis by F_1. This inhibitory role seems to be important for limiting ATP hydrolysis by F_1 that is not membrane-bound, as seen in ϵ mutant strains, which are barely viable (*25*−*27*). The mechanism by which ϵ acts upon the remaining F_1 subunits to cause inhibition is unknown, although evidence indicates that interactions with subunit β are important (*28*). The removal of ϵ from F_1 results in about a 6-fold increase in ATP hydrolysis activity (*29*, *30*). Subunit ϵ is thought to

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act by reducing the rate of phosphate release (*31*), and therefore it might be important for the coordination of rotary catalysis. Other studies have shown a nucleotide and DCCD[1] dependence for both the protease sensitivity of ϵ and the cross-linking to other subunits by ϵ, indicating conformational changes (*32, 33*).

Deletion mutants from both the carboxy terminus (*27*) and the amino terminus (*34*) of subunit ϵ from *E. coli* have been constructed and analyzed. A truncated form containing only the first 93 residues, plus 2 serine residues, was capable of partially inhibiting ATP hydrolysis and of promoting the binding of F_1 to F_o. Shorter subunits of about 80 residues were also capable of the binding function, but did not have inhibitory properties (*27*). Deletions of up to 15 residues at the amino terminus did not seriously impair function, although larger deletions did. Internal deletions have also been analyzed (*35*), providing consistent results. For spinach chloroplast subunit ϵ, it has been shown (*36*) that deletions of 5 and 10 residues at the carboxy terminus are much more deleterious than similar ones in *E. coli*, and that deletions at the amino terminus are also deleterious (*37*).

In a previous study from this laboratory (*38*), alanine-scanning mutagenesis was applied to subunit ϵ in an attempt to discover residues that are necessary for binding. Some alanine mutants had no effect, but those that did could be classified into two groups. The first group of mutants lost F_1 from the membranes, ϵ was not inhibitory toward ATP hydrolysis, but ATP hydrolysis remained fully sensitive to DCCD inhibition. The second group of mutants maintained F_1 binding to membranes and were highly inhibitory toward ATP hydrolysis, but the sensitivity of ATPase to DCCD was reduced.

In this report, we have attempted to quantitate the effects of both alanine mutants and truncated mutants on binding and inhibition of F_1 ATPase activity, through analysis of purified mutant subunits. Furthermore, these results have been interpreted in light of recently reported high-resolution structures of subunit ϵ.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, Inc. Taq DNA polymerase was obtained from Life Technologies, Inc., or from Promega Corp. NADH, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma Chemical Co. Synthetic oligonucleotides were obtained from Operon Technologies, Inc., or from National Biosciences, Inc. Urea was from International Biotechnologies, Inc. ATP was obtained from Boehringer Mannheim. Promega Corp. supplied Wizard-Minipreps for plasmid isolation for sequencing. ACMA was obtained from Molecular Probes. LDAO and DCCD were obtained from Fluka. Immunoblotting reagents were obtained from Bio-Rad, and anti-ϵ monoclonal antibodies, ϵI and ϵII, were a generous gift of Dr. Roderick Capaldi, University of Oregon (*39*). Some figures were produced from images generated by the molecular modeling software RasMol (*40*).

Bacterial Strains. Strain XL1-Blue (*rec*A1, *end*A1, *gyr*-A96, *thi*, *hsd*R17(r_k^-, m_k^+), *sup*E44, *rel*A1, λ^-,(*lac*), {F′, *pro*AB, *lac*I^q ZΔM15, Tn10(Tet^R)}) was obtained from Stratagene and was used for subclonings and mutagenesis. Strain XH1 (Δ*unc*C, *bgl*R, *thi*-1, *rel*-1, Hfr PO1) (*26*) was used to characterize mutations in *unc*C. It contains a deletion from −2 to 271 in *unc*C, and produces no detectable ϵ subunit. It is complemented by plasmid pXH302S, which contains a synthetic version of *unc*C (*26*). Cultures were grown at 37 °C, and cell density was monitored by the 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 A salts supplemented with succinate (0.2%) or with glucose, as indicated (*41*). Media were supplemented with ampicillin (100 mg/L) or tetracycline (12.5 mg/L) as appropriate.

