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Mutations of membrane boundary residues in *E. coli* ATP synthase

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Abstract

ATP synthase is the primary producer of adenosine triphosphate (ATP), a primary energy storage molecule in cells that is used in a variety of cellular processes. ATP synthase functions by using the proton gradient present across the cellular membrane to power a series of conformational changes resulting in the synthesis of ATP. E. coli ATP synthase has two major parts, F_1 and F_0, connected by the gamma stalk. F_0 is membrane embedded, and consists of subunits a, b, and ten copies of subunit c, typically referred to as the c-ring. High energy protons enter through a half channel in subunit a and are passed into the c-ring, which rotates in a way that has protons exit on the low energy side of the membrane through a second half-channel. Recent studies have shown mutations of residues in the half-channels can severely impact the functionality of the synthase itself, making these residues potentially important to structure. Studies have also shown residues Y73 and F76 on the ion channel are potential mutation sites, which has since been confirmed with cystine mutations at Y73 and F76. Valine, serine, and lysine mutants have been made and tested for Y73 to help determine whether size and hydrophobicity are factors in functionality. Results showed size, and potentially hydrogen bonding, to be strong factors in functionality at this site. The next step is to make and test the same set of mutants at F76 and compare results to Y73.

1. Background

ATP synthase is the primary producer of adenosine triphosphate (ATP), the primary source of energy storage in cells. It is present in various forms across almost all forms of life. ATP synthase uses the proton gradient present across cell membranes to power a series of conformational changes that catalyze the condensation of ADP and P_i. *E. coli* ATP synthase units are composed of two major parts, F_1 and F_0. Protons are translocated through the membrane bound F_0 sector, while ATP synthesis occurs in F_1. Since ATP synthase is the primary energy storage producer in cells, disruption of the ATP synthase causes cell death[1] Because of this, it is considered a potential target for new forms of antibiotics. By better understanding the structure and functions of ATP synthase, knowledge of potential targets for drugs can be improved and further refined.

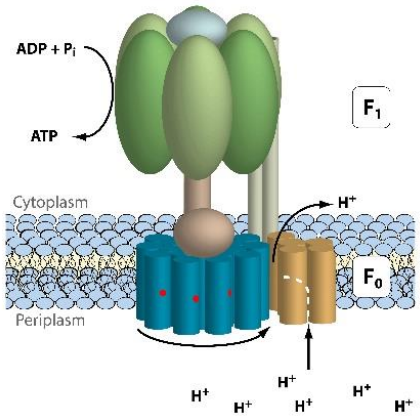

Figure 1. Structure of *E. coli* ATP synthase

E. coli ATP synthase is one of the simplest forms of ATP synthase, and as such is frequently used as a model system for ATP synthase of higher organisms. In *E. coli*, F_0 is membrane bound and composed of subunits a, b, and ten copies of subunit c, which form a ring. During ATP synthesis, a high energy proton enters through the entry half channel in subunit a and is passed to a proton binding site on the c-ring. The c-ring then rotates, taking in new high energy protons. When a proton nearly completes a full rotation, the now low energy proton exits through a second half channel in subunit a to the other side of the membrane. The c-ring is attached to the

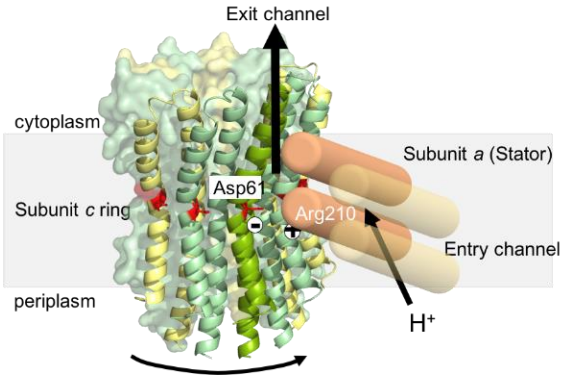

Figure 2. Subunit *a* and the *c*-ring, with entry and exit half-channels depicted.

gamma stalk in such a way that, when the c-ring rotates, the gamma stalk rotates as well, causing the conformational changes in the F_1 subunit necessary to produce ATP at the three catalytic sites.

