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The Importance of Arginine *c*50 to the Rotary Mechanism of F-type ATPase

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Abstract

F_1F_O ATP synthases catalyze the synthesis and hydrolysis of adenosine triphosphate (ATP), the universal biological energy carrier, and are thus integral to life. The membrane-embedded F_o sector utilizes the proton electrochemical gradient to generate rotation of the *c* ring in in the F_O sector, which drives ATP's chemical synthesis in the F_1 sector. Recent structural models of ATP synthase from cryo-electron microscopy have revealed the architecture of the rotor-stator interface. However, despite the higher resolution information, the intricacies of the rotation mechanism are not fully understood. Previous research identified specific residues of the *c* subunit of *E. coli* F_1F_O, including Arg50 on the cytoplasmic end of transmembrane helix 2, that may be necessary for function. Mutation of Arg50 to Cys abolishes ATP-driven H^+ pumping and blocks H^+-permeability, but these mutants are still able to grow in succinate minimal medium, suggesting that ATP synthesis activity is unaffected. Since positively-charged amino acids are conserved in this region of subunit *c* and have been proposed to form a salt bridge with subunit *a*, we tested the necessity of positive charge and steric bulk at this position using chemical and genetic modifications. Chemically appending side chains including ones containing a positive charge onto Cys50 with methanethiosulfonate did not restore H^+ pumping activity. However, mutation of position 50 to nonpolar and carboxyl groups as well as His partially supported ATP-driven H^+ pumping as well as ATP synthesis activity. And, mutation to Lys fully supported ATP-driven H^+ pumping activity. These results indicate that position 50 of subunit *c* is tolerant to mutations excluding cysteine, but that a positive change at the end of a four carbon linker is highly favorable.

1. Introduction

Adenosine triphosphate (ATP) is an energy-carrying molecule that is found in all forms of cellular life and can be described as the energy currency for cells. Hydrolysis of the terminal phosphate group is used in biochemical reactions such as glycolysis and the citric acid cycle to overcome energetic thresholds. After hydrolysis, the adenosine diphosphate (ADP) is rephosphorylated in order be reused by the cell. This process is performed by the enzyme ATP synthase (ATPase), a membrane-embedded protein complex. ATPase channels the electrochemical gradient of positive ions (H^+ or Na^+) generated by the electron transport chain in order to generate mechanical energy to enact conformational changes within itself and catalyze the synthesis of ATP. Thus, the chief purpose of ATPase is the transformation of electrochemical energy to chemical energy. In order to understand the mechanism of enzyme function, understanding the structure of the protein as well as the role of these positive ions is key.

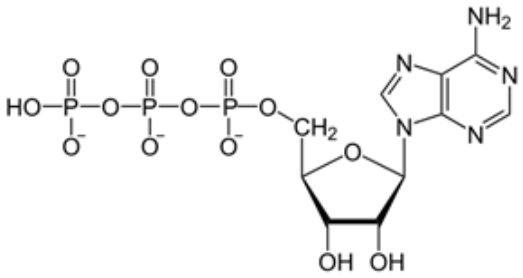

Figure 1: Adenosine Triphosphate (ATP)

ATPase is composed of two main sections, the F_O motor and the F_1 motor, each with their own unique subunit types.[1,2] The synthesis of ATP is performed via conformational changes of ATPase F_1 motor's alpha (α) and beta (β) subunits. These conformational changes are caused by processes performed in the F_O region of the protein, namely, the buildup of torque in the gamma subunit (γ). And this buildup is caused by the channeling of the electrochemical gradient through subunit *a's* aqueous channels and onto the *c* subunit ring. (Figure 2).

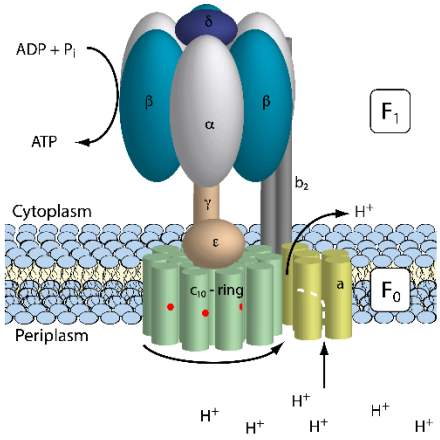

Figure 2: The subunits of ATP Synthase (republished with permission by P. Ryan Steed)

The structure of the F_O motor's subunits is most clearly understood by following the path of a proton through it. To begin, positively charged ions enter subunit *a* through a half channel on the periplasmic side of the membrane due to their higher concentration. Rather than moving through the entirety of subunit *a* and ending up on the cytoplasmic side of the membrane, the first half channel ends when the proton is transferred onto one of the *c* subunits. This interaction occurs between *a*Arg210 and *c*Asp61 at the *a*-*c* interface.[5,6] After this transfer, *c* ring spins and a deprotonated *c* subunit releases the one that was protonated. After a complete rotation attached to the carboxyl group of *c*Asp 61 the proton then is exported through the other cytoplasmic half channel of subunit *a* and the proton translocation process within F_O is complete. Thus, the gradient promotes unidirectional rotation.