Construction of Mutations. Plasmid pXH302S was used to construct the mutations using the cassette-mutagenesis technique, as described previously (*42*, *43*). The DNA sequences were determined as described previously (*38*). The truncated mutants were constructed using BAL-31 nuclease digestion after cleavage at the unique *Msc*I site, near residue 122, as described previously (*44*). A 6-histidine tag was introduced at the C-terminus of the ϵ subunit in the following way: A once-cut fragment was isolated (Bio-Rad, Prep-A-Gene) from partial digestion with *Bsr*FI. The fragment was further digested briefly with BAL-31 nuclease, followed by ligation to a synthetic oligonucleotide (CATCACCATCAC-CATCACTAGAATTC) that coded for six histidine residues, and a *Hin*dIII site. After DNA sequence analysis, a candidate was identified as containing an in-frame insertion of six histidine codons followed by a stop codon, and named pXH302H. This construct was missing the last three residues of the normal sequence of subunit ϵ (KAM).

Preparation of Cell Fractions and Assays. Fractionation of cells and isolation of membranes and stripped membranes were carried out as described previously (*45*). Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as standard. ATP hydrolysis assays were carried out as described (*38*) with 50 mM Tris−acetate, pH 7.5, 3 mM ATP, 5 mM $MgCl_2$, 5 mM KCN, 2 mM PEP, 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 ϵ, LDAO was added to the assay medium to a final concentration of 1% (*30*). Inhibition of ATP hydrolysis by DCCD was performed as previously described (*42*). Fluorescence quenching assays were performed using 300 μg/mL membrane protein in a solution of 50 mM MOPS, pH 7.3, 10 mM $MgCl_2$, 1 μM 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 μM FCCP. Gel electrophoresis and immunoblotting were carried out as described previously (*26*).

Purification of ϵ Subunit. To increase the expression of ϵ, *unc*C mutants were cloned into the overexpression plasmid pXH303S (*26*). The segments between restriction sites for *Ssp*I and *Spe*I from pXH302S, which includes the region of alanine mutations, were ligated to pXH303S, which was cut

[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; ACMA, 9-amino-6-chloro-2-methoxyacridine; LDAO, lauryldimethylamine oxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, *N*-morpholinopropanesulfonic acid; tr80(S), mutant truncated after the first 80 amino acids with an additional serine residue; Y63A, mutant in which tyrosine 63 is replaced by alanine.

with the same pair of enzymes. The samples containing recombinant ϵ were prepared as follows: ϵ-deletion XH1 was transformed with pXH303S or its derivatives bearing mutations. Membrane vesicles were prepared using a French press as described previously (*45*). After centrifugation for 1 h at 50 000 rpm (Beckman Ti 70.1 rotor), the supernatant fraction was saved. The membranes were stripped of F_1 by gentle agitation overnight in 1.0 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 2.5 mM β-mercaptoethanol, and 10% glycerol (v/v) at 4 °C. After centrifugation for 1 h at 50 000 rpm (Ti 70.1 rotor), the stripped membranes were discarded. The supernatant fraction was combined with the previous one and stored at −60 °C. The primary step in isolation of subunit ϵ was carried out by preparative isoelectric focusing electrophoresis using a Bio-Rad Rotofor Cell, according to the manufacturer's instructions. Samples were brought to 50 mL with 10% glycerol (v/v), 2% (w/v) ampholyte (Bio-Rad Bio-lytes, pH range 5−8), and filtered to remove particulates. The filtered solution was loaded into the Rotofor cell and focused without other treatment for 3 h at 15 W constant power. The apparatus was cooled to 10 °C by a circulating water bath. The initial conditions were approximately 400 V and 17 mA. At equilibrium, the values were approximately 880 V and 9 mA. Twenty fractions were collected and analyzed by polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue R-250 or subjected to immunoblotting using anti-ϵ monoclonal antibody. The major fraction containing pure ϵ was dialyzed for 24 h to remove the bound ampholyte, with one change of buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$, 200 mM NaCl). This fraction was further subjected to ultrafiltration on Amicon Centicon-30; 1 mL of the solution was added to the sample reservoir and centrifuged at 4000*g* for 30 min. The solution recovered in the filtrate cup (0.7 mL) was stored at −60 °C prior to use.

