University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
December 2021

Interactions of charged amino acids in the proton exit pathway of E. coli ATP synthase

Ariel Stewart
Biochemistry
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Ryan Steed

Abstract

In the cell, energy is required to carry out all necessary functions to keep the cell alive. The carrier for this vital energy is known as adenosine triphosphate, ATP. The ATP synthase complex, where a majority of ATP is synthesized, is composed of eight unique protein subunits in a stoichiometry of $ab_2c_{10}\alpha_3\beta_3\gamma\varepsilon\delta$, making up two units, F_1 and F_0. The F_0 sector moves protons, driven by the proton gradient, across the cell membrane to drive ATP production via a rotary mechanism. The main pathway by which protons are translocated through F_0 is through the a-c interface. In this pathway, we are investigating several conserved residues, including arginine (R) 50 in subunit c and glutamate (E) 196 and aspartate (D) 92 in subunit a, to determine what makes them essential to function. Previous work has suggested that the R50 could be a part of a functionally important salt bridge with E196 or D92 on subunit a, or involved in a water wire within the proton channel. To clarify the role of these residues, mutations were generated and their functionality examined through biochemical assays. In general, mutations made at aD92 significantly impacted functionality and were not well tolerated. We observed substantial functional defects in the aD92A/cR50A mutant, where both positions are replaced with alanine (A), and the aD92R/cR50D mutant, where the positions are swapped. While more investigation is needed, this sensitivity may be due to the disruption of an electrostatic interaction between rotor and stator. The mutations made at the aE196 position showed a mild effect on ATP-driven proton pumping, while moderately inhibiting ATP synthesis. These results indicate that aE196 is not involved in a salt bridge with cR50 but do suggest that aE196 may have a greater role in the synthesis direction.

1. Introduction

All cells must have an energy input to power biological reactions and cellular functions. ATP has three high-energy phosphorous bonds, that when broken, release the energy necessary for many biological reactions that are necessary for cellular function. The main producer of ATP, the energy currency of the cell, is ATP synthase. Understanding the way that ATP synthase functions could help lead research in the field of drug development and modification, as ATP synthase serves as a good medicinal target due to its biological importance and complexity. This can be seen through the example of the medication Bedaquiline, which is used to treat multidrug-resistant tuberculosis. Multidrug-resistant tuberculosis accounted for 1.5 million deaths in 2013, thus the importance for this kind of medication to be developed to combat the progression of multidrug-resistant bacteria.[1] ATP synthase has also been used as a target for novel cancer therapies that suppress tumor growth in various cancers such as breast cancer, colon cancer, lung cancer, etc.[2] Therefore, we must build off of the foundation of knowledge of ATP synthase and ask more questions about it so that the foundation can be expanded and be applied in other areas of research.

The energy necessary for biochemical reactions to occur is generated in the mitochondria in eukaryotic cells and in the cell wall of prokaryotic cells. This study was performed on *Escherichia coli* (*E. coli)* cells, which contain relatively

simple versions of the electron transport chain and the ATP synthase complex and are typically used as a model system for these processes. The electron transport chain is important because it takes the electron carriers from the reactions that occur in the cell and breaks them down, releasing or pumping protons across the membrane and into the inner membrane space. After this pumping of protons has occurred, there is a charge difference across the membrane with the inner membrane space being more positive and the inner membrane being more negative. This is where ATP synthase comes in.

ATP synthase, powered by this charge difference, is a machine composed of two molecular motors, F_0 and F_1. The protons in the cytoplasm enter the F_0 subunit through what is known as the a subunit, as shown in orange in Figure 1. Then the proton enters the c ring, and the c ring rotates. As the c ring rotates it causes the stator, or the gamma (□) subunit, in brown, to rotate as well. This then causes the F_1 to turn and causes the alpha (α) and beta (□) subunits, in dark and light green respectively, that makeup F_1 to go through conformational changes. When the α and □ subunits go through the conformational change, adenosine diphosphate (ADP) and inorganic phosphate (P_i) enter the F_1 subunit and are condensed to produce ATP.[3]

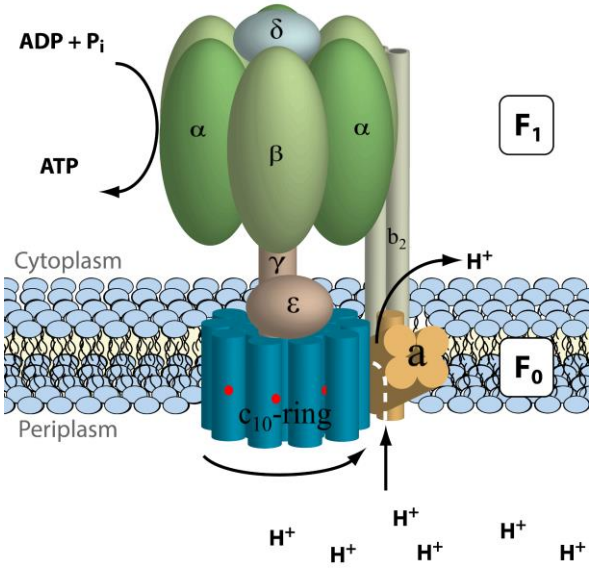

Figure 1: The structure of ATP synthase

The question then becomes what causes the F_0 to rotate and how does the function of ATP synthase produce energy. This is the question that has been and is being investigated. It is suspected that the answer may lie in the interactions between the a and c subunits.[4] In recent years, it was discovered that there are aqueous half channels between the two subunits that the protons flow through as they go between the a and c subunits. Since it has been suspected that the answer for rotation lies between these two subunits, the first step in research was to closer examine the residues within the two subunits.

This was done by mutating what is known as the wild type (WT), or the ATP synthase that is found in "normal" cells. The mutation was to take the wild type and change all of the residues in the a and c subunits to cysteines and examine function through the use of a fluorescent maleimide, or a fluorescent indicator.[5] Through this process the essential residues where determined. Because rotation has been linked to the interactions between subunits a and c, more research is being executed on these important residues to determine the reason behind the rotation of ATP synthase and how this rotation produces energy.

Currently at UNC Asheville, a great deal of research is being carried out to learn more about the reason behind the rotation of the F_0 subunit and to understand the role of some of the residues previously determined as important for function.[5] The residues of interest in this study are arginine 50 on subunit *c* and its possible electrostatic partners, glutamate 196 and aspartate 92 residues on subunit a (Figure 2). Previous work by Founds and Rothrock (unpublished) determined that the positive charge of Arg50 might be necessary for function, but that the steric bulkiness of the group does not affect overall function at this position.