Depending on the organism and type of ATPase there are between 8 and 15 *c* subunits within the ring, and when in motion, the torque, which twists the *γ* subunit of F_1. [1,6] The rotation of *γ* then causes conformational changes in the F_1 motor thus allowing for the synthesis of ATP. Within ATP synthase, the three α/β active sites of the F_1 motor complete the reaction converting ADP and inorganic phosphate (P_i) into ATP. These subunits have three conformational states that they cycle through; each active site is in a different conformation than the others at any given time. These conformations include open, tight, and closed. One full cycle of these in the forward direction results in one ATP formed from ADP and P_i. A full 360° turn of the *c* ring results in the each of the three active site going through all three conformations resulting in 3 ATP. The number of cations from the electrochemical gradient necessary to complete this process is determined by the F_O motor, or more specifically the number of *c* subunits present in the rotor ring. Therefore, the number of protons needed to synthesize an ATP is dependent on the number of c subunits and can be expressed as (3ATP/number of c subunits) or the ion to ATP ratio.[1,6,7] While much is known about the overall mechanism of this process there are still aspects of it that are yet to be fully explained.

For example, ATP synthase not only functions in forward direction, synthesizing ATP it can also run in reverse by hydrolyzing ATP and acting like a proton pump, creating an electrochemical gradient. This unique reversibility of the enzyme's functionality makes it of particular interest to researchers as it is unclear if these two processes function in similar ways or if there are different pathways for each process.[14,15]

Although basic structural and functional information of E. coli ATP synthase have been determined, only recently has an experimentally-generated, medium-resolution model been made. This model is a result advancements in Cryo-electron microscopy, and while this model does have the resolution to determine position of α helices, a model has yet to surface that resolves individual residues.[2,8] The crucial residues are Asp 61 in subunit *c* and Arg210 on helix 4 for subunit *a*, which are proven to be necessary for normal enzyme function, are indeed located in a manner which corroborates claims regarding their functionality.[13] This model also visualized the protein in its three F_1 conformational states: open, half closed, and closed. Even though this model was able to resolve the structure at 6.9-8.5 angstroms the researchers were unable to differentiate any individual helices, leading to a lack of resolution specifically in the *a*-*c* interface. (Figure 3)[2]

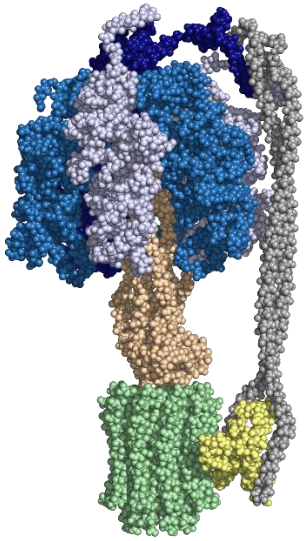

Figure 3: The Cryo EM Structure of F-type ATPase as determined by Sobti et al.[2] (PDBID:5T4O)

Understanding the interactions at the *a*-*c* interface is important as this site is the source of the transformation of electrical to mechanical energy. The turning of the *c* ring, and thus the synthesis of ATP, is dependent on the interactions that occur at this site. The elucidation of ATP synthase's hydrolysis and synthesis mechanisms at the *a*-c interface could affect the structural dynamics of the enzyme's proton driven transition states, especially within the F_O subunit, where this research is being conducted. If new critical residues that aid in the proton translocation process are discovered, drugs can be designed to target specific interactions to inhibit one of the two pathways the enzyme works in. Investigation into the chemical properties of residues within the polar loop of subunit *c* also remains largely unexplored leaving this area as prime candidate to help bridge the gaps in the understanding of subunit *a/c* interactions. The goal of this research is to investigate *a*-*c* interactions via the genetic and chemical modification of *c*R50. By altering this residue's chain length and hydrogen bonding ability and then preforming in vitro biochemical assays to determine protein functionality in the hydrolysis and synthesis directions, this research seeks to determine if *c*R50 plays an important role in the interactions that cause c-ring rotation and whether those interactions are used in both synthesis and hydrolysis.

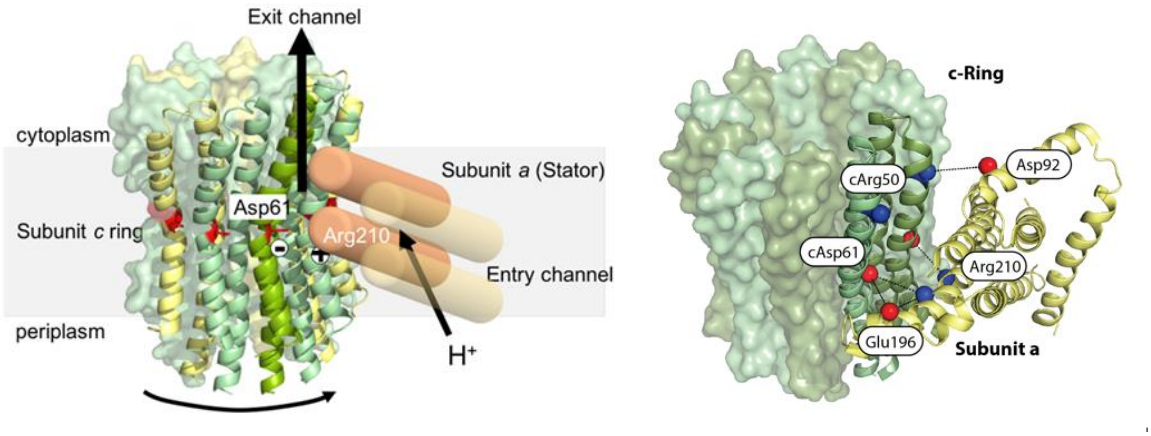

Figure 4: A. The subunit *a*-*c* interface detailing proton transport path. B.cR50's (blue) potential salt bridge interactions with acidic residues subunit *a* (red). (Republished with permission by P. Ryan Steed)