Purification of ϵ-Free F_1. Strain XH1 was transformed with pXH302H, and F_1 was prepared by stripping everted membrane vesicles as described above. A 10 mL sample, prepared from 500 mL of culture, was dialyzed against 25 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$ overnight at 4 °C and diluted 5-fold before the chromatography. Three milliliters of Ni−NTA resin was incubated with the above sample for 1 h. The mixture was packed into a 1 cm × 5 cm column. The column was washed by the dialysis buffer until A_{280} returned to less than 0.01. The column was washed further with buffer containing 25 mM boric acid, pH 9.0, 20 mM imidazole until A_{280} returned to less than 0.01. F_1 depleted of ϵ was eluted with 25 mM Tris-HCl, pH 8.0, 0.4% LDAO (v/v), 20% glycerol (v/v). Fractions were collected and assayed for ATPase activity. The enzyme was stored at −60 °C prior to use.

RESULTS

In a previous study (*38*), a series of alanine-substitution mutants in subunit ϵ were constructed and analyzed for effects on F_1 binding and inhibition of ATP hydrolysis in membrane preparations. In this study, subunit ϵ was purified from seven of the previous mutants, and from six newly constructed mutants (see Figure 1). This permitted a more direct analysis of the binding affinity for F_1, and for the extent of inhibition of F_1 ATPase. Three new alanine-substitution mutants were constructed: Y63A, D81A, and T82A. The first residue was chosen because it lies at the interface of the two domains. The last two were prepared because in the previous study, mutations at 81 and 82 reduced binding and inhibition, but they had been isolated only in combination with a third mutation, R85A. A series of C-terminal truncated subunits was also constructed, and three were chosen for analysis. The shortest, tr80(S), is truncated at residue 80, in the ninth β-strand, and contains an additional serine. The next, tr94(LAS), is truncated at residue 94, in the first long α-helix, and contains three additional amino acids at the C-terminus. The longest is tr117(AS), which is truncated in the last α-helix, and contains an additional two amino acids.

Table 1: Growth Properties of Epsilon Mutants

strain[a]	growth on succinate	growth yield[b] (%)
1100	++	100
XH1	−	26
XH1/pXH302S	++	86
XH1/pXH302S(T82A)	++	84
XH1/pXH302S(D81A)	++	85
XH1/pXH302S(Y63A)	++	52
XH1/pXH302S(tr80(S))	−	26
XH1/pXH302S(tr94(LAS))	++	64
XH1/pXH302S(tr117(AS))	++	70

[a] Growth on succinate is judged by appearance on succinate minimal plates after 48 h at 37 °C. [b] Growth yield is expressed relative to the growth yield of strain 1100. These results are the averages of at least two determinations that did not differ by more than 4%.

