Spring 5-2016

Structure-Guided Site-Directed Mutagenesis of the Bacterial ATP Synthase's Epsilon Subunit

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A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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May 2016

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Abstract

Adenosine triphosphate (ATP) contains energy-rich phosphoanhydride bonds that provide the energy needed for many cellular processes. F-type ATP synthase is found in bacteria, chloroplasts, and mitochondria, having a conserved function to catalyze the synthesis and hydrolysis of ATP. ATP synthase is a membrane bound rotary motor enzyme, with coupled rotation between it's two distinct complexes F_o and F_1. In bacteria and chloroplasts, the ε-subunit's C-terminal Domain (εCTD) has a distinct regulatory function that is absent in mitochondria. Determining the inhibitory interactions of ε is important in understanding it's physiological functions and for potential targeting of ε's bacteria-specific inhibition for development of new antibiotics. Guided by a high-resolution structure of ε inhibition catalytic complex, in this study I use site-specific mutagenesis of the εCTD in *Escherichia coli* (*E. coli*) to investigate interactions and make mutations at regions important for ε inhibition. I then analyze the effects of these mutants through phenotypic growth and ATP hydrolysis assays.

Executive Summary

Adenosine triphosphate (ATP) is a primary energy carrier in cells of all known kingdoms. ATP synthase, an evolutionarily conserved membrane bound enzyme, is responsible for making most cellular ATP in animals, plants, and in bacteria. The ATP synthase is essential for a number of pathogenic bacteria. Although the bacterial and human ATP synthase share homologs, the C-terminal domain of bacterial epsilon subunit (εCTD) has an inhibitory interaction that is absent in humans. If we can understand how this bacterial-specific inhibition works, we can target the enzyme to kill bacteria without harming our own enzyme. This makes the εCTD a good potential target for antibiotic development.

The model organism being investigated in this study is *Escherichia coli* (*E. coli*), a gram-negative bacteria found in the human gut, and used for over 30 years to study ATP synthase. All subunits of *E. coli* ATP synthase have homologs in ATP synthases of other bacteria, chloroplasts, and mitochondria. Exploring the interactions between εCTD of F_1-ATPase is crucial to understanding the inhibition of ATP synthesis and hydrolysis in bacteria. In this study, I will investigate two sites on the εCTD that may be important in inhibiting ATP synthesis and hydrolysis in bacteria.

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Introduction

The synthesis of ATP from ADP and P_i is facilitated by a proton transport mechanism found in the membrane embedded F_O complex. Proton motive force (pmf) is the electrochemical gradient of protons utilized by F_o to drive synthesis of ATP at catalytic sites located in the F_1 complex (Fig. 1; Duncan 2004). *In vitro*, F_1 can be dissociated from the membrane as a water-soluble form that can only hydrolyze ATP.

The F_1 complex is composed of 5 distinct subunits: $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The F_o complex, located in the membrane lipid bilayer, is composed of 3 distinct subunits: a_1, b_2, and c_{10}. Specifically, the *a*- and *c*-subunit play a role in catalyzing proton transport through proton binding at the c-subunit ring proton transport sites, facilitating rotation relative to the *a*- and *b*- subunits.

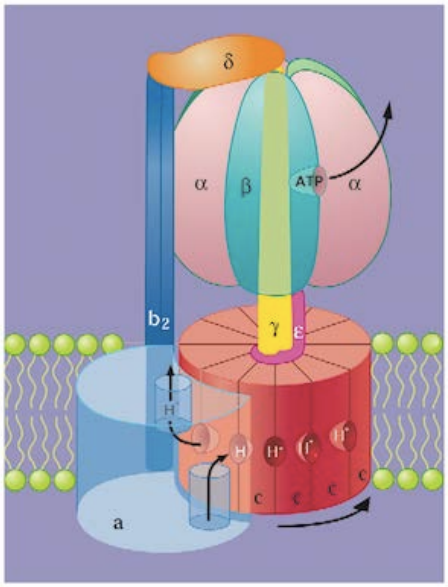

Figure 1: Model of *E. coli* ATP synthase rotary motor enzyme: Black arrows show the direction of proton transport and subunit rotation during ATP synthesis (Duncan 2014 review).

The ε-subunit of F_1 has been further studied for its role in inhibition of enzyme activity. Epsilon consists of two domains: the N-terminal domain (εNTD) and the C-terminal domain (εCTD) (Fig. 2). Studies have revealed that the εCTD is not required for oxidative phosphorylation but instead is involved in the regulation and inhibition of enzyme activity (Feniouk 2006). The εCTD has two known conformations: extended and compact. The extended conformation or inhibitory state of εCTD (ε_x state) inserts into the central catalytic cavity of the F_1 complex, interacting with 5 other subunits (Cingoloni and Duncan 2011). The εCTD in ε_x state inhibits the enzyme by preventing the rotation of the γ-subunit. The active form of the enzyme has a compact conformation of the εCTD (ε_c state) (Wilkens et al. 1998). When soluble F_1 is diluted to low concentrations, ε can dissociate from the enzyme, which releases the inhibition. The primary focus of my research project is to use site-directed mutagenesis to identify residues in the εCTD that are most important for inhibitory interactions of the εCTD with the β- and γ- subunits.