1.1 Previous Findings and Experimental Justification

The potential for two different mechanistic modes involving the *a*-*c* interactions was observed by Fillingame and Steed in their experiment to characterize the aqueous accessible areas of the *a*-*c* interface, by changing the native residues of the protein to cysteine. Among these residues in the polar loop of the c subunit was Arginine 50. During the characterization of protein functionality, E. *coli* with cys mutant ATP synthases were able to survive in a succinate minimal media, but failed to show proton pumping activity. This result is unexpected as E. *coli* should only be able to grow in succinate medium if they have functioning ATPase as succinate metabolism requires functional ATP synthase. If the mutant cells had died in this medium and failed to pump protons in the ATP hydrolysis assay then this would suggest the two pathways have the same mechanism. However, the surviving mutants that are not able to pump protons suggest that there are differing mechanisms for synthesis and hydrolysis.[3,4,5] The mechanistic importance of cR50 is now being investigated due the unique interactions the guanidinium group can provide, namely its positive charge, due to high pKa.

Arginine at this position on the c ring form potential ionic contacts to subunit *a*, particularly with aD92 and aE196. The postive chage of the guanadinium group has been proposed to interact with the carboxyl groups within subunit *a* at positions D92 and E196. This potential electrostatic interaction could play a key role in the rotation of the *c* ring. This salt bridge functionality is occasionally referred to as the "leash" mechanism.[15]

As the cysteine mutant was already created, the first step of the project to use a cysteine modification scheme to quickly test a number of different side chains. Probing function using cysteine modification using chemical reagents has been performed previously, a notable example being a Cl^-/H^+ exchanger protein.[9] Similarly, this projects seeks to generate a variety of residue substitutions to determine the functional parameters of the enzyme. In the case of the exchanger protein, side chains with differing length and acidity were introduced chemically to the residue responsible for proton transport. Results confirmed that the residue's protonation was necessary for complete functionality of the enzyme and validated this experimental strategy.[9]

The goal of this research is to investigate the impact the residues of the polar loop have on the *a*-*c* proton pumping interface. Previous work indicated that when residues in this area were changed to Cys, proton pumping was compromised but not ATP synthesis ability.[1,11] Therefore, the objective of the proposed project is to investigate the functional role of subunit *c*'s Arg 50 via site directed, genetic and chemical mutagenesis. Specifically, the project seeks not only to test the hydrolysis of ATP but also its synthesis. Mutagenesis of the *c*Arg 50 to test for the importance of the charge, steric bulk and hydrogen bonding ability of the residue will be used to determine its characteristic functionality in the polar loop of subunit *c* in F-type E. *coli* ATP synthase.

1.2 Experimental Approach

This project use PCR with mutagenic primers to generate mutant ATP synthases. This method has been previously used in the group to mutate the *uncE* gene, which encodes the *c* subunit. The mutated DNA is then cut and pasted into a plasmid using restriction endonucleases. Once the plasmid is successfully constructed it is inserted into a *E. coli* cells, which read the DNA and synthesize the mutated protein complex. The cells are then homogenized to create inverted membrane vesicles (ISOv) containing the transmembrane ATP synthase. After these vesicles are collected the functionality of the mutated ATP synthases can finally be assessed.[1,3,4] Cysteine mutants were modified using methanthiosulfonates (MTS) (Figure 6). These modifications will not be naturally occurring amino acids but instead will change the side chain composition of a Cys mutation by creating a disulfide bridge between the sulfur present in the residue and that of the MTS. This process has been used in the past to probe the functionality of enzymes by mimicking a natural mutation or introducing a novel functional group.[8,16] (Figure 6)

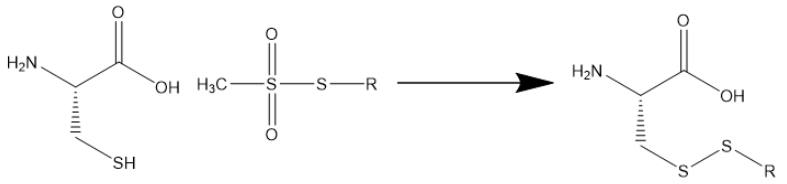

Figure 6: MTS cysteine modification Reaction Scheme

After mutant creation and chemical modification, the ATP-driven proton pumping assay to determine indirectly uses the fluorescence of 9-Amino-6-Chloro-2-Methoxyacridine (ACMA) to measure proton pumping ability. The deprotonated state of ACMA can freely diffuse through the vesicle membrane while its protonated charged state is unable to do so. As a result the lower the pH of the vesicle due to ATP hydrolysis pumping protons results in a higher concentration of ACMA in the vesicle. At low concentrations, such as those present while ACMA is able to diffuse, the molecule fluoresces. However, as the ACMA concertation increases it fluoresce quenching occurs. Thus, a reduction in fluorescence indicates that more ACMA is trapped in the vesicle due to the protons pumped in by ATP synthase while it hydrolyzes ATP. Lastly the ionophore nigericin (Nig) is added to the reaction to exchange protons from the interior of the vesicle replacing them with potassium ion, resulting in the restoration of ACMA's fluorescence and confirming protein function.[4,5,11] (Figure 7) Assay Synthesis function of mutated protein was also determined via the detection of luminance of firefly luciferase as described in the methods section.