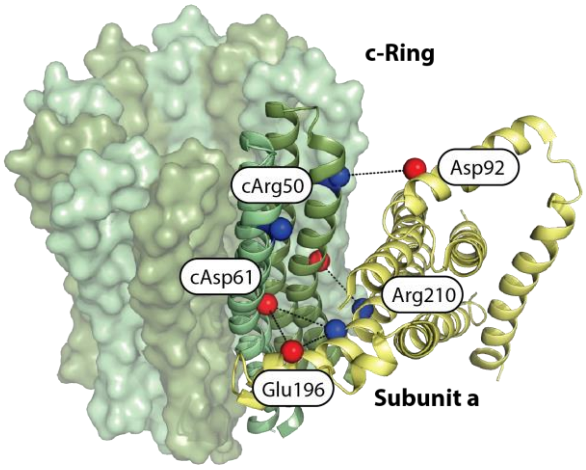

Figure 2: Possible interaction along the a-c interface

Previous research by others in the field has made fast progress at expanding the amount of information to be built on, from the first perceptions that it exists until today.[6] In 2010, Pogoryelov et al. used DCCD modifications with a DCCD labeling assay and free-energy molecular dynamic calculations to find the mechanism behind the movement of ions across the membrane and the arrangement of the a subunit to the c subunit. This was later looked at to determine the likelihood of aqueous channels. This is important because it could be the cause of rotation of the F_0 subunit and is being used today to find the function of the residues behind this interaction between the a and c subunits.[7]

Fillingame and Steed, in 2014, established that there are, in fact, aqueous channels between the a and c subunits that is responsible for the movement of protons between them and cause rotation of the F_0 subunit, but in this the direction and mechanism of rotation is also discussed. This is also where a picture of the necessary residues becomes clearer. The way that Fillingame and Steed proved the existence of this channel and what residues lie around it by using cysteine mutations and assays to examine function and then crystallization was completed and an X- ray taken.[8]

In the same year, Fillingame and Steed also discussed the residues in the c subunit that are in what is known as the polar loop or the part of the c ring that is exposed to the aqueous channels and is necessary for ATP synthase function. These residues in the c subunit were discovered using the Barik technique for cysteine substitutions that was purified and then used to replace a section that was in the wild type ATP synthase. These mutants were then left to grow on agar plates to examine ATP synthase in vitro. Later, fluorescence quenching was performed to examine the amount of nigericin from the baseline which indicated the amount of mutant ATP synthase. Then a series of steps were carried out to prepare the sample for an assay to be taken to look at ATP synthase function. The sample was purified using a column after preparatory steps and liposomes were created. Then the F_0 liposomes were used in electrical gradient ACMA fluorescence and initiated with valinomycin. The measurements taken from this method gave the researchers more information on the effect of cysteine substitutions and metal ions Ag^+ and Cd^{2+} on the function of the complex.[9]

However, it was shown a couple of years later in 2016 by Kühlbrandt and Davies found through the use of electron cryomicroscopy, otherwise known as cryo-EM, that the a subunit is tilted at a 30° angle when it was originally thought to be vertical. Cryo-EM is a way of determining the structures of macromolecules with very high resolution without having to crystalize them.[10]

In 2017 it was proposed by Yanagisawa et al., the possibility of a salt bridge occurring between the a and c subunits involving E196.[11] 2018 Nakanishi et al. established through improved cryo-EM techniques the way that ATP synthase maintains the structure of its stator and how proton translocation occurs.[12] However, in 2021, Yanagisawa et al. determined through recent cryo-EM structures that most residues along the aqueous half channels were to far apart to form hydrogen bonds and instead proposed a possible water wire along this pathway instead, possibly involving E196.[13]

Since the idea of ATP synthase was proposed, many have undertaken the task of understanding how this tiny machine works. From the beginning when the structure could only be guessed at till now where it has been determined through years of research what the overall structure looks like and what residues are necessary for function. In these years of research, the importance of understanding how ATP synthase functions has come into the light when examining multidrug resistant tuberculosis. Now we seek to look more closely at this complex to learn more of the

secrets behind its mechanism of function starting with arginine 50 and the residues it may interact with to induce rotation of the *c* subunit and subsequently the F_1 unit.

2. Experimental Methodology

2.1 Mutagenesis

Mutagenesis was performed by ligating synthetic mutant gene fragments (Twist Bioscience) into the template plasmid pFV2, which contains a cys-less construct of the unc operon that encodes ATP synthase. Plasmid DNA was isolated from *E. coli* cultures using a Monarch plasmid miniprep kit (NEB) and concentration was determined using A_{260}. Template plasmid and synthetic gene fragments were digested with PflM1 and BsrG1 restriction enzymes. DNA fragments were separated by electrophoresis, and the DNA was extracted from the gel using a Monarch gel extraction kit (NEB). Fragments were ligated together, and the resulting plasmids were used to transform *E. coli* DK8 cells, which lack the UNC operon. The sequences of the newly created plasmids were confirmed by Sanger sequencing.[14]

2.2 Inside- Out (ISO) Vesicle Preparation

Mutant *E. coli* cells were prepared by growing them in 1L LB medium with 100µg/mL ampicillin. After growth for ~8h, cultures were centrifuged at 4000 xg for 15 minutes to collect cells. Cells were resuspended in TMG buffer (50 mM Tris-HCl, 5.0 mM $MgCl_2$, 10% (v/v) glycerol, pH at 7.5) supplemented with 1mM DTT and 1mM PMSF. Then the cells were lysed in the homogenizer (≥15,000 psi) five times and membranes were collected through centrifugation at 9000 xg for 10 minutes where the supernatant was collected and centrifuged once more at 193,000 xg for 1h. Cells were resuspended using a glass dounce in ~3mL TMG. Once this was complete, cells were stored at -80℃. Protein concentrations were determined through a Lowry assay as previously described.[15]