Table 2: ATPase Properties of Epsilon Mutants

	ATP hydrolysis by membranes		
mutant	sp act.[a]	sp act. + LDAO[b] (*x*-fold stimulation)	sensitivity to DCCD[c] (%)
wild type	0.59	1.77 (3.0)	69
T82A	0.69	2.08 (3.0)	70
D81A	1.05	1.60 (1.5)	80
Y63A	0.25	1.86 (7.4)	39
tr80(S)	0.14	0.22 (1.6)	15
tr94(LAS)	0.72	1.21 (1.7)	68
tr117(AS)	0.49	1.06 (2.2)	67

[a] Specific activities are expressed in units of μmol min^{-1} (mg of protein)$^{-1}$. All results are the average of at least two determinations that did not differ more than 6%. [b] Rates were measured in the presence of 1% LDAO. Fold stimulation is the ratio of the rate (+LDAO) to that without LDAO (column 1). [c] These rates are expressed as a percentage of the activity that is lost after a preincubation with 50 μM DCCD for 15 min at 37 °C.

Growth Properties. Plasmids bearing the six mutations were used to transform the ϵ-deletion strain XH1, and the resulting strains were analyzed for growth on minimal medium supplemented with succinate, and for growth yield in minimal medium with limiting glucose. The results are shown in Table 1. The mutants D81A and T82A grew similar to the wild type, while Y63A, tr94(LAS), and tr117-(AS) had somewhat lower growth yields. Only tr80(S) was unable to grow on succinate minimal medium, and was indistinguishable from XH1 in terms of growth yield.

Analysis of Membranes. Everted membrane vesicles were prepared from each of the six mutants, and the results of assays for ATP hydrolysis are shown in Table 2. ATP hydrolysis rates are shown, along with the determination of fold stimulation by LDAO and sensitivity to DCCD. LDAO

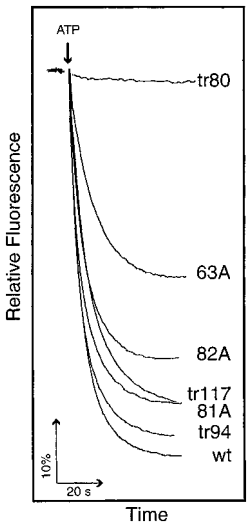

FIGURE 2: ATP-driven proton translocation by ϵ mutants. Proton gradient formation in membrane vesicles prepared from the ϵ mutants is indicated by quenching of the fluorescence of ACMA, as described under Experimental Procedures. Quenching was abolished by the addition of 1 μM FCCP (not shown).

is known to stimulate the ATP hydrolysis of F_1 from *E. coli*, primarily by competing with subunit ϵ. Therefore, stimulation of ATPase by LDAO is an indicator of inhibition by ϵ. DCCD reacts with subunit *c* of the membrane sector under the conditions used here. Therefore, inhibition of ATP hydrolysis correlates with F_1F_o integrity. The Y63A mutant was highly inhibitory as indicated by the fold stimulation by LDAO, but was less sensitive to inhibition by DCCD than was the wild type. The D81A and T82A mutants were both highly sensitive to DCCD, but D81A was only marginally stimulated by LDAO, while T82A was normal. The two longer truncated mutants had rather similar properties: decreased levels of membrane-bound ATPase activity, low stimulation by LDAO, and normal sensitivity to DCCD. The shortest of the truncated mutants, tr80(S), also showed low stimulation by LDAO, but in addition had very low levels of membrane-bound ATPase activity, and low sensitivity to DCCD. The membranes from these six mutants were also tested for ATP-driven proton translocation, and the results are shown in Figure 2. Only the tr80(S) mutant was unable to pump protons, although Y63A was diminished in rate.

Purification. ϵ subunits from wild-type and 13 mutant strains were purified by preparative isoelectric focusing. The purity of all mutants was confirmed on polyacrylamide gels after electrophoresis (results not shown). Immunoblotting using the ϵ_{II} monoclonal antibody showed some reaction with all purified mutants except D81A and the double mutant D81A/R85A (results not shown). A partially purified, ϵ-free F_1 was obtained from the strain XH1/pXH302H using immobilized Ni-affinity chromatography, as described under Experimental Procedures. This preparation was confirmed to be devoid of ϵ by immunoblotting, using two monoclonal antibodies (results not shown). The ϵ-free F_1 has a specific activity of about 30 μmol min^{-1} (mg of protein)$^{-1}$ and can be inhibited by 85% by preincubating with saturating amounts of ϵ (Figure 3). Assuming a molecular weight of 15 000, an apparent dissociation constant of about 5 nM was estimated, consistent with previous studies (*17*).