The purposes behind the residues at the a-c interface is currently unclear. Previous studies have shown the aqueous half channels leading to the c-ring and the respective proton binding sites to be sensitive to inhibition and mutation. [1, 2]. Disruption of the binding site through mutation is particularly viable at the a-c subunit interface, where the conservation of amino acids is particularly high.[3] Current findings show mutations of residues on the ion channels of an ATP synthase can severely impact the functionality of the synthase itself. Different mutations of residues result in different options for proton binding sites and interactions, leading to potential alterations in ion selectivity and loss of function.[3]

Previous studies[4] have determined which residues contribute to forming the aqueous half channels. One of these studies[5] identified positions Y73 and F76 on subunit c as sensitive to mutations due to cystine substitution reducing or eliminating proton pumping activity. Since these two residues are located at the a-c subunit interface, they have the potential to impact interactions between subunit a and the c-ring. However, the potential impact on function of further mutations at those two sites is still unknown.

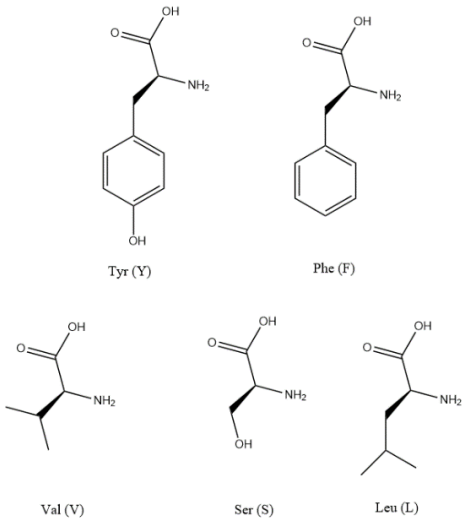

Figure 3. The two original residues (Y73 and F76) and the three major mutations tested.

In order to examine the chemical properties required at these positions, biochemical assays were used to test mutations at Y73 for functionality. Methodology used typically begins with growing the desired mutant stains of E. coli either created by mutagenesis or grown from stock, and then using the mutant cells to create inside-out (ISO) vesicles, which can then be used to run functional assays to determine different effects of the mutant on the enzyme. These assays included ATP-driven proton pumping, ATP synthesis, and H^+ permeability. Based on the results of these assays, the function of the residue can be better determined, as well as what sorts of mutations would be most likely to affect that site. Since other work on residues in a similar position on the other side of the membrane (F54 and I55) showed that these positions are most impacted by size and hydrophobicity, we hypothesize that these residues will behave similarly. The mutation of sites Y73 and F76 with cystine in order to test function impact, and then Y73 with valine, serine, and lysine in order to test the impact of size and hydrophobicity will allow for a better understanding of the importance of that location and what aspects are most vital along that membrane boundary.

2. Methodology

2.1 Preparation of ISO Vesicles

E. coli mutants stored at -80℃ were grown overnight at 37 ℃ in 5 mL of LB with 100 µg/mL ampicillin added. The small culture was then used to inoculate 1 liter of LB with ampicillin and incubated for 8 hours at 37℃ with shaking. The cells were then harvested by centrifugation for 15 minutes at 4000 rpm. The pellet was then resuspended in TMDG buffer (50 mM Tris-HCl, 5 mM MgCl2, 1 mM DTT, 1mM PMSF, 10% v/v glycerol, pH 7.5) and lysed at 18000 psi in an Avestin B15 homogenizer. Lysate was cleared by centrifuging for 10 minutes at 9000X g. The supernatant was then decanted into ultracentrifuge tubes and centrifuged at 193,000X g for 60 minutes. The pellet was resuspended in 3 mL of TMG buffer (50 mM Tris-HCl pH 7.5, 2 mM MgCl2, 10% glycerol) using a dounce homogenizer. The protein concentration in the vesicles was determined by a modified Lowry assay.[6]

2.2 ATP Driven Proton Pumping

Proton pumping activity was measured by fluorescence spectroscopy. Membrane vesicles were diluted to 10 mg/mL with TMG and 160 microliters of the diluted vesicles were added to 3.2 mL of HMK buffer (50 mM HEPES-KOH pH 7.5, 2 mM $MgCl_2$, 300 mM KCl) and 8 microliters of 1.2 mg/mL ACMA (9-Amino-6-Chloro-2-Methoxyacridine) fluorescent dye (λ_{ex} = 415 nm, λ_{em} = 485 nm). Thirty microliters of 25 mM ATP were added 20 seconds after beginning the assay to initiate pumping, and 8 microliters of 0.1 mg/mL nigericin were added 100 seconds after beginning the assay to halt pumping. Recording was ended at 120 seconds.