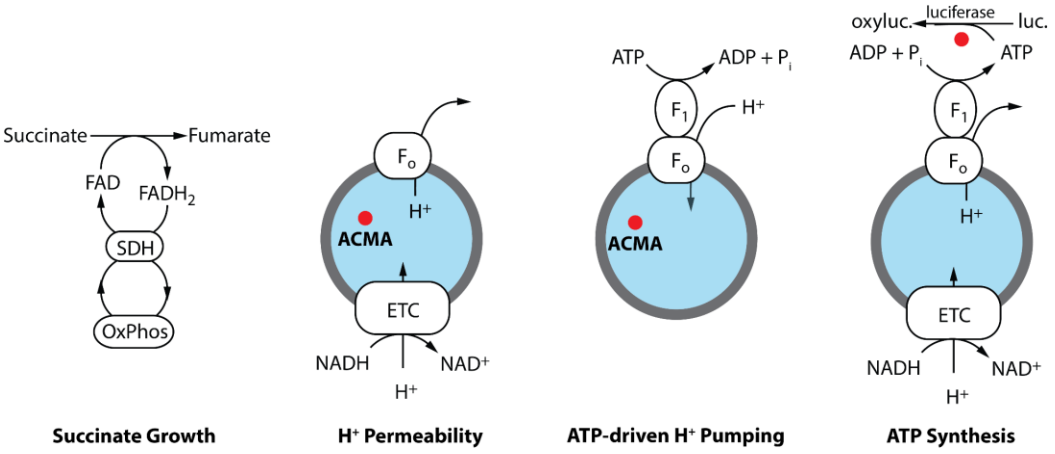

Figure 3: Methods used (except for H^+ permeability)

2.3 ATP- Driven Proton Pumping Assay

ISO vesicles were diluted to 10 mg/mL in TMG buffer and 160 µL was added to a test tube containing 3.2 mL HMK (50 mM HEPES-KOH pH 7.5, 2 mM magnesium chloride, 300 mM potassium chloride). Then 8 µL of 1.2 mg/mL ACMA was added and the test tube was vortexed before being transferred into a fluorescence cuvette containing a stir bar. Fluorescence (415 nm/485nm) was read by a Shimadzu RF-6000 fluorescence spectrometer for 20 seconds before 30 µL of 25 mM ATP, pH 7, was added, quenching fluorescence. After quenching was allowed take place for 80 seconds, 8 µL of 0.20 mg/mL nigericin was added, restoring fluorescence. Readings were terminated 20 seconds after the addition of nigericin and data was normalized to maximum fluorescence.

2.4 ATP-synthesis

ATP synthesis was measured using a luciferin/luciferase system where luminescence correlates with ATP production. The assay was performed in a white 96-well plate. Each well contained 280 µL of well solution (5 mM Tricine-KOH, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM ADP, 3.75 mM KH_2PO_3, 125 µM luciferin, 100 ng luciferase, and 2.5 mM NADH). The plate was brought to 30 °C in a BioTek Synergy plate reader and background luminescence was measured. The reaction was initiated by adding 10 µg ISO vesicles. Luminescence was read every 40 seconds for 15 minutes. Results are reported as luminescence traces and maximum slope relative to wildtype.

2.5 Succinate Growth

A prewarmed LB-amp agar plate was streaked with glycerol stocks of each mutant and incubated for ~17h.
Single colonies were used to inoculate 5 mL cultures of M63-TIV medium () supplemented with 100 µg/mL ampicillin, glucose, and 5% (v/v) LB broth. Ampicillin was present in all the tubes except for the one meant for DK8, which was inoculated with the glycerol stock of DK8. The cultures were then grown for ~17h.

A sterile 96-well plate was prepared with triplicates of wells containing 250 µL M63-TIV supplemented with 100 µg/mL (except for DK8 wells) and either glucose or succinate. Each well in the sterile 96-well clear plate was then inoculated with 5µL of the overnight culture. The plate was incubated at 37 °C with shaking, and optical density at 550 nm was read every 30-120 min for 26 h.

2.6 Safety Issues

Toxic chemicals used were ethidium bromide and nigericin. Both of which were handled using standard PPE. Live *E. coli* cells were handled according to biosafety level 1 procedures.

2.7 Methodology

Methodology used was primarily described in the works of Steed and Fillingame.

3. Results

In order to investigate the function of arginine 50 (R50) in subunit c and aspartate 92 (D92) and glutamate 196 (E196) in subunit a and to investigate whether or not there is in fact an electrostatic salt bridge made, mutants were made of D92 and E196 and double mutants with R50. ISO vesicles were prepared from the mutant E. coli cells, and several assays were performed. An ATP-driven proton pumping assay was performed to examine how well the ATP synthase pumped protons across the membrane into the cell. ATP synthesis was performed to examine how well the ATP synthase was able to use the ADP and P_i to create ATP powered by a proton gradient (generated by NADH oxidation). Lastly, the ability of mutants to grow on succinate was determined since succinate forces cells to use the oxidative phosphorylation pathway and ATP synthase. In this assay, growth examined on glucose is used as a control, where even DK8 cells can exhibit growth of about 60% through an alternate pathway.

3.1 Effects of mutations at the D92 position:

Mutations of D92 were not well tolerated as there is a significant decrease in function in both the ATP-driven proton pumping direction as well as in the synthesis direction. In the proton pumping assay (Figure 4), there was about a 60% loss of function when the most functioning mutant, D92A in orange, was compared to WT in dark blue. When comparing the other mutations to WT in this assay, D92R had even less function, and D92A/R50A and D92R/R50D had a loss of about 70% function. When examining D92 mutants in their ability to synthesize ATP (Figure 5), there seemed to be a slightly better tolerance for D92 single mutants where D92A and D92R showed about a 40% loss. However, D92A/R50A showed a 70% loss and D92R/R50D showed an 80% loss of function. These results, summarized in Figure 6, indicate that mutations made at the D92 position are not well tolerated, implying its significant role in proton translocation.

D92 mutations were also examined through a succinate growth assay, where their ability to grow on succinate versus glucose was examined (Figures 7 and 8). When succinate growth was examined, D92R/R50D showed less growth than the negative control, DK8. Interestingly enough though, D92A/ R50A and D92A had about the same level of growth and D92R had more growth than WT. Indeed, when the percent growth of each mutant was compared to WT (Figure 9), D92A had about a 36% growth and D92A/R50A had about 30%, while D92R had about 135%. Lastly, D92R/R50D had a very slight negative growth indicating a slight cell death. However, this assay was only preformed once and these results should be confirmed.

When the D92 mutants' ATP-driven proton pumping and ATP synthesis activities were comparted, there was a significant loss of function in both directions, but the impact of the mutations seemed to be greater in the pumping direction than in the synthesis direction. There was also a significant impact on the mutations' ability to grow on succinate as well, with the exception of D92R. These results indicate that D92 plays a significant role in proton translocation and ATP synthase function and that the negatively charged aspartate side chain is likely key to these functions.