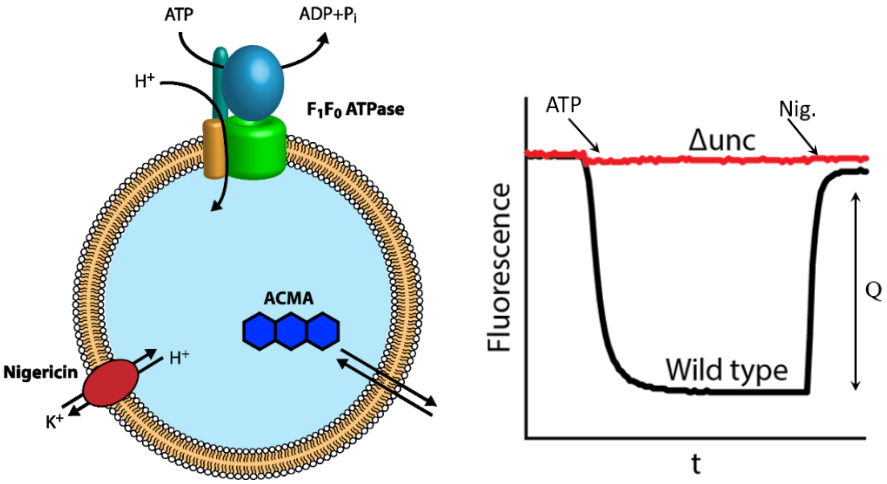

Figure 7: Proton Pumping Assay- Left representative diagram of ISOv undergoing Proton pumping experiment. Right- a representative graph of data collected from this assay. The addition of ATP reduces fluoresce as ACMA is protonated and trapped in the vesicle; fluorescence is restored with Nig. Bar graph data of this assay relies on the metric of percent

quenching which is defined as Q = 1- (minimum fluorescence after T_{ATP}) / (maximum fluorescence after T_{Nig})x100%. (Republished with permission by P. Ryan Steed)

2. Experimental Methods

2.1 Safety

Gloves and safety glasses were utilized within the laboratory as PPE. Ehthidium bromide, nigericin and heavy metal waste were disposed of separately due to environmental concerns. Lastly, biological waste was sanitized via bleach and/or autoclaving to destroy cultures and cell debris.

2.2 Mutagenesis

A two-step PCR reaction using forward, reverse and mutagenic primers was used to change one codon corresponding to the desired mutation in cysteine less pCMA113 plasmid, which encodes the entirety of ATPase. One percent agarose gels were used to determine the success of generating the desired mutant DNA fragment for cut and paste mutagenesis. After gel confirmation, restriction endonucleases BsrG1 and PpuM1 were to prepare a cut fragment for ligation into the pCMA113. Subunit *a* mutations utilized PflM-1 and BsrG1 restriction endonucleases. The mutant plasmid was then transformed into competent DH5α E. *coli* cells via heat shock, and the cells were grown on a LB agar plate with 100 µg/mL ampicillin. Plasmids were purified from small LB cultures using a plasmid miniprep kit (NEB). Creation of mutant plasmids was confirmed via DNA Sanger sequencing through the ligation sites. Mutant plasmids were then transferred into JWP 292 E. *coli* cells, which lacks genes for ATPase. Cultures containing 12% glycerol were stored at -80ºC.

2.3 Preparation of inside out vesicles (ISOv)

JWP292 cells encoding mutant ATP synthase were grown in 1L of M63 minimal medium (w/v-0.1% NaCl, 0.1% Tryptone,, 0.05% Yeast Extract 618 mM KH2PO4(mono), 382 mM K2HPO4 (dibasic), 10 mM MgSO4, 150 mM (NH4)2SO4, 5 mg/L FeSO4-(5 mg/mL thiamine, 100 mM arginine, 13.3 µM uracil, 20 mM dihydroxybenzoate). Cells are then collected from growth by centrifugation at 4000 x g and resuspended in TMDG buffer (50 mM Tris-HCl, 5.0mM $MgCl_2$, 10% glycerol, pH 7.5) with addition of 1.0mM DTT to prevent formation of disulfide bridges. The suspended cells are then passed through an Avestin B15 high pressure homogenizer under 15,000 psi to lyse the cells and generate ISOv. Cell debris was removed by centrifugation at 9000 x g. The vesicles are then collected via centrifugation at 200,000 x g, suspended via douncing in TMG and stored at -80ºC.