2.3 ATP Synthesis

ATP synthesis activity was measured by luciferin/luciferase reporter assay.[6] A white microplate was prepared with each used well containing reaction buffer. The conditions were 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, and 100 ng luciferase. Membrane vesicles were prepared in two micro tubes, each containing reaction buffer and 50 micrograms of ISO vesicles. Background luminescence of the plate without added vesicles was taken, then ISO vesicles were pre-energized with 2.5 mM NADH before adding to the wells. Plate luminescence was then read at 25 second intervals for 5 minutes. Initial rate (luminescence/second) was calculated from the slope of the linear portion of the graph.

2.4 Proton Permeability

Proton permeability activity was measured by fluorescence spectroscopy. Membrane vesicles were diluted to 10 mg/mL with TMG and 160 microliters of the diluted vesicles were added to 3.2 mL of HMK buffer (50 mM HEPES-KOH pH 7.5, 2 mM MgCl2, 300 mM KC) and 8 microliters of diluted ACMA (9-Amino-6-Chloro-2-Methoxyacridine) fluorescent dye (λ_{ex} = 415 nm, λ_{em} = 485 nm). 16 microliters of NADH were added 20 seconds after beginning the assay to initiate pumping, and 8 microliters of 0.1 mg/mL nigericin were added 100 seconds after beginning the assay to halt pumping. Recording was ended at 120 seconds.

2.5 Succinate Growth

Strains were streaked out and incubated at 37ºC overnight. Minimal glucose medium was then prepared (10X M63 buffer, 10X M63 salts, LB, 20% glucose, 1000X TIV) before adding ampicillin to all tubes except the negative control. All tubes were then inoculated and incubated overnight at 37ºC. Glucose (10X M63 buffer, 10X M63 salts, LB, 20% glucose, 1000X TIV) and succinate (10X M63 buffer, 10X M63 salts, LB, 20% succinate, 1000X TIV) mediums were then made and ampicillin added before distributing onto a clear 96-well plate. The plate was held at 37°C with shaking and OD_{550} was read every 30 min for 12 hours, then every 2 hours for 24 hours. Growth curves would then be plotted and compared for maximums.

2.6 Western Blot

Membrane vesicles were diluted to 10 mg/mL with TMG before adding 1 volume of Laemmli buffer and running on an SDS-PAGE gel with non-mutated variant and protein markers. Protein in the gel was then transferred onto PVDF membrane by wet transfer in cold Towbin buffer at 70 V for 90 minutes. The membrane was immersed in 5% dry milk in TBST buffer (Tris-buffered saline with 0.1% Tween-20) and incubated for 1 hour at room temperature with gentle shaking. The membrane was then rinsed with TBST and incubated 45 min with rabbit anti-His_6 monoclonal antibody (BioVision). It was then rinsed twice for 10 seconds and then twice for 8 minutes with TBST buffer. The membrane was then incubated 45 minutes with HRP-conjugated donkey anti-rabbit antibody (Promega). It was then rinsed twice for 10 seconds and then twice for 8 minutes with TBST buffer. The membrane was then placed onto plastic wrap and BCIP/NBT (Southern Biotech) was added to just cover the surface. Bands were allowed to develop for a few minutes, then the membrane was removed from the solution before being placed on a white background and photographed.[8]

3. Results

Cystine mutations were first tested at both sites, as previous papers[3] used cystine mutations to determine which residues were required for functionality in mapping the proton pathways present in subunit a. Once loss of function was confirmed at both sites with cystine mutants present, valine, serine, and leucine mutants were made to test required chemical properties at site Y73. Both sites used residues with aromatic rings, and residues along the membrane had previously[5] been shown to require steric bulk to maintain function, so the proposed mutants were intended to test gradually increasing size without including the aromatic ring in order to confirm or deny the importance of the greater bulk.