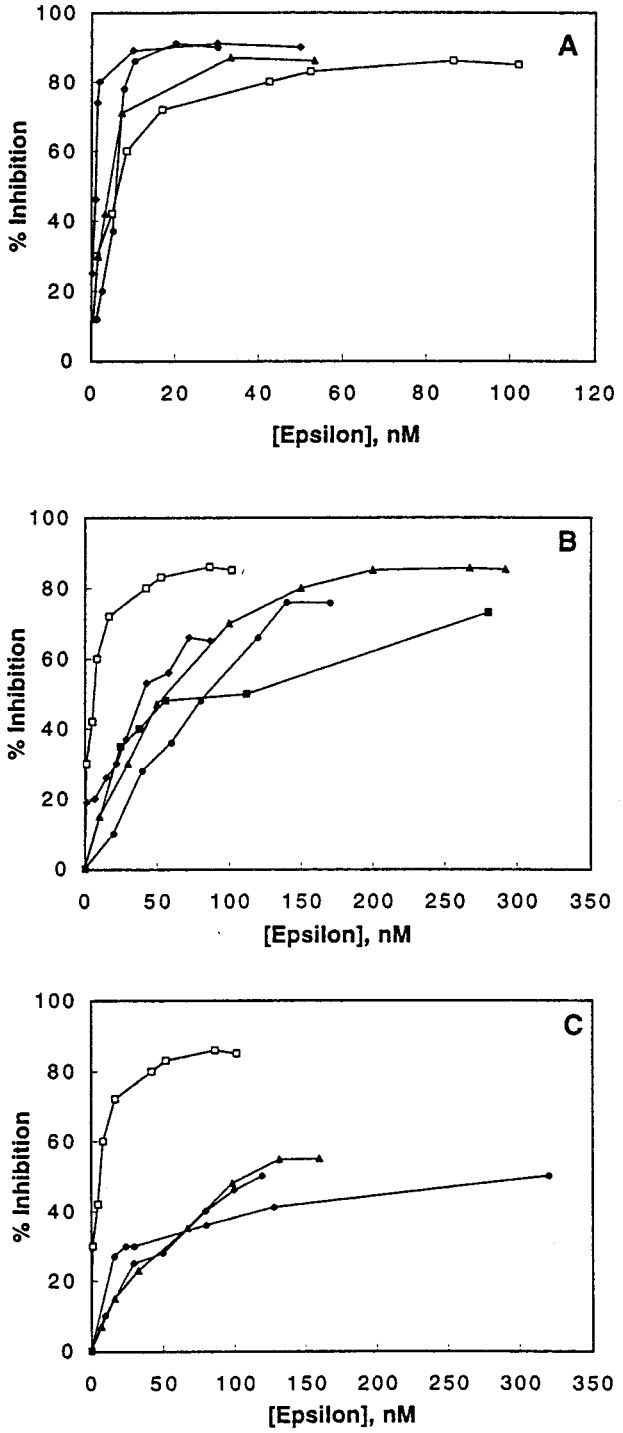

FIGURE 3: Inhibition of ATP hydrolysis. Assays were carried out as described under Experimental Procedures using ϵ-free F_1 with various concentrations of purified ϵ subunits. (A) Wild-type ϵ (open squares), E70A (filled diamonds), S65A (filled circles), E31A (filled triangles). (B) Wild-type ϵ (open squares), E59A (filled triangles), T77A (filled circles), Y63A (filled diamonds), tr117(AS) (filled squares). (C) Wild-type ϵ (open squares), R85A (filled triangles), T82A (filled diamonds), tr94(LAS) (filled circles).