2.4 Testing for Protein Concentration

A modified Lowry assay was used to determine the protein concentration in ISOv. To begin the assay the cell's proteins were denatured with sodium dodecyl sulfate (SDS) and exposed to $CuSO_4$ using a solution A (.02% Na_2CO_3 ,1% SDS, $1x10^{-4}$% Cu^{2+}) and incubated for 10 minutes at room temperature. Folin-Ciocalteu's phenol reagent was then added to a final volume of 300uL (16% IOV, 16% SDS, 33% Floin reagent 35% ddH_2O) and left to react for 30 minutes. The absorbance of each sample was read at 650nm. A calibration curve was created from stock solutions of bovine serum albumin (BSA) to determine a final protein concentration of ISOv.

2.5 ATP-driven H^+ Pumping Assay

Membrane vesicles were diluted to 10mg/mL using TMG, and 160uL of ISOv was added to 3.2mL HMK buffer (10mM HEPES-KOH pH7.5, 5.0mM $MgCl_2$*$6H_2O$, 300mM KCl) with 8uL ACMA (.12mg/mL). A baseline fluorescence of the sample was collected with excitation at 415nm and emission at 485nm. After 20sec of data collection, 30uL of 25mM ATP was added. At 100sec, 8uL of 2mg/mL nigericin was added to restore fluorescence and then data collection was stopped at 125 sec. Alternate buffers at 10 mM at were used to test proton pumping at different pH values, including pH 5.5 (MES-KOH), pH 6.5 (MES-KOH) or pH8.5 (TRIS-KOH).

2.6 Cysteine Chemical Modification

Modification to cR50C with chemical reagents was performed by dissolving reagents at 100 mM in either DMSO, DMF, or Ethanol followed by addition to ISOv at a final concentration of 1mM. The reaction was incubated for 15 minutes at room temperature. After the reaction, functional assays were performed on vesicles as described. Reagents used for Chemical modification included: methyl methanethiosulfonate (MMTS), propyl methanthiosulfonate (PMTS), tertbutyl methanthiosulfonate (MTS TERT), benzyl methanthiosulfonate, (MTS BENZ), phenyl methanthiosulfonate (MTS PHEN), 2-hydroxyethyl methanthiosulfonate (MTS 2HE), ethyltertbutyl methanthiosulfonate (MTS ETB) 2-aminoethyl methanethiosulfonate (MTSAE), (2-(Trimethylammonium)ethyl methanethiosulfonate (MTSET), N-ethylmaleimide (NEM) and iodoacetemide (IA).

2.7 DTNB Ellmans Assay

To confirm cysteine reaction with MTS reagents, ISOv were diluted to 0.5 mg/mL and added to a 96 well plate with 1%SDS and 0.2mM dithio-bis (2-nitrobenzoic acid) (DTNB). Absorbance of DTNB was read at 512 nm.

2.8 Succinate Growth

To determine mutants' ability to grow on succinate medium, small culture were grown overnight at 37ºC from glycerol stocks in glucose media. (M63-TAUD, 6.0% glucose,0.1mg/mL ampicillin). Cultures were then diluted 10,000 fold in M63 medium and 100uL of this dilution was plated on both an LB agar plate (LB, 0.1 mg/ml ampicillin) and an M63 succinate plate (M63-TAUD, 20 mM succinate, 0.1 mg/ml ampicillin). Plates were incubated at 37 ºC. Growth was confirmed on LB plate after 16hrs. After 72hrs, growth was confirmed and colony size was measured on the M63 succinate plate.

2.9 ATP Synthesis Assay (Biovison staybrite Kit):

ISOv's were diluted to 5mg/mL and incubated for 1hr. on ice in TMG buffer with 0.02mM malonate. Luciferase enzyme and luciferin-luciferase reaction buffer (Biovison) were incubated at room temperature and protected from light for 1.5 hrs. After incubations, 10uL of IOV's were added to phosphate reaction buffer (20 mM tricine-KOH (pH 7.5), 0.1 M NaCl, 5 mM KPi , 5 mM $MgCl_2$), 2.5mM ADP and lastly 375mM succinate (pH 7.5) to begin synthesis reaction. At time 0 and then 1 minute intervals 10uL was removed from the reaction and added to the luciferin-luciferase plate. Once all time points were taken the luciferin-luciferase plate was read for luminescence.

3. Results and Discussion

3.1 Mutagenesis

The mutations that were generated and assayed are listed in table 1. All completed mutants are preserved as glycerol stocks of DH5α, used for generation of pure mutant plasmid or JWP 292, which are used for all protein characterization assays.

Table 1: List of mutations and their ability to grow on succinate minimal media

Mutation	Growth On Succinate	ATP Synthesis Assay
WT	+	+
cR50A	+	+
cR50C	+	-
cR50D	+	+
cR50E	+	+
cR50H	+	n/d
cR50M	+	+
cR50K	+	n/d
aD92R	+	n/d
aE196R	+	n/d
aD92R/cR50D	+	n/d

3.2 Genetic Mutants Grow in Succinate Media and have a Working Synthesis Pathway

The results of the succinate growth show that all mutation made to cR50 were able to grow on a succinate medium (Table 1). This data in conjunction with the data collected on with the synthesis assay (figure 8) displays that functionality in succinate growth likely corresponds to ATPase activity as expected, with the exception of the cysteine mutant.