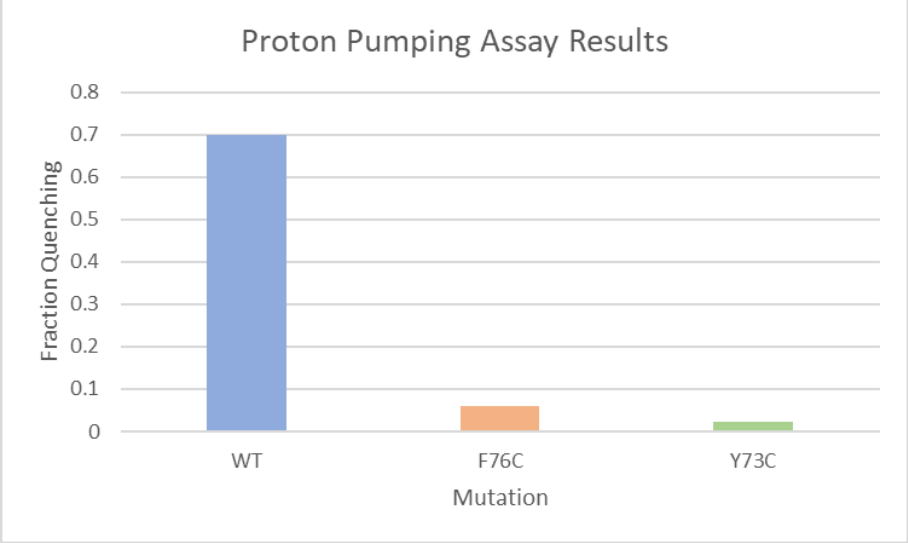

Figure 4: Proton pumping activity of mutant ISO vesicles expressed as percent quenching of ACMA fluorescence.

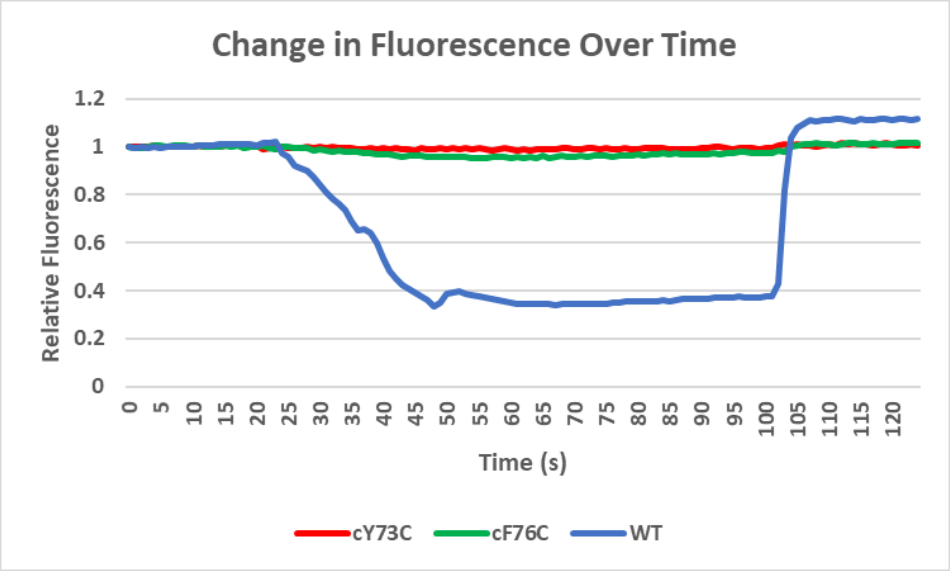

Figure 5: Representative fluorescence traces showing proton pumping activity. ATP was added at 20s and nigericin was added at 100s. Change in fluorescence of the mutants and the wild type over the observed period.

Figure 4 shows the results of the proton pumping assay, comparing the two cystine mutants to the wild type. The wild type is shown with a relatively high percent quenching (70%) as compared to the cystine mutants (2% and 6%

respectively), showing the cystine mutants to have much lower overall activity than the wild type. Figure 5 shows the proton pumping assay results across the full 120 seconds, illustrating the relative changes in fluorescence manifestation across all tested options.