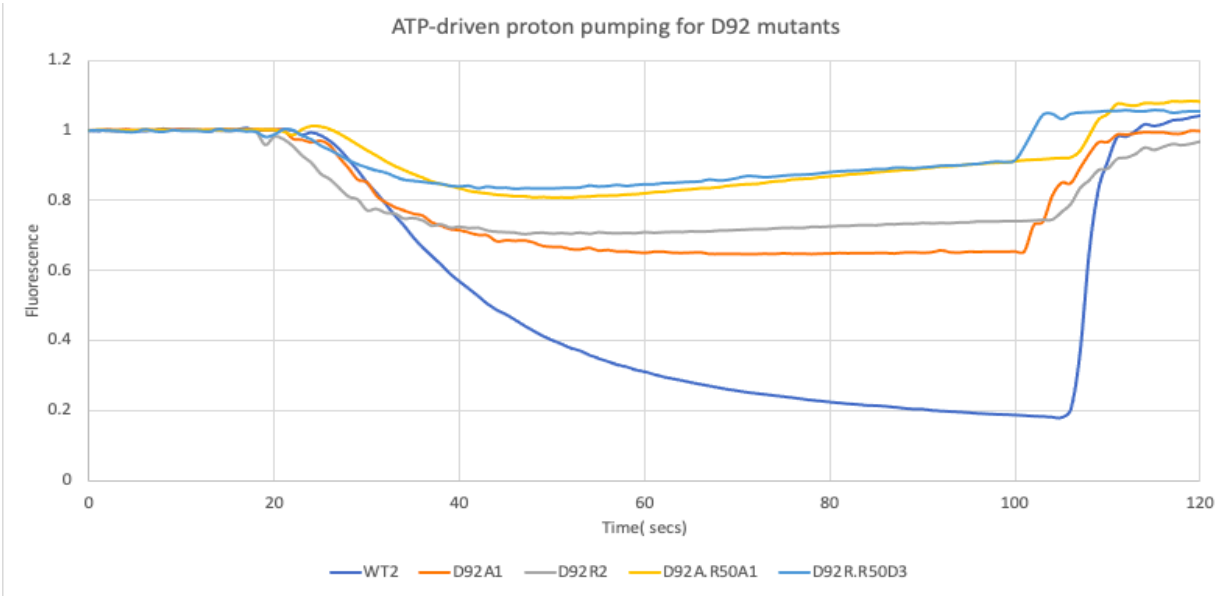

Figure 4: Summary of ATP-driven proton pumping for the D92 residue

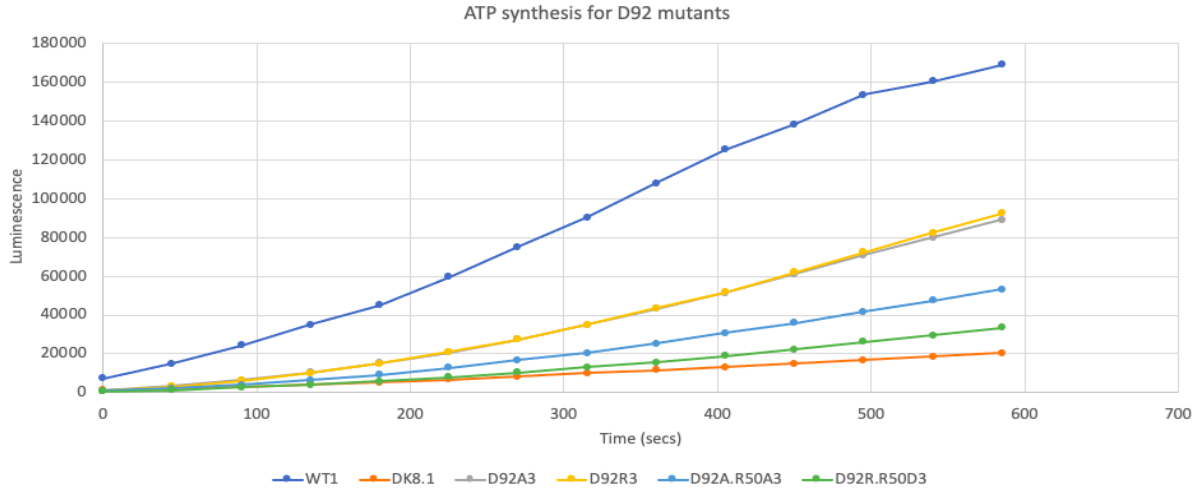

Figure 5: Summary of ATP synthesis for the D92 residue

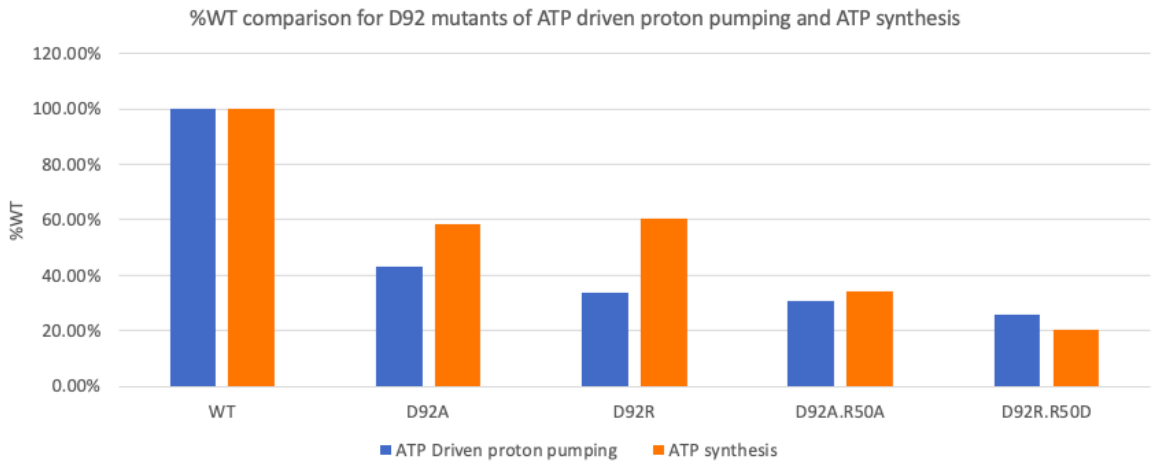

Figure 6: Comparison of ATP-driven proton pumping and ATP synthesis for D92 mutants