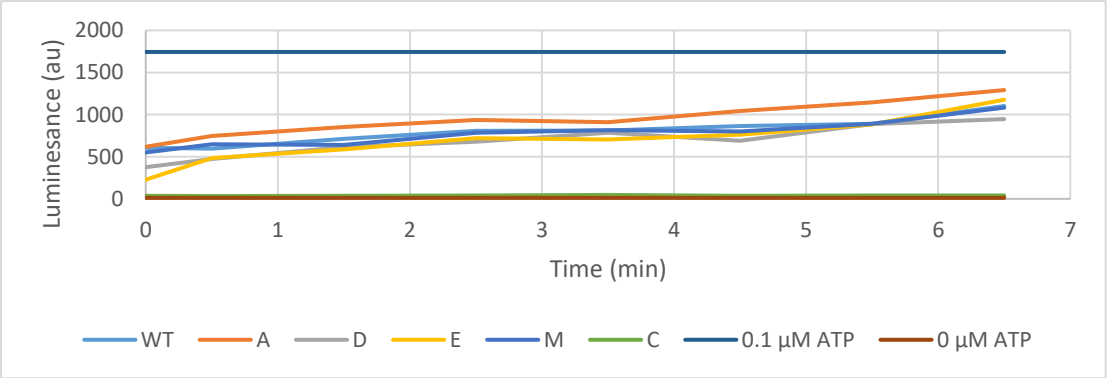

Figure 8: As the ATP concentration is raised the luminance of luciferin increases as it is phosphorylated by the luciferase enzyme thus displaying functionality of the protein. 1.0µM ATP standard and a water blank are also shown as controls.

3.3 Chemical Modification does not Restore Proton Pumping Activity

Existing cysteine mutant was modified with MTS reagents to test non-naturally occurring functional groups. Results show that no proton pumping functionality was returned to the enzyme after reaction with any of the introduced chemical modifications. The native arginine residue is characterized by its positive charge as well as its ability to donate and accept in hydrogen bonding. The methyl and propyl MTS modifications were designed to strip the site of its hydrogen bonding ability and introduce hydrophobicity. These two modifications were expected not to restore functionality as they lack any of Arginine's chemical properties. The mutant generated using MTSET was designed to mimic lys as it contains a positive charge albeit while having the added steric bulk of three methyl groups. It is possible that the extra bulk may be what is inhibiting the protein, however, only a Lys mutant could prove this hypothesis. The MTS amino ethyl modification also mimics lysine, but it lacks the positively charged side chain of the natural amino acid but does allow for the donation of hydrogen bonds. The only mutant created that could accept hydrogen bonds was the iodoacetamide modification. And lastly the N-ethyl-maleamide was used as a means of adding steric bulk to the site. Each of the chemical modifications to the cysteine mutant failed to restore functionality to the

protein. However, despite the diversity of modifications and conformation via DTNB assay, none of them recovered proton pumping activity from the nonfictional cysteine mutant. (Figure 9)

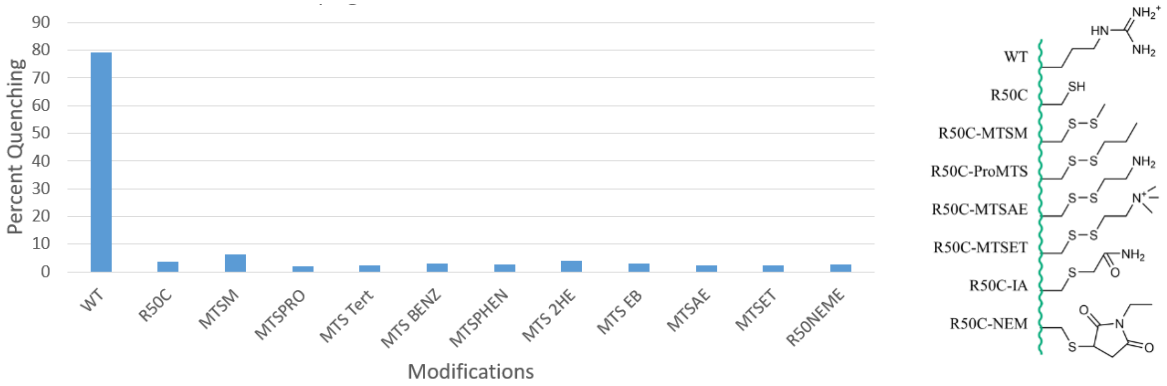

Figure 9: Left-Percent quenching of relative fluorescence during the proton pumping assay. Right- chemical modification: MTS PRO- Propyl ,MTS Tert- tertbutyl MTS Phen- PhenylMTS 2hE- 2-hydroxy ethyl (not pictured)