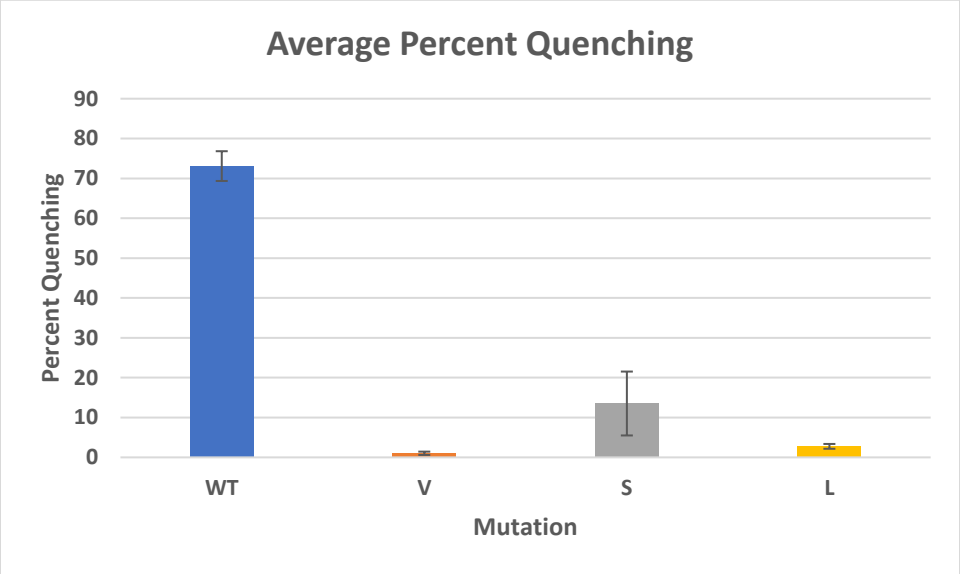

Figure 6: Percent quenching of the three tested Y73 mutations as compared to the wild type.

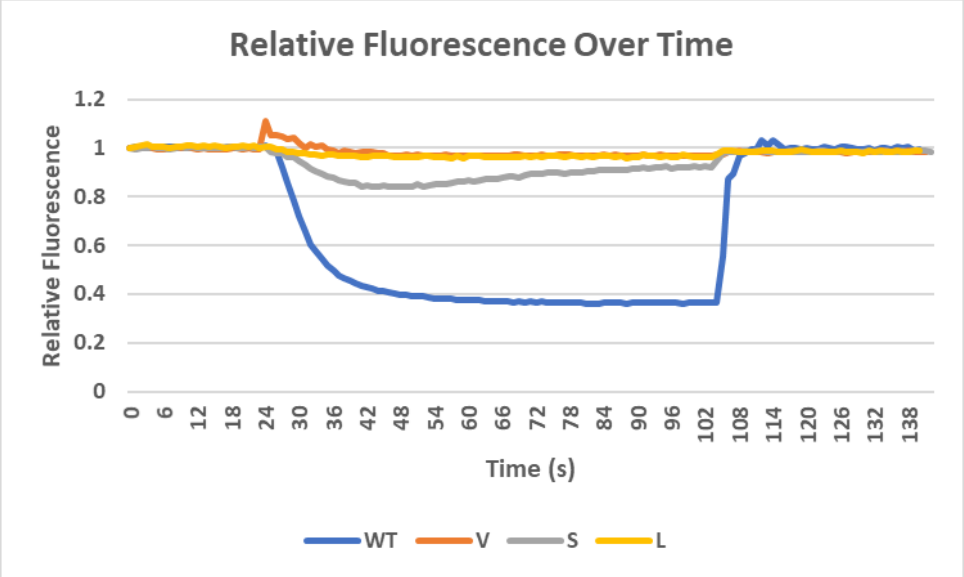

Figure 7: Change in fluorescence of the mutants and the wild type over the observed period.