Inhibition Assays. The purified ϵ subunits from the 13 mutants were also tested for the ability to inhibit ATP hydrolysis by the ϵ-free F_1, over a broad range of concentration. Two of the new mutants, tr80(S) and D81A, were completely unable to inhibit ATP hydrolysis, as was the double mutant D81A/R85A from the previous study. Results

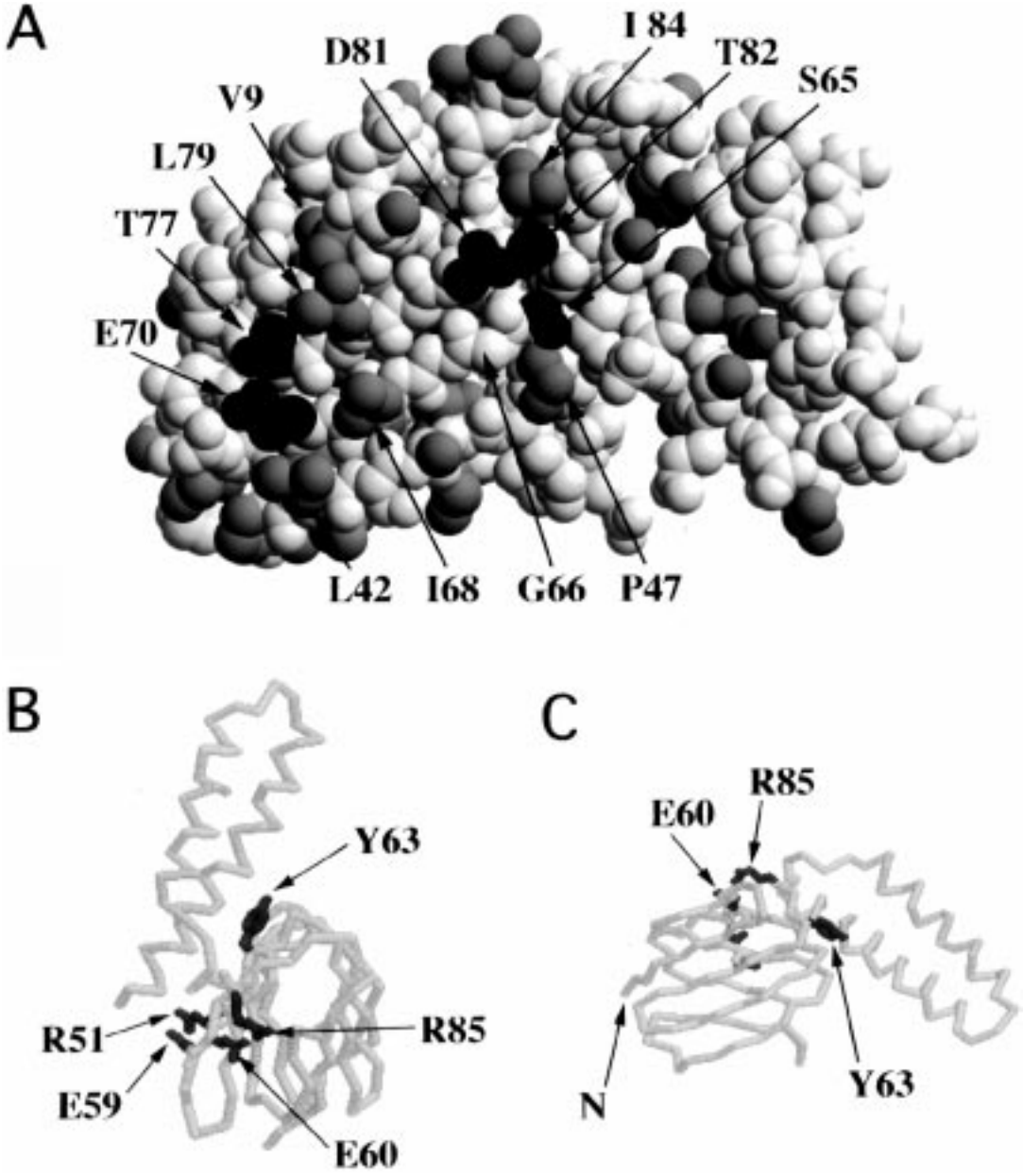

FIGURE 4: (A) The face of ϵ likely to interact with γ. The structure of subunit ϵ is shown in the space-filling mode. The side chains of key residues Ser65, Glu70, Thr77, Asp81, and Thr82 are colored black, and the side chains of all hydrophobic residues are colored dark gray. Asp81, Thr82, and Ser65 are clustered, along with Ile84 and Pro47. Thr77 and Glu70 are also clustered, along with Leu79, Ile68, Val9, and Leu42. Between the two clusters is a cavity due to Gly66. (B) A backbone model of subunit ϵ, showing the side chains of Y63, R51, E59, E60, and R85 in black. Y63 is packed between the two domains, and is largely buried. Salt bridges are formed between the side chains of R85 and E60, and between the side chains of R51 and E59. (C) The model from panel B, viewed from the perspective of panel A. This shows that the highlighted residues do not lie on the face that is proposed to bind subunit γ.

from the remaining 10 mutants are organized into 3 groups and presented in Figure 3A,B,C.

In the first group, shown in Figure 3A, are E31A, S65A, and E70A. These mutants have a slightly higher affinity and slightly greater extent of inhibition, as compared to the wild type. This is especially so for E70A and S65A. The second group, shown in Figure 3B, contains E59A, Y63A, T77A, and tr117(AS). These mutants all have a clearly reduced affinity (about 10-fold), but seem to inhibit to about the same extent as wild type at the highest concentrations. The third group, shown in Figure 3C, contains T82A, R85A, and tr94(LAS). These mutants, in particular tr94(LAS), appear unable to inhibit ATP hydrolysis to the same extent as wild type, over the concentration range tested.