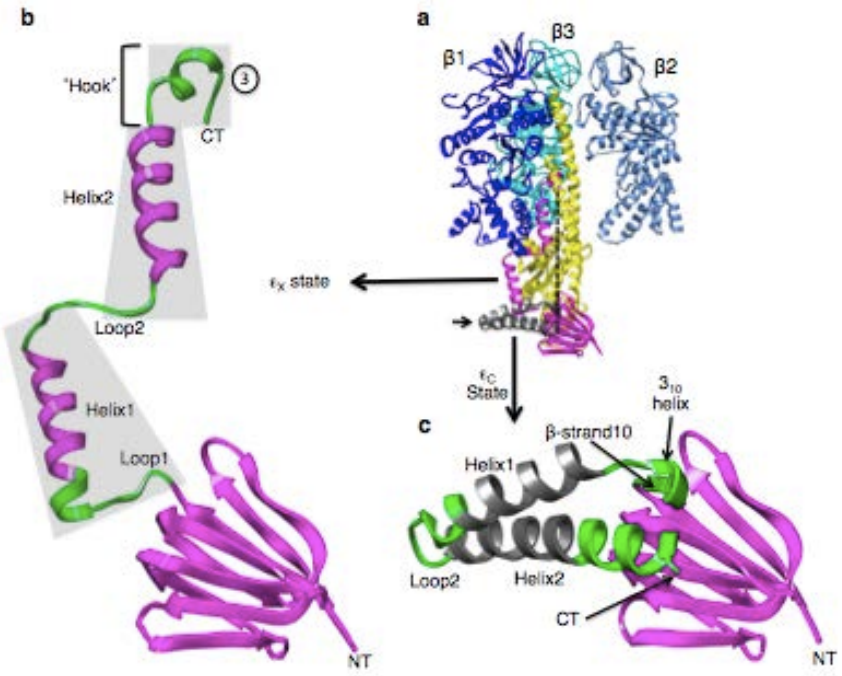

Figure 2, Extended and compact conformations of epsilon. Image **a** shows the F_1 complex with α subunits omitted to visualize ε's extended state (magenta), and the position of the εCTD in the compact state (gray helices). Image **b** shows ε alone with the two helices in the extended conformation. Image **c** shows the compact conformation of ε. Adapted from Cingolani and Duncan (2011).

A recent structure of F_1 from *E. coli* has provided relevant insights on the catalytic sites and subunit interactions of the enzyme (Cingolani and Duncan 2011). In Tom Duncan's lab, previous research demonstrated that the ε_x state is prevented from forming until hydrolysis of ATP at a catalytic site. This conformational change is significant in showing how εCTD inhibits F_1 (Shah et al. 2013). Since ε-inhibition is bacterial-specific, the εCTD could be a good target for antibiotic development by discovering compounds that mimic or strengthen inhibition on ATP synthase by the εCTD.

The aim of this research project is to understand the inhibitory behavior of the εCTD in the F_1 complex of F_OF_1. Guided by a high-resolution structure of the ε-inhibited catalytic complex (Cingolani and Duncan 2011), site-specific mutagenesis of the εCTD will be used to investigate interactions thought to be important for inhibition. Mutating residues of the enzyme can provide information on specific interactions necessary for ATP synthesis, hydrolysis, and inhibition. Using information available from the *E. coli* F_1 structure, I will explore interactions between the εCTD and other F_1 subunits of the enzyme that are likely to play a role in inhibition. The target sites for mutagenesis are in the C-terminal domain of the ε-subunit.

Previous existing mutations include εΔ5, with the last five residues of the ε-CTD deleted and ε88stop, with the whole inhibitory domain deleted (Shah 2015). These mutants will be useful for comparison with new mutants. Specific residues in two sites on the εCTD will be mutated to observe changes in functional and possibly disruptive interactions with other subunits. Three single

point mutations will be made at alanine97 (εA97) residue in helix 1. The second site is on helix 2, lysine123 (εK123). The primary focus is to use site-directed mutagenesis to identify residues in the ε-CTD that are most important for inhibitory interactions of ε-CTD with the γ- and β- subunits.

Methods

Construction of pMBε01 plasmid:

The vector for separate expression of ε-subunit is modified version from pXH302s (Xiong and Vik 1995). An insert encoding an affinity tag (6xHis.Nsi) was introduced into the vector at the start of the atpC gene for ε. The final plasmid pXH302s+NsiI.6xHis was named pMBε01. The NsiI site will be useful for moving the 6xHis-tagged ε into other constructs. The method used to introduce the insert was fusion PCR (Ho et al 1989). Two initial PCR reactions (PCR 1 and PCR 2) amplified the upstream and downstream regions of the plasmid. The final fusion PCR reaction (PCR 3) created the desired insert. Then a ligation reaction (Wu and Wallace 1989) with vector and insert generated pMBε01. The unique NsiI site was used to screen plasmids from transformed colonies by restriction digest: a linearized plasmid showed successful insertion of the NsiI-containing fusion product. DNA sequence analysis (Upstate Medical University core Facility) confirmed that the plasmid contained the correct atpC gene with the added region including the NsiI site and encoding the 6xHis tag.