The three mutants tested at Y73 are shown to have considerably lower quenching in comparison to the wild type, indicating a lesser presence of proton pumping (Figure 6, 7), though the serine mutant is shown to have greater but not full functionality as compared to the wild type and other mutants.

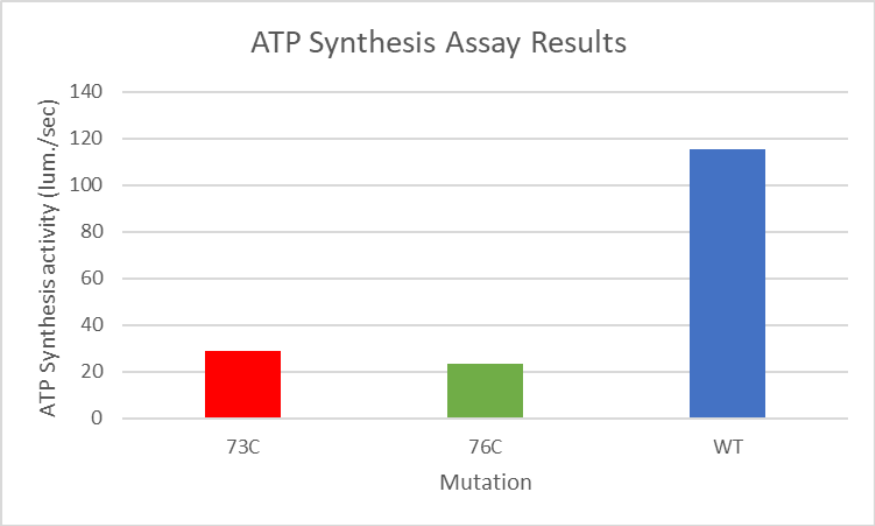

Figure 8: Average initial rate of ATP synthesis expressed as luminescence change per second.

Figure 8 shows the results of the ATP synthesis assay comparing the rates of luminescence production of the cystine mutants to the wild type. The wild type is shown to have a considerably higher rate (115lum/sec) compared to the rates for the cysteine mutants (28 lum/sec and 23 lum/sec, respectively). Which indicates the interface is being affected by these mutations in both directions of proton pumping.

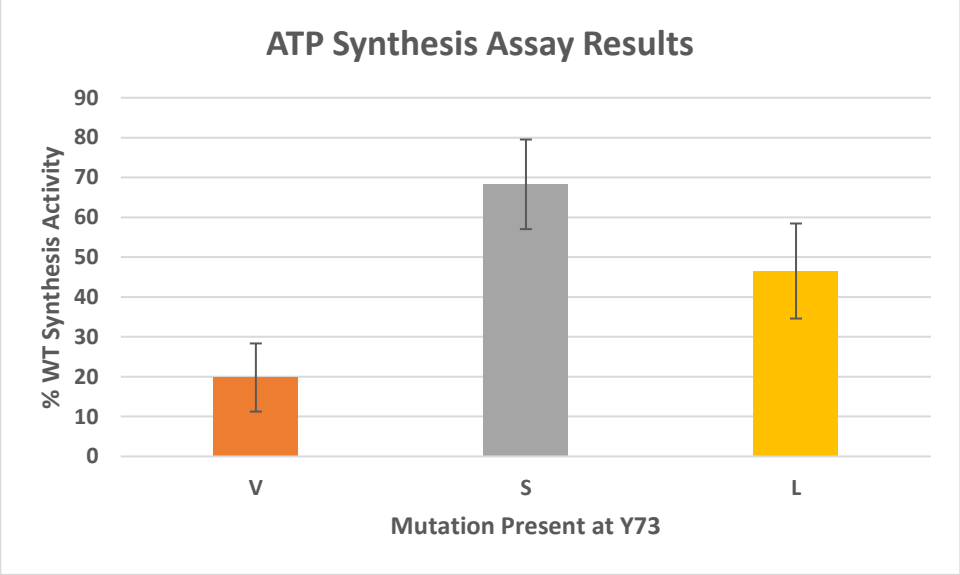

Figure 9: Average change in luminescence of tested mutants as normalized to the wild type.