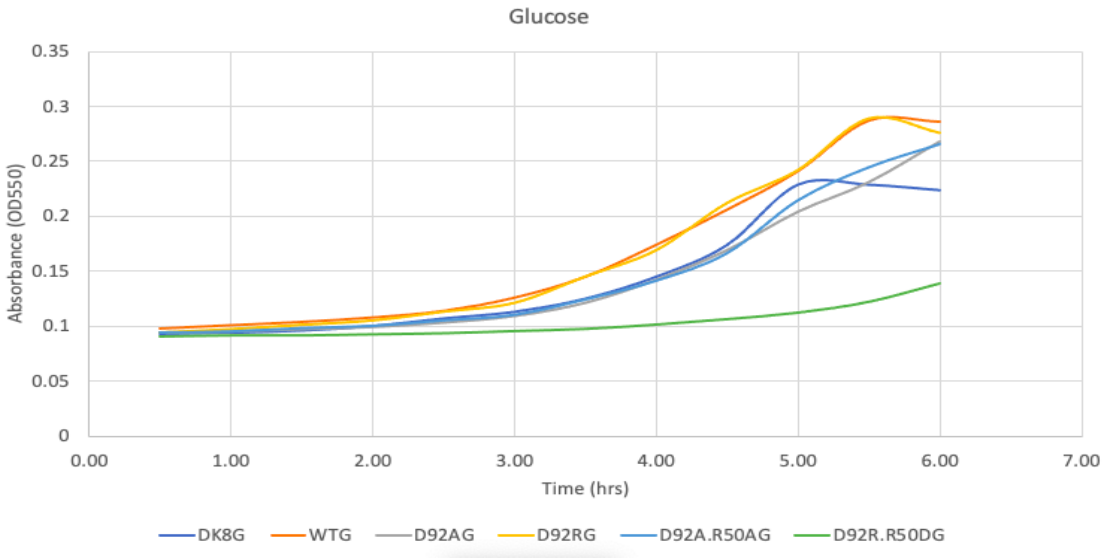

Figure 7: Sample data for the glucose control of the succinate assay involving D92 mutants

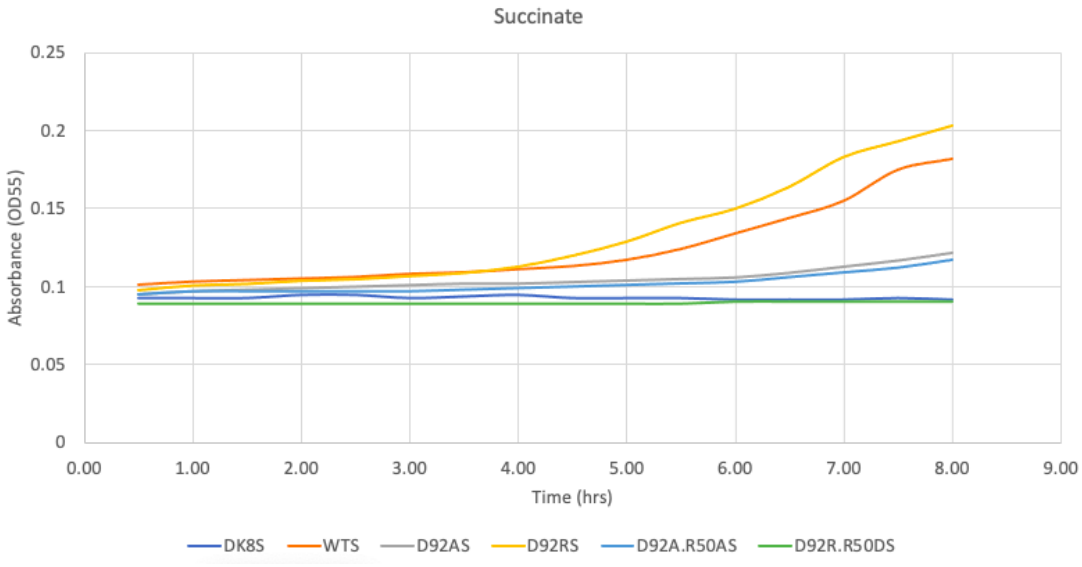

Figure 8: Sample data for succinate growth of D92 mutants

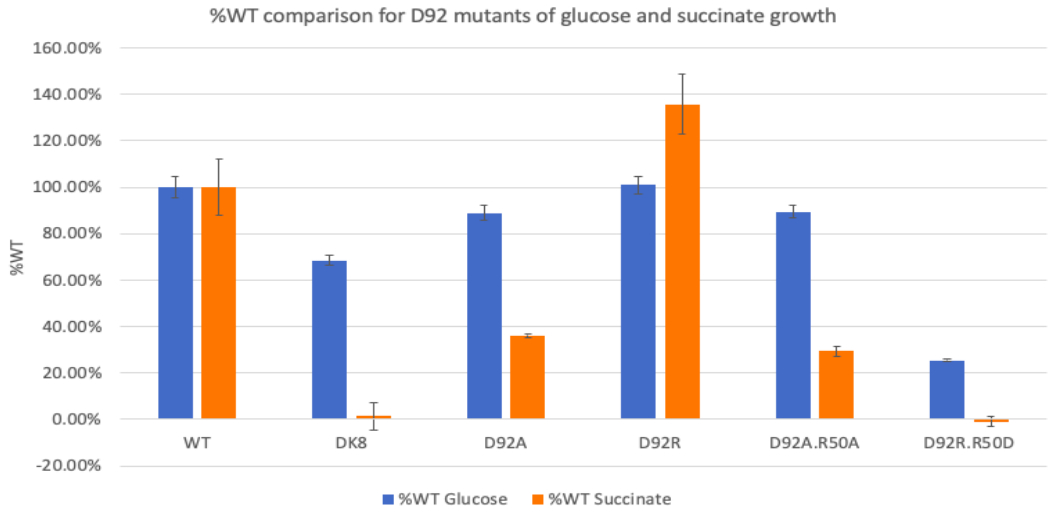

Figure 9: Summary graph comparing glucose and succinate growth for D92 mutants

3.2 Effects of mutations at the E196 position:

Mutations of E196 were better tolerated when compared to mutations made at the D92, however there was still some loss of function. This difference could indicate that the E196 position is not as involved in the movement of protons across the membrane. When the proton pumping activity of mutations were examined, the mutation with the highest activity, E196A/R50A, lost only 10% of function. E196A was close with about 18% loss, while E196R had about 30% loss and E196R/R50E had the greatest loss of about 45% . However, when the ATP synthesis activity of mutations was examined, it appears that mutations had a greater effect on synthesis, as seen in Figure 12. E196A and E196A/R50A had similar function with only about 45% loss in synthesis when compared to WT (Figures 11 and 12), while E196R/R50E had a 60% loss, and E196R had the greater loss in synthesis capability with a loss of about 70%.