SEQ:

```
ε:     M   H   H   H   H   H   H   G   H   M>Epsilon
atpC: ATG CAT CAC CAT CAT CAC CAC GGT CAT ATG
      (NsiI)                              (NdeI)
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Structural identification of residues to mutate in the εCTD:

Using the molecular modeling program Chimera (Petterson et al, 2004), I was able to analyze the epsilon-inhibited structure of the F_1 complex. Individual amino acids in the sequence were studied based on distances between individual atoms, angles/torsions, hydrogen bonds, and any clashes/contacts that may be important in the structure and function of the F_1 complex. I identified two residues, based on what is currently known about the molecular structure of ATP synthase, that appear to play important roles in εCTD: εA97 and εK123. "In silico" mutagenesis was used to introduce possible amino acid substitutions (rotomers) and study the contacts/clashes to determine the substitution that should be most effective at each residue.

Site-Directed Mutagenesis of the *atpC* gene for Epsilon:

Using mutagenic primers for εA97, the following mutants were made in pMBε01: εA97M, εA97Q, εA97L. Mutagenic primers were designed by first checking common codon usage in *E. coli*. The plasmid was linearized in a double-digest reaction with AfeI and PstI, and 5' and 3' PCR fragments were generated. Gibson Assembly (Gibson 2009) was used to combine the vector and insert for the εA97L mutant. A more traditional cloning method as described for pMBε01 construction was used for εA97Q, εA97M, ε88stop, and εΔ5 mutants. Mutant plasmids were transformed into competent DH5α cells and transformant plasmids were screened by DNA sequencing to confirm the presence of the desired mutation and the absence of any undesired changes. Outstanding mutagenesis projects are εL123M, εL123Q, εL123E.

Phenotypic Assay measuring Respiratory Growth:

Phenotypic effects of the mutations were observed after transforming each pMBε01 mutant into the XH1 expression strain (Xiong and Vik 1995). The XH1 expression strain has a deletion of the chromosomal epsilon gene but expresses all other subunits of the enzyme. This phenotypic assay measures growth of the mutants in liquid culture with succinate as a non-fermentable carbon source (Shah and Duncan 2015). *E. coli* is a facultative anaerobe, and can grow by aerobic respiration or by fermentation through glycolysis. Growth in liquid culture can provide a more sensitive ranking of the effects of the mutations on the *in vivo* assembly of functional ATP synthases. ATP synthesis function is required for growth on succinate. Thus, if cells do not grow, assembly of ATP synthase has been disturbed or enzymatic activity is compromised. For XH1 cells transformed with pMBε01 (wild-type or each ε mutant), bacterial colonies were inoculated in 10 mL of Luria Bertani broth (LB) + ampicillin (0.1 mg/ml) overnight. Cells were then diluted again into 10 mL LB + ampicillin to an initial A_{600} = 0.1. Cultures were grown at 37 °C until A_{600} = 0.8, then cells were diluted 100-fold in minimal medium containing succinate as carbon source. Growth was measured every 15 minutes using a Biotek Synergy HT plate reader at 37 °C, with 0.4 mL triplicates of each strain in a 48-well microplate (Shah and Duncan 2015).

Preparation of inverted membrane vesicles for functional assays:

E. coli cells can be put under high pressure in a French Pressure cell and, as the pressure is slowly released, the pressure shift disrupts the cells (protocol in Shah and Duncan 2015). Cell membrane fragments turn inside out as a result, and the F_1 complex is exposed to the external