3.4 Proton Pumping Assay with genetic mutations of R50C in the c subunit

After chemical modification testing concluded, genetic mutants were characterized, the mutants of figure 9a (R50H and R50K) both should be able to provide a positive charge similar the Arg. Function in the His mutant it was expected to be greater as it has the potential to carry a positive charge. However, the environment around it at pH 7.5 could be preventing the protonated state to be readily present, depending on microenvironment. The Lysine is functional due to its larger pK_a suggesting that the protonation of this residue is important. The mutations in figure 9b also support that the positive charge is necessary for full functionality as they also lack residue which can be protonated resulting in a positive charge. The only outlier in this group is the R50A mutation, but this lack of steric bulk could leave space for another charged particle or residue to fill the gap. (Figure 10)

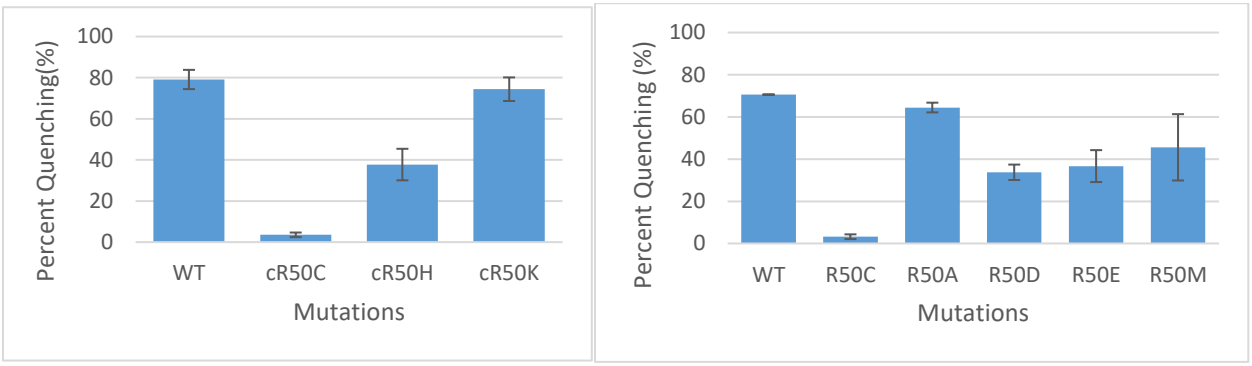

Figure 10: Functionality of each mutant is expressed in percent quenching with higher percent quenching corresponding greater proton pumping activity. Mutants which have a potentially positive residue are depicted on the left while non-positively charge mutation are depicted on the right. Wild type and Cys mutant are also shown as positive and negative controls respectively.

3.5 pH variable Proton Pumping Assay

To further probe the mutant with titratable positively charged residues, several buffer systems were created to test proton pumping over a range of biological pHs to specifically determine if the protonation states of residues which have a titratable positive charge could be manipulated to restore function more effectively. (Figure 11) It was found that pH did not provide a charge specific trend as the cR50H was consistently semifunctional in pH's above its

expected pK_a of around 6.0. Surprisingly wild type had a dramatic drop in functionality at pH 5.5. This could be due to large amount of ACMA becoming pre-protonated (pK_a 8.6) outside the membrane and have difficulty penetrating in the first place thought this does not explain the His mutant's functionality at high pH's. However, The consistent functionality of the Lys mutant shows that positive charge is favorable at this position as chain lengths provided by the Arg and Lys side chains.

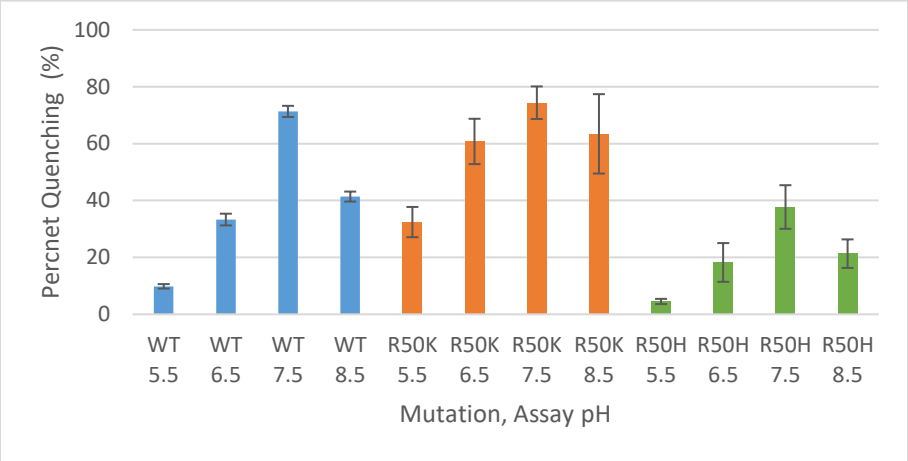

Figure 11: pH dependent Proton Pumping activity: WT (blue) retains functionality in all pH's except 5.5 and does Lys (orange), though there is reduction in function, likely due to the pH sensitivity of ACMA. cR50H (green) should be protonated at lower pH's suggesting the placement of the positive charge is key in functionality.

3.6 Determining the existence of "Leash-Like" Salt Bridges.

The proton pumping activity of the single point mutation is residues implicated in salt bridge formation between subunit *a* and *c*R50 appear to show that this interaction is not key in driving or regulating movement of the *c* ring. If the salt bridge formation was crucial to function it would be observed that both single subunit *a/c* mutations were comparably non-functional to their double mutant counterparts. And while this is true for the single *c* subunit mutations, the functionality of the single *a* subunit mutations based on the high pK_a of the Arginine residue it can be expected to produce a repulsive positive charge as the assays pH of 7.5. Thus, it appears that the formation of a ionic interaction between the carboxyl groups of subunit *a* and cR50 are nonexistent (figure 12).