However, E196 mutants' growth on succinate showed a significant on growing capacity, see figures 14 and 15. E196A continued to show the best functionality of the mutants, but still showed a % growth, when compared to WT,

of only 33%. The others showed a 16%, 10%, and 2% growth for E196R, E196A/R50A, and E196R/R50E respectively. However, DK8 showed less than 60% growth in glucose, indicating that these results are not conclusive due to the control being inconsistent, as well as the assay having only been preformed the one time.

In the examination of the E196, the impact of mutation on function at this position was less than what was examined at the D92 position but there was still loss of function. Mutations seemed to have greater impact in the synthesis direction than in the pumping direction. In contrast, however, there was a significant impact on succinate growth where the single mutations at the E196 position showed the greater growth density but only up to 33%. However, ATP-driven proton pumping and synthesis results indicate that E196 might play a less significant role in ATP synthase function.

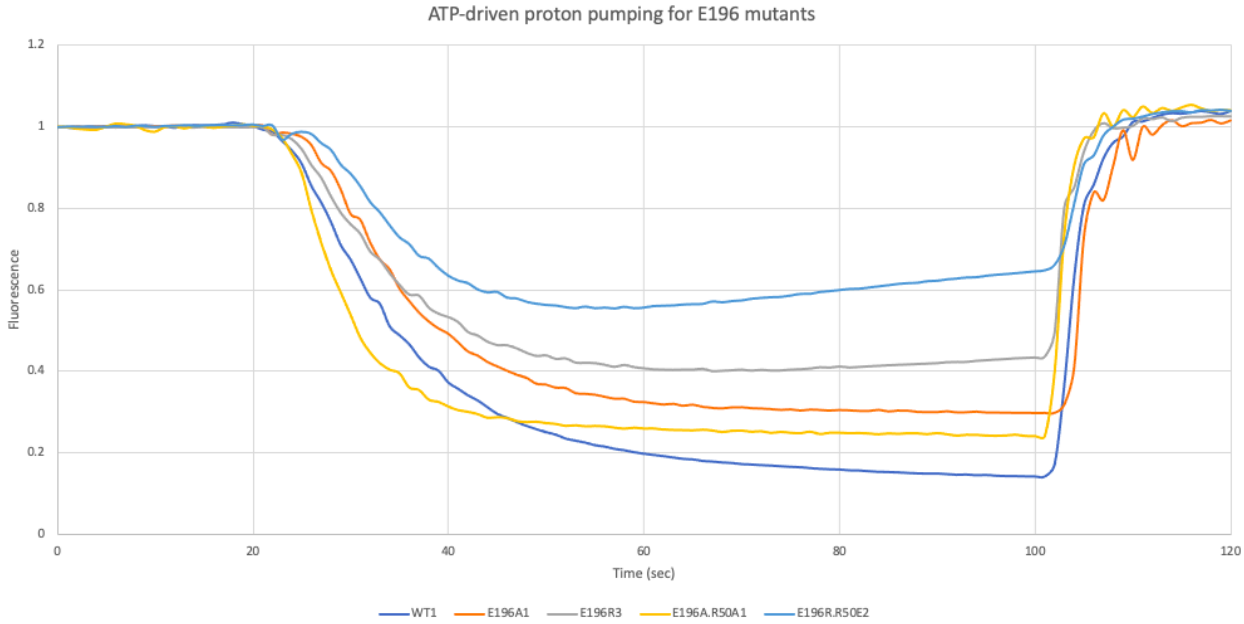

Figure 10: Summary of ATP-driven proton pumping for the E196 residue

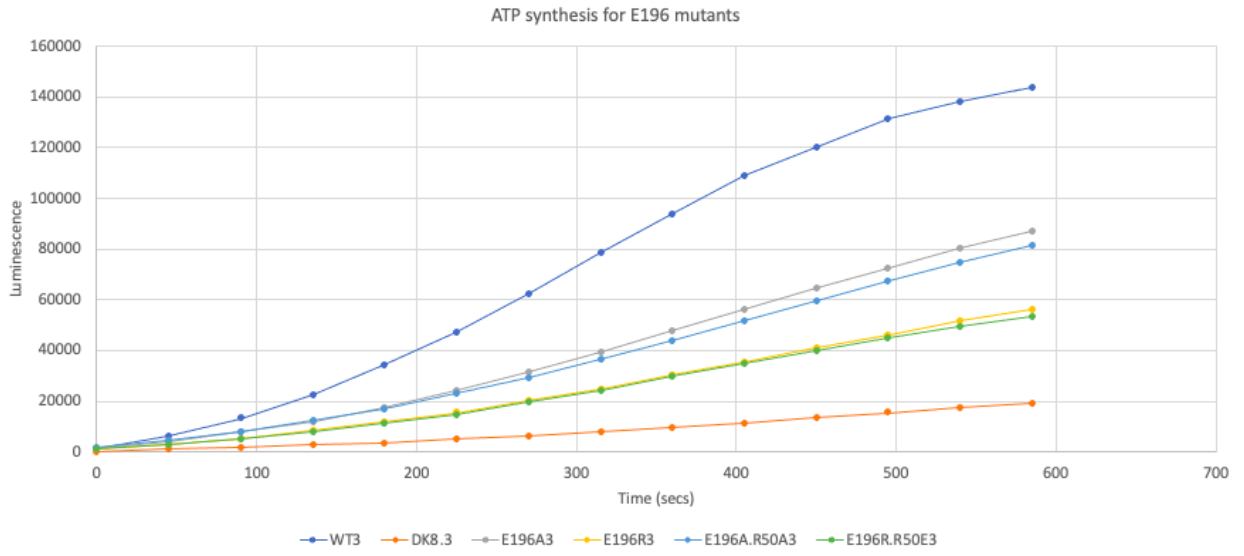

Figure 11: Summary of ATP synthesis for the E196 residue

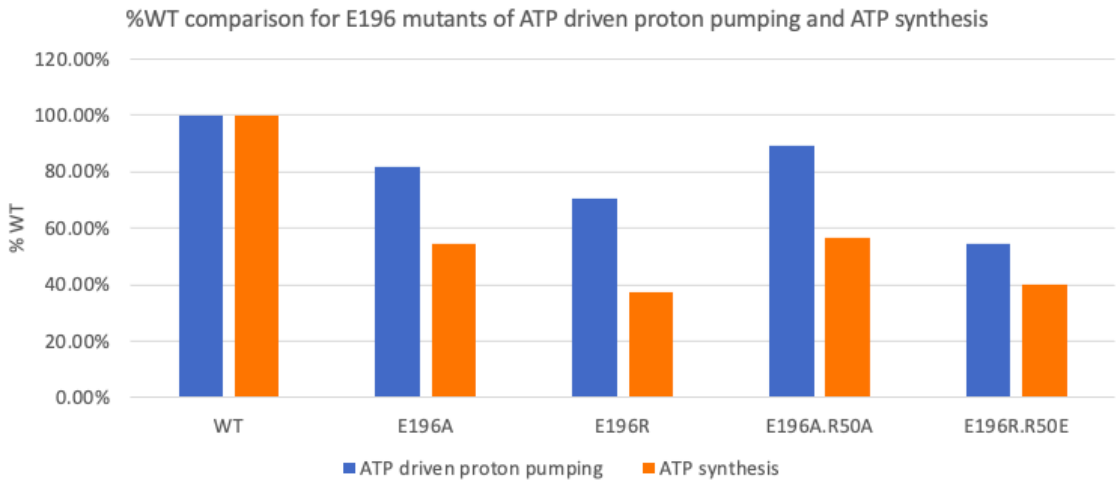

Figure 12: Comparison of ATP-driven proton pumping and ATP synthesis for E196 mutants

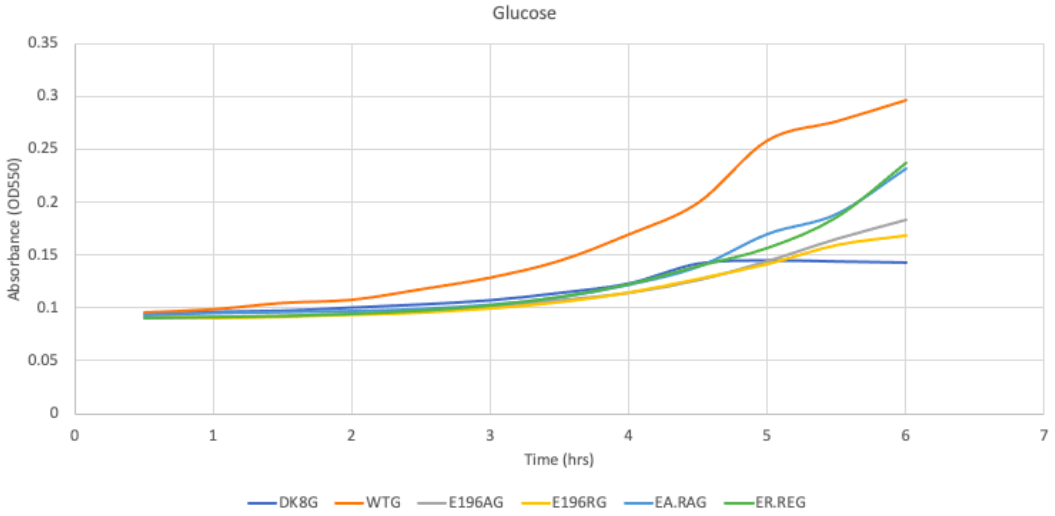

Figure 13: Sample data for the glucose control of the succinate assay involving E196 mutants