environment. Modified Lowry assay protocol was used to determine membrane protein concentration (Peterson 1977).

ATP Hydrolysis:

Assays to measure ATP hydrolysis by membranes were done by a photometric "coupled enzymes" assay (protocol in Shah and Duncan 2015). Assays testing the effects of lauryl dimethylamine-N-oxide (LDAO) and Dicyclohexylcarbodiimide (DCCD) were included as before (Shah and Duncan 2015). LDAO is known to alleviate epsilon inhibition, therefore activating the enzyme. DCCD covalently modifies aspartate61 residues in the c-ring of F_O, which are required for proton transport. Therefore, DCCD irreversibly inhibits ATP synthesis and hydrolysis by blocking proton transport through F_o.

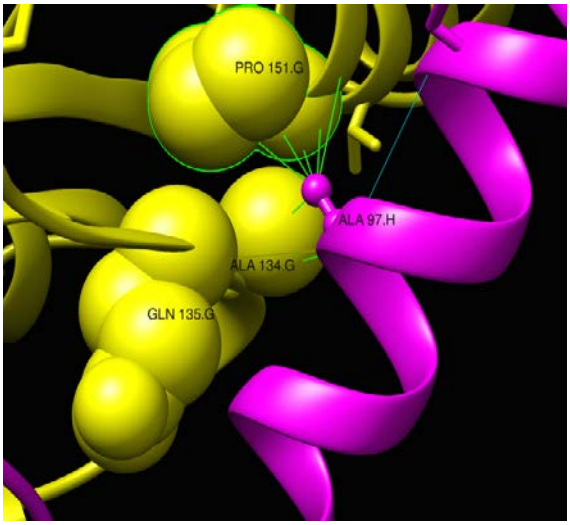
Table 1: Primers used in site-directed mutagenesis

Construct	Primers used in PCR: Sequence 5' to 3'
pMBε01	**PCR reaction 1:** pMBε01 forward: C AAA CTG GAG ACT GTC ATG CAT CAC CAT CAT CAC CAC GGT GGC CAT ATG GCA ATG ACT TAC CAC XH downstream: GAC TGG CTT TTG TGC TTT TCA AGC CGG **PCR reaction 2:** pMBε01 reverse: GTG GTA AGT CAT TGC CAT ATG GCC ACC GTG GTG ATG ATG GTG ATG CAT GAC AGT CTC CAG TTT G XH upstream: GAG CGT CGA TTT TTG TGA TGC TCG TC **FPCR reaction 3:** pMBε01 forward and pMBε01 reverse
εA97L	εA97L forward: GAA GCG GCC ATG GAA CTG AAA CGT AAG GCT GAA GAG εA97L reverse: CTC TTC AGC CTT ACG TTT CAG TTC CAT GGC GCG CGC TTC
εA97M	εA97M forward: GAA GCG CGC GCC ATG GAA ATG AAA CGT AAG GCT GAA GAG εA97M reverse: CTC TTC AGC CTT ACG TTT CAT TTC CAT GGC GCG CGC TTC
εA97Q	εA97Q forward: GAA GCG CGC GCC ATG GAA CAG AAA CGT AAG GCT GAA GAG εA97Q reverse: CTC TTC AGC CTT ACG TTT CTG TTC CAT GGC GCG CGC TTC
εΔ5	εΔ5 forward: GCT GCG CGT TAT CGA GTT GTA ACA CCG GCT TGA AAA GCA C εΔ5 reverse: GTG CTT TTC AAG CCG GTG TTA CAA CTC GAT AAC GCG CAG C
ε88stop	ε88stop forward: GCC GAC ACC GCA ATT CGT GGC CAA TAA CAC CGG CTT GAA AAG C ε88stop reverse: GCT TTT CAA GCC GGT GTT ATT GGC CAC GAA TTG CGG TGT CGG C

Results

Epsilon Mutants:

In order to examine the role of ε-helix1/γ interaction for forming inhibited states, we targeted alanine97 (εA97) in the εCTD's first helix. Mutations were avoided that would likely disrupt the compact state. In ε's extended state, εA97 side chain contacts four residues of γ (γP151, γQ135, γA134, γG150). The structure of ε-inhibited F_1 indicates tight packing of the εA97 side chain in a pocket formed by these γ residues (Fig. 3). Mutations at this first target site should address whether the γ/ε-helix 1 contact is important for transition into the ε-inhibited state, and I will test this by introducing bulky residues that should disrupt this interface. Mutating εA97 to an amino acid with a larger side chain should disrupt this tight packing. Hydrophobic/nonpolar amino acid like leucine or methionine and a polar uncharged amino acid such as glutamine were considered as possible mutants. In the compact state, εA97 does not contact ε's second helix or the εNTD (Fig. 4). Mutating εA97 should not disrupt the compact conformation of ε.



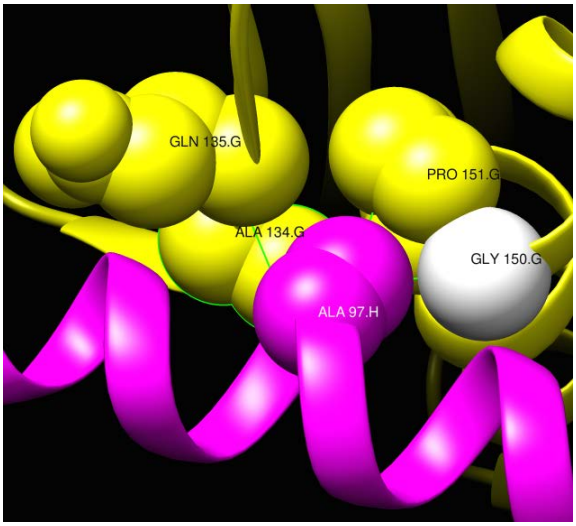


Figure 3, In ε's extended conformation (magenta)**, εA97 (ball/stick) packs against several γ residues (yellow, space-filling).** Van der Waal contacts of εA97 with γ are shown with green lines. The position of εA97 indicates tight packing with four residues of gamma. A larger side chain should disrupt the packing in this region.

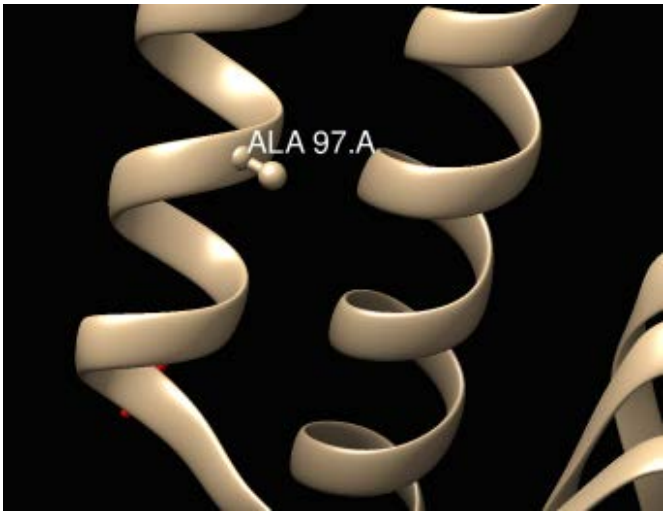


Figure 4, εA97 compact conformation. The side chain of εA97 has no contacts in the compact conformation, and faces away from the second helix. A mutation in this region should not disrupt the compact conformation of ε.