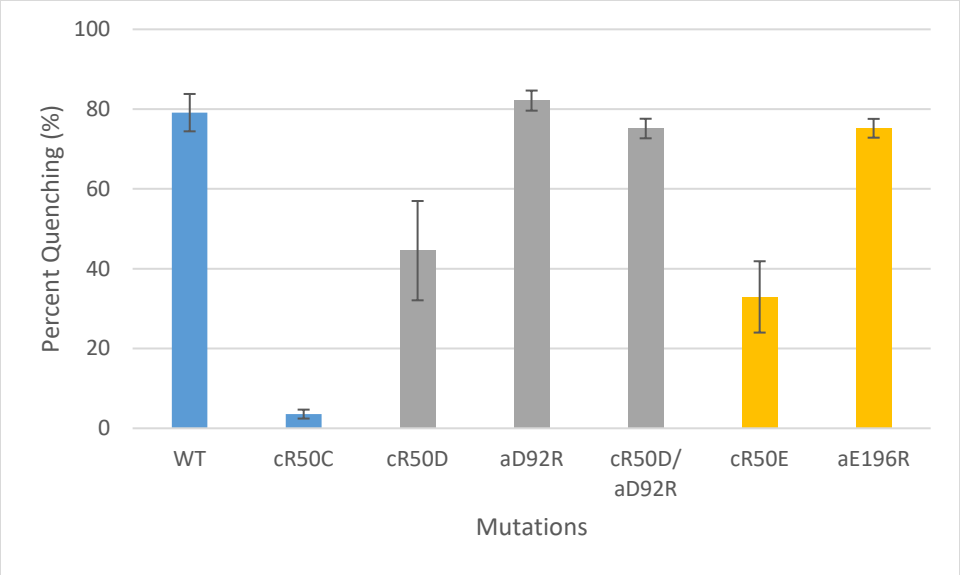

Figure 12: Positive and negative controls WT and R50C respectively are shown in blue. While mutations relevant to each proposed salt bridge are presented in grey for cR50-aD92 interaction and yellow for cR50-aE196 interaction. The percent quenching suggests on all mutants show functionality; so, there is likely necessary ionic interaction between these residues and cR50.

4. Conclusion

The overall conclusion from this research is that thought the Arginine 50 is a highly conserved in the polar loop of the *c* subunit of F-type ATPase's its presence is necessary for functionality of the hydrolysis and synthetic of ATP. After investigating the effects of several mutations, it is clear that the position of the positive charge is highly specific. This locational dependency of a positive charge is best observed by the functionality of the Lys mutant and the diminished functionality of the His mutant. The lack of functionality of the MTSET Cys modification can also be explained by this phenomenon as this modification has an additional three methyl groups which may prevent the charge from being positioned in the correct location. This result was observed in the generation of the mutants which lack positive charge, R50D,E,M and those that provide other H-bonding groups(R50D,E). However, the functionality of cR50A should be further explored to determine if other residues compensate for the lack of charge by taking advantage of increase in "free space" provided by the alanine side chain.

As for the other chemical modifications of cR50C, none of them displayed functionally in the H^+ pumping assay. Additionally, with the variety of genetic mutations which allowed for semi-functionality it is suppressing that no chemical modifications restored function even after DTNB treatment to test for reactivity. However, it is possible that the R50C mutation is binding to a metal which damages or deforms the complex even after removal and reaction with MTS reagents. Therefore, making it appear that chemical modification makes the protein nonfunctional when in reality the lack of function is a relic of sample preparation. Currently we are investigating using a chelating agent to rule out this possibility. Additionally, it is also possible that the presence of the disulfide bridge creating to connect the chemical modification is causing too much steric hindrance or creating a non-optimal chemical environment at when placed at position 50. Both of these hypothesizes are unlikely, but are a possibility when scrutinizing the results of this project.

In terms of the investigation into the interaction of cR50 and aD92 and aE196, the double mutations and pH dependent assay show that salt bridge formation does not occur between these residues. Though the addition of carboxyl groups at position 50 may still allow for hydrogen bonding interactions with aD92 and aE196 in the proposed *a*-*c* leash interaction, the functionality of non-charged mutants would not allow for the formation of this interaction and provide evidence against its existence as a regulatory mechanism. Additionally, the replacement of the subunit *a* carboxyl group with the guanadinium group of Arg would likely cause charge repulsion if these residues interacted with R50; so, a decrease in functionality would be expected but, this is not the case.

Finally, due to a lack of ATP synthesis data for all known mutations it is unclear whether or not the residue at position 50 has any bearing mechanism differentiation from ATP synthesis and hydrolysis. However, succinate plate data shows that all mutants grow to the same size as wild type within the succinate media, indicating that that these mutants should have functionality within ATP synthesis assays. Therefore, if there are alternate mechanisms for *a-c* interactions in synthesis and hydrolysis R50 is not a contributor to their differentiation, answering the question which began this research.

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6. References

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