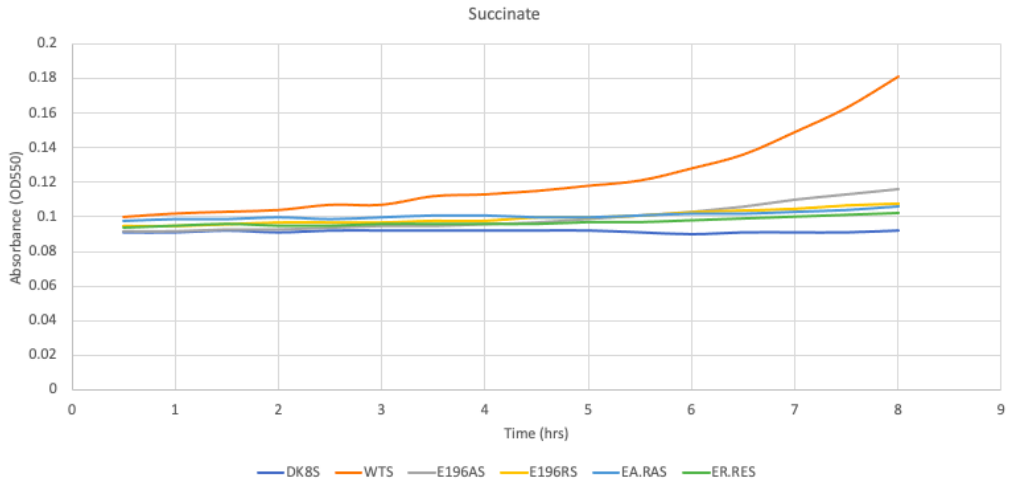

Figure 14: Sample data for succinate growth of E196 mutants

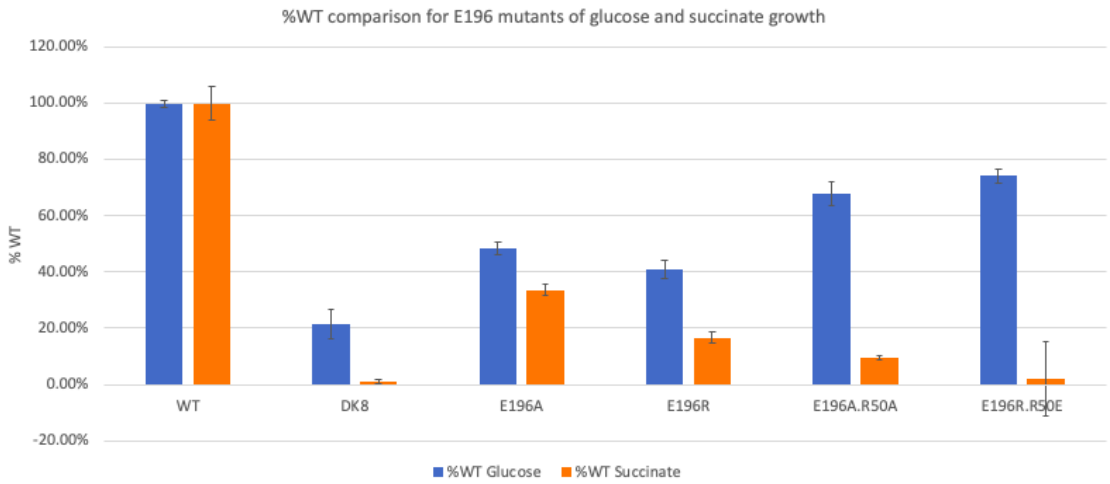

Figure 15: Summary graph comparing glucose and succinate growth for E196 mutants

4. Conclusion

In conclusion, ATP synthase is the primary producer of ATP, the carrier for the energy necessary for biological function in many living organisms. Understanding how ATP synthase functions is useful in the development of more and better medication for various diseases, from those caused by bacteria to cancer cells. In this study, the residues cR50, aE196, and aD92 were investigated due to their genetic conservation. Previously, a functionally important salt bridge was proposed to exist between the residues cR50 and aE196, but, based on Cryo-EM structure, aD92 offers a better candidate for interaction with cR50 due to their proximity in space. To investigate these proposed interactions, 8 mutations were made and tested. Results indicate that both D92 and E196 are functionally important, but D92 was more sensitive to mutation. Among the 8 mutations made were double mutations involving R50 to probe the possible interactions between cR50 and aD92 or cR50 and aE196. Neither revealed any additional information about the function since their function did not differ from that of the single mutations. However, results do indicate that the D92 residue is a little more important for the proton pumping function than synthesis and vice versa for E196. These results further confirm the importance of the acidic residues at the 92 and 196 positions in the c subunit. Future directions are to clarify the results for succinate growth, as the glucose controls were not as expected, and to synthesize the results

presented here with those from R50 mutations that were tested by others in order to fully understand the roles of these three residues in proton translocation and torque generation.

5. Acknowledgements

I would like to thank Dr. Steed for his patience, guidance, and kindness throughout the research process and my time at UNCA. I would also like to thank the entire UNCA chemistry department for their support and teaching throughout my time at UNCA. Thank you to GlaxoSmithKline for their funding of the department and my research.

6. Bibliography

(1) He, C.; Preiss, L.; Wang, B.; Fu, L.; Wen, H.; Zhang, X.; Cui, H.; Meier, T.; Yin, D. Structural Simplification of Bedaquiline: The Discovery of 3-(4-(N,N-Dimethylaminomethyl)Phenyl)Quinoline-Derived Antitubercular Lead Compounds. *ChemMedChem* **2017**, *12* (2), 106–119.

(2) Wang, T.; Ma, F.; Qian, H.-li. Defueling the Cancer: ATP Synthase as an Emerging Target in Cancer Therapy. *Molecular Therapy - Oncolytics* **2021**, *23*, 82–95.

(3) Pogoryelov, D.; Krah, A.; Langer, J. D.; Yildiz, Özkan; Faraldo-Gómez, J. D.; Meier, T. Microscopic Rotary Mechanism of Ion Translocation in the Fo Complex of ATP Synthases. Nat. Chem. Biol. 2010, 6 (12), 891–899.

(4) Steed, P. R.; Fillingame, R. H. Aqueous Accessibility to the Transmembrane Regions of Subunit c of the Escherichia Coli F1F0 ATP Synthase. *J. Biol. Chem.* **2009**, *284* (35), 23243–23250.

(5) Deleon-Rangel, J.; Ishmukhametov, R. R.; Jiang, W.; Fillingame, R. H.; Vik, S. B. Interactions between Subunits a and b in the Rotary ATP Synthase as Determined by Cross-Linking. *FEBS Lett.* **2013**, *587* (7), 892–897.

(6) Walker, J. E. The ATP Synthase: The Understood, the Uncertain and the Unknown. Biochem. Soc. Trans. 2013, 41 (1), 1–16.

(7) Pogoryelov, D.; Krah, A.; Langer, J. D.; Yildiz, Özkan; Faraldo-Gómez, J. D.; Meier, T. Microscopic Rotary Mechanism of Ion Translocation in the Fo Complex of ATP Synthases. Nat. Chem. Biol. 2010, 6 (12), 891–899.

(8) Steed, P. R.; Fillingame, R. H. Aqueous Accessibility to the Transmembrane Regions of Subunit c of the Escherichia Coli F1F0 ATP Synthase. *J. Biol. Chem.* **2009**, *284* (35), 23243–23250.

(9) Steed, P. R.; Fillingame, R. H. Residues in the Polar Loop of Subunit c in Escherichia Coli ATP Synthase Function in Gating Proton Transport to the Cytoplasm. J. Biol. Chem. 2014, 289 (4), 2127–2138.

(10) Kühlbrandt, W.; Davies, K. M. Rotary ATPases: A New Twist to an Ancient Machine. *Trends Biochem. Sci.***2016**, *41*(1), 106–116.

(11) Yanagisawa, S.; Frasch, W. D. Protonation-Dependent Stepped Rotation of the F-Type ATP Synthase C-Ring Observed by Single-Molecule Measurements. *Journal of Biological Chemistry* **2017**, *292* (41), 17093–17100.

(12) Nakanishi, A.; Kishikawa, J.; Tamakoshi, M.; Mitsuoka, K.; Yokoyama, K. Cryo EM Structure of Intact Rotary H+-ATPase/Synthase from Thermus Thermophilus. Nat. Commun. 2018, 9 (1), 89.

(13) Yanagisawa, S.; Frasch, W. D. Ph-Dependent 11° F1FO ATP Synthase Sub-Steps Reveal Insight into the FO Torque Generating Mechanism. *bioRxiv* **2021**.

(14) Steed, P. R.; Fillingame, R. H. Subunit a Facilitates Aqueous Access to a Membrane-Embedded Region of Subunit c in Escherichia Coli F1F0 ATP Synthase. *J. Biol. Chem.***2008**, *283*(18), 12365–12372.

(15) Fillingame, R. H. Identification of the Dicyclohexylcarbodiimide-Reactive Protein Component of the Adenosine 5'-Triphosphate Energy-Transducing System of Escherichia Coli. *Journal of Bacteriology* **1975**, *124* (2), 870–883.