Using Chimera, three potential mutants at εA97 were examined: leucine (εA97L), glutamine (εA97Q), and methionine (εA97M). In the extended state, εA97L and εA97Q have many predicted clashes. Leucine indicates 6-9 clashes, with 1 potential clash in the compact conformation. Glutamine indicates 8-15 clashes with residues on γ. Methionine is also a possible mutant, as it is not too hydrophobic and should not destabilize the α-helix. Methionine in extended conformation indicates 8-14 clashes. There is a high probability of 0 clashes in the compact conformation for all three amino acid substitutions.

Another target site includes the second helix of ε and its predominant role in inhibition. A few distinct residues of the εCTD appear to form specific hydrogen bonds that may be critical for stabilizing the ε-inhibited state. Epsilon Lysine123 (εK123) in helix two contacts βD372 of subunit β_1 and αA405 of subunit α_3. There is likely a hydrogen bond and a charge interaction between the side chains of εK123 and βD372 (Fig. 5). Lysine is positively charged, so introducing a long non-

polar amino acid like methionine or polar uncharged glutamine would disrupt this bond. Mutating lysine to a negatively charged amino acid like glutamate would disrupt this bond and introduce an electrostatic repulsion with βD372.

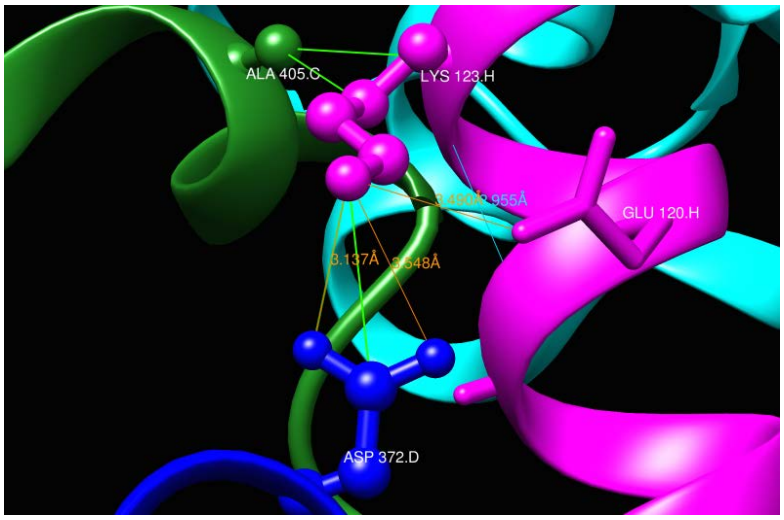


Figure 5, εK123 in ε's extended conformation. Ball and stick models of contacting residues are shown with green lines and apparent hydrogen bonds are shown with orange lines.

In ε's compact conformation, the first two carbons of the εK123 side chain contact Tyrosine53 and Proline47 in the εNTD (Fig. 6). Structural analysis indicates methionine, glutamine, and glutamate should not alter these interactions in the compact conformation. Additionally, these mutants should not disrupt the α-helical structure of ε.

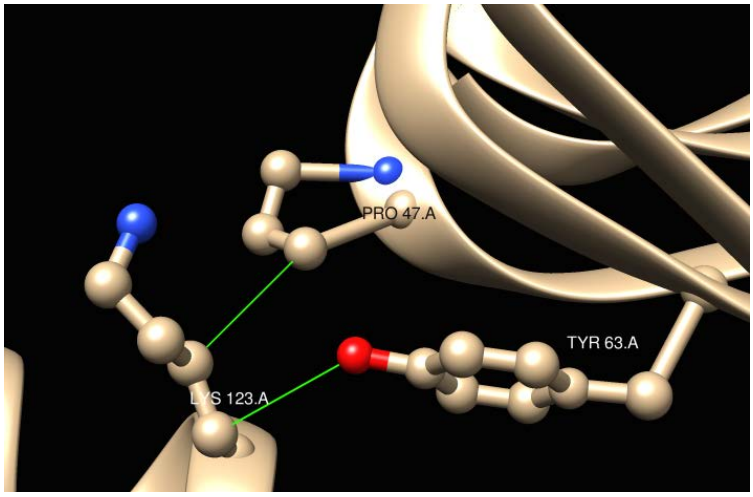


Figure 6, Contacts of εK123 in the compact conformation. The Cβ and Cγ carbons of the εK123 side chain have contacts (green lines) with Tyr53 and Pro47 in the εNTD. The most favorable rotamers of methionine, glutamine, and glutamate would have their Cβ and Cγ carbons in similar positions, so these mutations should not alter these interactions in the compact conformation.

Using structure editing in Chimera, three potential mutants were modeled at εK123: methionine (εK123M), glutamine (εK123Q), and glutamate (εK123E). In the extended conformation, 1 likely rotamer of εK123M had a high probability to clash with αA405-Cβ of subunit α_3. Another favorable εK123M rotamer is oriented away from this αA405 and from βD372 of β_1, indicating the εK123M substitution may occur without significant steric disruptions. In ε's extended state, the εK123Q mutation indicates only one clash with αA405 of subunit α_3. The εK123Q mutation in ε's extended conformation indicates a high probability of having no clashes, but a small probability of having clashes with αA405. In the compact conformation, εK123M indicates low probability of clashes with the εNTD, while εK123Q indicates a low probability of clashing with Tyrosine53 on the εNTD and Glutamine127 on ε's second helix. However, the compact conformation also indicates probability of forming clashes with Tyr 53 on εNTD and Glutamine 127 on ε's second *a* helix. Compact confirmation also indicates probability of forming clashes with Tyr 53 on εNTD. Thus, the potential for mutagenesis of εK123 in ε's second α-helix is promising. However, the εK123 mutants have not been created yet, so the remaining results will focus on the εA97 mutants.

Phenotypic Assay measuring Respiratory Growth