DISCUSSION

In this study, we have extended our previous analysis (*38*) of a collection of alanine-substitution mutants by developing an inhibition assay using purified ϵ subunits and ϵ-free F_1. In addition, six new mutants have been constructed and analyzed. In the previous study, ϵ mutants were analyzed in membrane preparations only. One limitation of that approach was that a lowered affinity of the mutant subunit could not be distinguished from a reduced ability to inhibit ATP hydrolysis. In the current approach, one can measure changes both in affinity and in extent of inhibition.

The role of the C-terminus of ϵ was addressed by analyzing three truncated mutants: tr80(S), tr94(LAS), and tr117(AS).

Based on the structural models, these mutants are truncated in the ninth β-strand, the first long α-helix, and the second α-helix, respectively (see Figure 1). A similar series of truncated ϵ subunits was analyzed previously (*27*). Our results are largely consistent with those results, but purification helps clarify the role of the C-terminus in ϵ function. The tr80(S) mutant causes a large reduction in membrane-bound ATPase activity, and it does not inhibit ATP hydrolysis. Therefore, it is more similar to the tr73(WG) mutant of Kuki et al. (*27*), than their tr78(VAS) mutant. Based on considerations of the structural models, the measured properties of such mutants may be largely a consequence of their stabilities. Small differences in primary structure, and in genetic background, may explain any differences in properties between these mutants.