As shown in a recent study, aerobic growth is inhibited when five C-terminal residues are deleted from the ε subunit of *E. coli*'s ATP synthase (Shah and Duncan 2015). This growth inhibition is due to a reduced capacity for ATP synthesis *in vivo*. Whereas the prior study over-

expressed the entire ATP synthase, our results (Figs. 7, 8) confirm a similar or slightly greater reduction in growth yield when εΔ5 is expressed separately and all other F_OF_1 subunits are expressed from the chromosomal *atp* operon. The new εA97 mutants tested here are expected to disrupt the interaction of ε-helix1 with γ. If the interaction of ε-helix1 with γ is critical for epsilon to achieve the extended state, disrupting it will prevent inhibition of the enzyme. Aerobic growth assays of εA97 mutants in liquid medium with succinate as the sole carbon source show that the εA97 mutants grew almost as well as wild-type cells (Figs. 7, 8). The εA97 mutants grew as well as or better than the ε88stop mutant, which has its entire C-terminal domain deleted.. Overall, these results indicate that F_oF_1 is assembled properly in εA97 mutants and retains near-normal ATP synthesis *in vivo*. Hence, εA97 mutations do not lead to increased inhibition of the enzyme as seen with εΔ5.

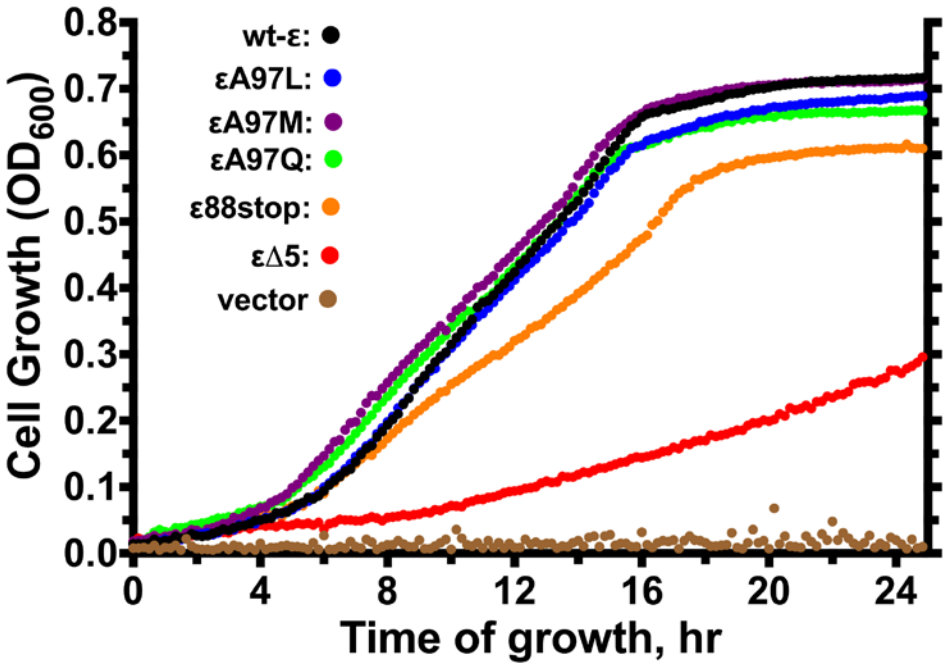


Fig 7, Phenotypic Respiratory Growth Curves. Growth kinetics are shown in defined medium that contains succinate as sole carbon source. Cells were grown for at least 24 hours, with OD measurements taken every 15 minutes.

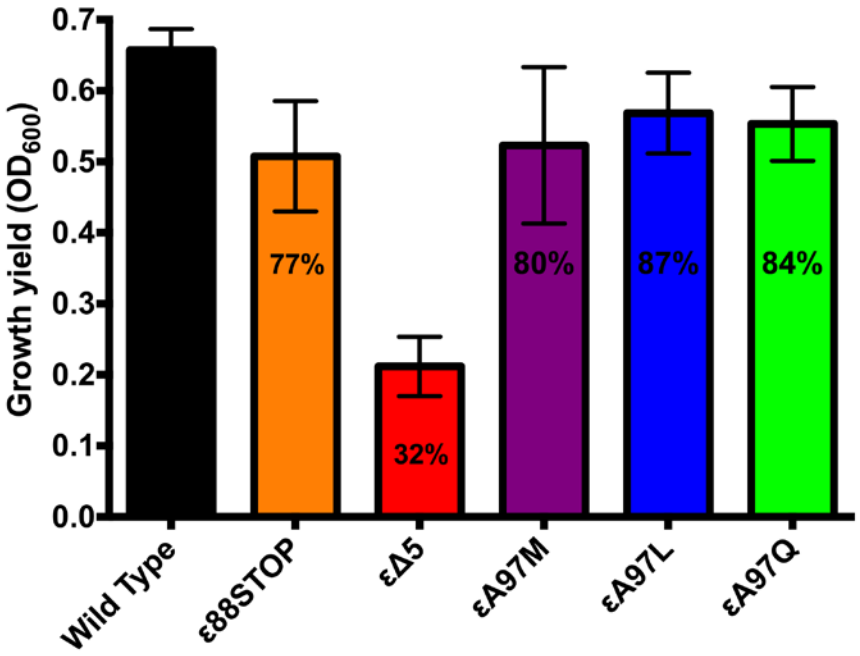


Fig 8, Relative Growth Yield of Mutants as compared to wild-type: Wild-type's growth plateau was set as 100% and values for the relative growth yields of mutants are shown. Error bars represent standard error of the mean (SEM) from 4 separate assays.

ATPase Activities of wild-type and mutant membranes:

ε97 mutants show higher activity than WT, this could be because mutants are inherently activated compared to WT, but might also be due to higher levels of F_OF_1 expressed in the mutant membranes. LDAO is an amine oxide detergent that has been shown to increase ATPase activity by disrupting the epsilon inhibition of the enzyme. Therefore, it can indicate the extent of inhibition by ε. We subjected all membranes to coupled ATPase assays with and without LDAO to estimate the levels of ε-mediated inhibition. WT showed 1.9x activation by LDAO ($p= 0.002$), which is consistent with previous studies. The εA97M and εA97Q mutants showed almost no activation by LDAO, suggesting those membranes lack intrinsic capacity for ε

inhibition.. The εA97L data were too scattered to conclude if it is more similar to WT or the other two εA97 mutants. LDAO activation data for εΔ5 and ε88stop are scattered but similar to results with those mutants shown by Shah and Duncan, 2015 (2X for εΔ5, 1.4X for ε88stop).

DCCD is an inhibitor of ATP synthase that covalently binds to the Aspartate 61 (Asp61) residue of the c-subunits. This residue is essential for proton transport through F_O. Thus, pre-incubation of membranes with DCCD for 30 min inhibits proton transport through F_O and, when F_1 is well coupled to F_O in the membranes, inhibits the majority of membrane ATPase activity. We found that the ATPase activity of all mutant membranes is as sensitive to inhibition by DCCD as are WT membranes. Thus, the εA97 mutations do not cause any significant uncoupling between the F_1 and F_O motors in the ATP synthase.

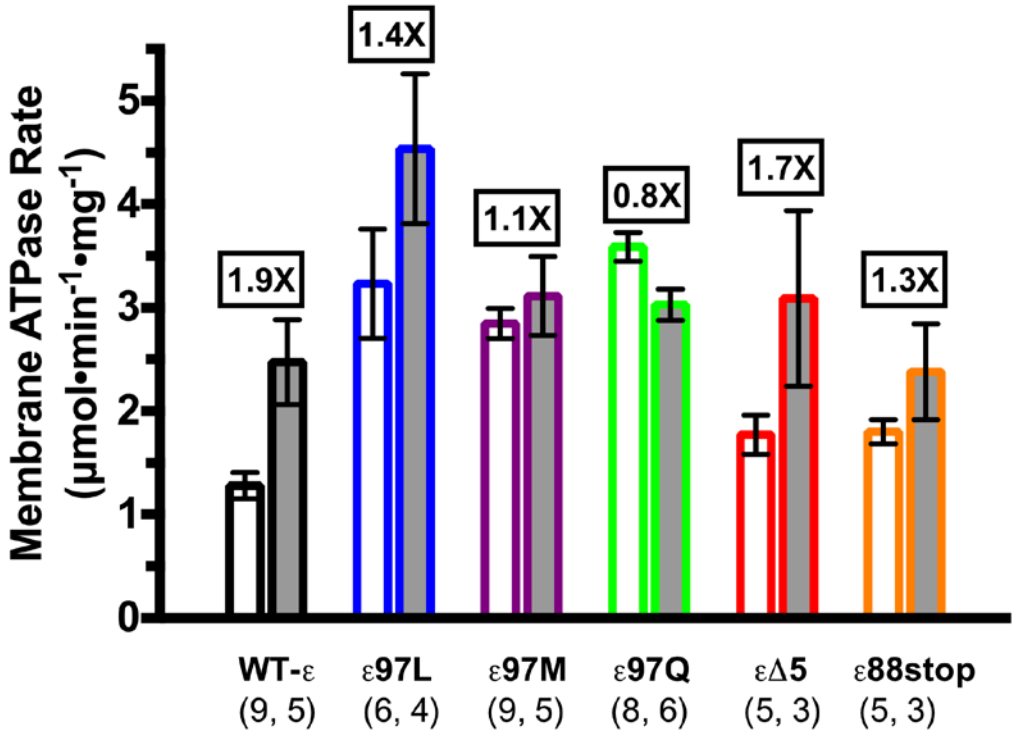


Figure 9, *In-vitro* membrane ATPase activities. Direct assays (clear bars) and LDAO activation (shaded bars) are shown. Below each sample, the number of separate assays is shown in

parentheses.(direct, +LDAO). Error bars represent the SEM. For each membrane sample, the value above the bars is the ratio of activity +LDAO to the direct activity.

Discussion

My project focused on understanding the inter-subunit and intra-subunit interactions of the epsilon subunit in bacterial F-type ATP synthase. We generated three mutants that had single point mutations at εA97 with the help of site-directed mutagenesis. The mutants were subjected to phenotypic growth assays in medium containing a non-fermentable carbon source, succinate. Since non-fermentative growth requires ATP synthesis by F_OF_1, any defect in growth would be a result of impaired ATP synthase function. Our assays did not show any major defect in growth in the εA97 mutants. The growth yield was slightly reduced but was still better than ε88stop, which has the entire εCTD deleted.

ATP hydrolysis activity of the mutants was examined with the help of a coupled enzyme assay. In order to take a closer look at ε-mediated inhibition, the samples were treated with LDAO, which relieves ε-mediated inhibition, increasing the ATPase activity. The three mutants showed less activation than WT membranes. The reduced activation of εA97 mutants was similar to that seen with ε88stop, which suggests that εA97 mutations may disrupt the ability of ε to achieve the inhibited state. In order to take a closer look at the effect of the εA97 mutants on the enzymatic activity, the inhibition of ATPase activity by εA97M/Q/L should be directly measured by quantifying F_O F_1 in membranes by immuno-blotting (as in Shah and Duncan, 2015).

Residue εA97 is present in helix1, and contacts the γ subunit in the ε-inhibited state or ε-helix2 in ε's compact conformation. Residue εK123 is present in ε-helix2, which contacts α

and β subunits in the inhibitory state, but contacts ε-helix1 in ε's compact state. The proton flow through F_O is coupled with enzymatic reactions by F_1. However, in reality, there is some amount of slippage observed even in WT enzyme whereby some protons 'slip' through without resulting in work. The mutations in the above mentioned locations might result in increased leakage of protons or decreased coupling. As a result, the mutant enzymes may have an impaired capacity to generate proton motive force. This is unlikely for two reasons: first, mutant cells showed nearly normal growth on the respiratory substrate succinate; second, DCCD modification of F_O inhibited mutant membrane ATPase as effectively as for WT, indicating normal F_O-F_1 coupling. To further confirm this, a fluorescent assay could be used to directly measure the kinetics of proton pumping by the mutant membranes (as in Shah and Duncan 2015).

In order to study the effects of the mutations on interaction of the εCTD with F_1, another approach should be used for future studies: to correlate inhibition with F_1 binding and dissociation of ε. F_1 can be isolated from F_oF_1-ATPase in a soluble form and ε becomes a dissociable inhibitor. F_1 can be depleted of endogenous ε by an anti-ε immuno-affinity column. Thus, inhibition of isolated F_1(–ε)can be quantified by adding each isolated ε mutant. As shown by Shah and Duncan (2013), affinity-tagged ε (+/- mutants) can be overexpressed for this purpose. Thus, the inhibitory constant (K_I) can be compared between wild type and mutant forms of ε. The εA97 mutations may lead to altered binding of ε to other F_1 subunits. In wild-type enzyme, ε's C-terminal α-helices alternate rapidly between the compact and extended conformations. In the compact conformation, the helices are in a coiled-coil conformation with each other; whereas in the extended conformation, helix1 establishes contacts with the γ subunit and helix2 establishes contacts with 2 α, 2 β and γ subunits. With isolated WT F_1, ε favors the extended conformation and the enzyme is strongly inhibited. This conformational change may be