The second largest truncated ϵ subunit, tr94(LAS), is striking in its diminished extent of inhibition of soluble F_1. The low level of stimulation of membrane-bound ATPase by LDAO is consistent with the low inhibition. Under some conditions, LDAO has been shown to stimulate ATPase activity, even in the absence of ϵ, up to about 2-fold (*28*). Similar results were obtained by analysis of deletions at the C-terminus of ϵ from spinach chloroplast (*36*). In that study, deletions of 4 and of 10 amino acids from the C-terminus showed significantly reduced inhibition of soluble F_1, even at ratios of at least 7.5:1. Other studies (*20, 46*) have indicated that residues 106 and 108 of ϵ can be cross-linked to α and β subunits, and that residue 138 can be cross-linked to γ (*18*). Therefore, such interactions involving the C-terminus of ϵ are likely to be important for inhibition.

In contrast, the truncated ϵ, tr94(LAS), is nearly normal with respect to ATP-driven proton translocation and sensitivity to DCCD, in membrane preparations. The presence of F_o in membrane preparations to stabilize bound ϵ could account for the rather normally functioning ATPase with truncated ϵ. The demonstration of cross-linking of residue 31 in ϵ to the *c* subunit of the *E. coli* F_o has defined one element of this interaction (*22*). Similar conclusions about interactions between ϵ and F_o have been drawn from studies of N-terminal deletions of the spinach chloroplast ϵ (*37*).

Primary contact between ϵ and F_1 subunits is likely to be with the γ subunit (*17*). Cross-linking between γ and residues in ϵ, S10C (*46*), M138C (*18*), T43C (*18*), and H38C (*19, 47*), has established a periphery of this interface. A clustering of residues that when changed to alanine have altered affinity for F_1 is shown in Figure 4A. These residues, S65, E70, T77, D81, and T82, are surrounded by hydrophobic residues, some of which have been previously suggested to reside at the $\epsilon-\gamma$ interface (*16*). It is interesting to note that T82 is in close proximity to S65, although T82A has reduced affinity, and S65A has increased affinity. The same situation holds for T77A and E70A. One possible explanation is that T77, T82, and D81 interact directly with the γ subunit, and that these interactions are strengthened when the polar side chains of S65 or E70 are removed. In addition, the side chains of S65 and E70 may be important for interactions with γ by a particular conformation of ϵ, for example, one that interacts favorably with F_o. This is suggested by the loss of DCCD sensitivity seen with S65A and E70A, but not with D81A or T82A. An increased level of inhibition of ATP hydolysis by S65C has been reported recently (*18*), which is very similar to that reported here for S65A. Without further structural analysis of the purified D81A, it is not clear if the lack of inhibition is due to improper folding.

The R85A mutant is very similar to tr94(LAS) in all properties measured. Residue 85 is found in the tenth β-strand, just before a loop that connects the β-sheet domain to the α-helical domain. In the X-ray structure, shown in Figure 4B, the side chain of R85 forms a salt bridge with the side chain of E60 from the sixth β-strand. The main chain oxygen of R85 forms a multicentered hydrogen bond with main chain nitrogens of D88 and L89. In the mutant R85A, these interactions will likely be disturbed due to loss of the side chain. These changes might impact the normal interactions of the C-terminal α-helices with F_1.

Mutants E59A and Y63A represent residues that have clear structural roles: E59 forms a salt bridge with R51 in an adjacent β-strand, and Y63 is largely buried at the interface between the two domains (Figure 4B,C). Results indicate that the loss of charge in E59A influences the strength of the binding, but at saturation, it inhibits to the normal extent. The Y63A mutant is more profoundly altered in function, and resembles a maleimide-reacted H38C mutant (*19*), because of its high stimulation by LDAO. This could be explained if the mutation permitted the two domains of ϵ to move apart, thereby allowing more extensive, simultaneous interactions with both F_1 (subunits α/β) and F_o (subunit *c*) in membrane preparations.

Finally, it should be noted that none of the residues mutated in this study are conserved among all species, and, therefore, the effects seen here may be largely restricted to *E. coli*, and closely related species. This sequence variability among homologues is consistent with the variation in functional properties of this subunit, as has been seen in thermophilic PS3 (*48, 49*), chloroplast (*36, 50*), and rat liver (*51*) subunits.

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