prevented if the εA97 mutations disrupt the ε-helix1/γ interaction, and that interaction is necessary for ε-helix2 to insert inside F_1 to achieve the fully-inhibited state. To test this directly, an established optical method can be used to measure kinetics of binding and dissociation for F_1 and mutant ε. The extended state of the εCTD limits the F_1/ε dissociation rate so, for example, F_1/ε88stop dissociates ~80X faster than F_1/wt-ε (Shah and Duncan, 2013). Thus, measuring F_1/ε with the εA97 mutants should confirm whether the mutations disrupt access to the extended, inhibitory conformation.

ATP synthesis assays will also be needed for key interpretation of the εA97 mutants. These assays can be easily done with the same membrane samples that were used for ATP hydrolysis assays. Other future experiments should include carrying out immunoblotting experiments to determine expression level of ε in the strains and the content of F_OF_1 in the membranes (anti-β blot, as in Shah et al., 2015). A western blot will quantify activity per mg of enzyme rather than activity per mg of membrane. Additionally, outstanding projects include mutations at εK123. This site contains possible hydrogen bond and side chain specific interactions that may be important for ε inhibition..

Overall, my work continues the lab's focus on understanding the complete role of the ε subunit in the functioning of the bacterial ATP synthase. It is established that ε is responsible for auto–inhibition of the enzyme. With ATP synthase approved as a target for drugs against pathogenic bacteria like *Mycobacterium tuberculosis*, targeting the auto–inhibitory properties of ε subunit in pathogens may provide another way of inhibiting their growth and working around the problem of drug resistance. In order to do so, further studies need to be done to elucidate the unique role of the ε subunit in regulating function of bacterial ATP synthases.

Acknowledgements

First, I would like to thank my advisor, Thomas Duncan for accepting me into his lab my sophomore year. I would not have been able to complete my capstone without your guidance. Thank you for sacrificing weekends to do membrane preps and assay work with me, which was crucial to progress my capstone.

I want to thank my mentor, Nancy Walker who helped me with most of my cloning and mutagenesis work. Thank you for teaching me new strategies and teaching me to be patient with research.

I also want to acknowledge my post-doc Naman Shah for helping me during my initial years in the lab and always being willing to give me feedback and advice.

Thank you Renee Crown Honors program and faculty for choosing to fund my research this past summer and recognizing the potential in my research project.

Thank you Professor Chan, for being my capstone reader and teaching me many of the concepts that I needed to advance my research in your Biochemistry II course that I took my junior year.

I am grateful for all the support I have received from advisors, mentors, and peers during this process. Without the help of all my mentors and advisors at Syracuse University, including Kari Segraves and Kate Hanson, I would not have been able to complete my capstone and I greatly appreciate your help.

Finally, I want to thank my family and friends for their constant support and encouragement.

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