Similarly, Figure 9 shows higher rates of proton pumping among the three tested mutants in the opposite direction, but still lower than seen in the wild type. The serine mutant was highest, but it still did not reach above 70% function in comparison to the wild type. This is still higher than the proton pumping results, but there is still enough loss of functionality in comparison to the wild type for it to be clear the mutations are affecting function.

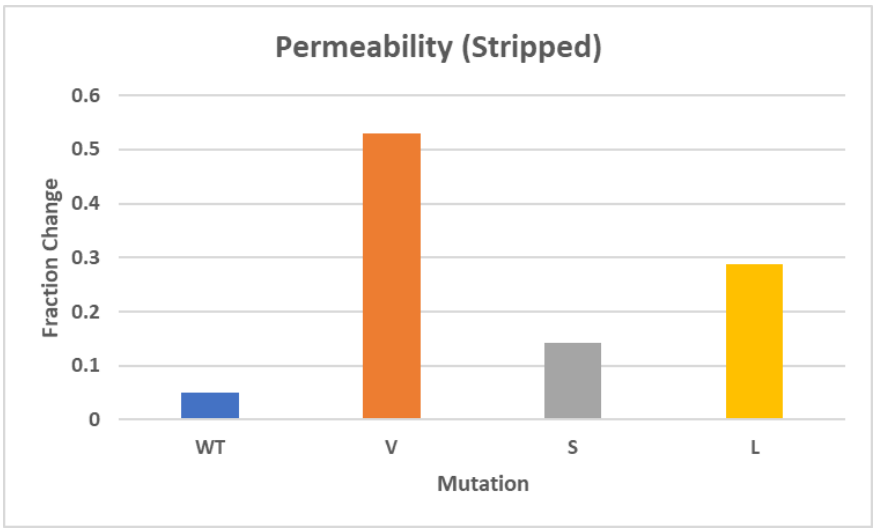

Figure 10: Percent change in luminescence as compared to the wild type in stripped ISO vesicles. Note: higher percent quenching indicates less permeability

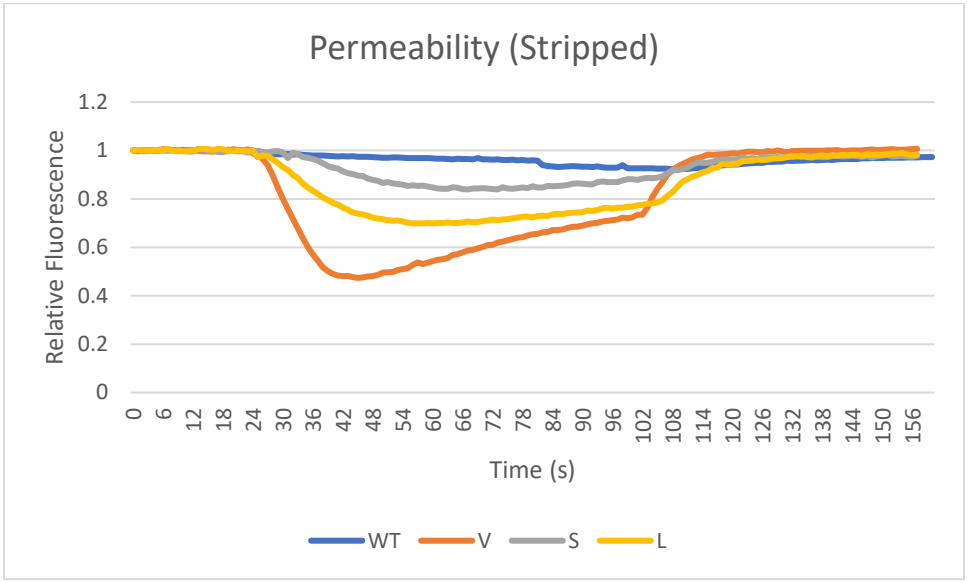

Figure 11: Change in fluorescence of the stripped mutated vesicles as compared to the wild type over a given period of time.

The results from figures 10 and 11 show the Y73S mutant continues to be considerably closer to the WT standard in term of florescence, while Y73L and Y73V are nearly the inverse, indicating very high concentrations of protons in Y73V, and that they are being blocked from exiting as those present in the wild type do, absent the F_1 subunit.

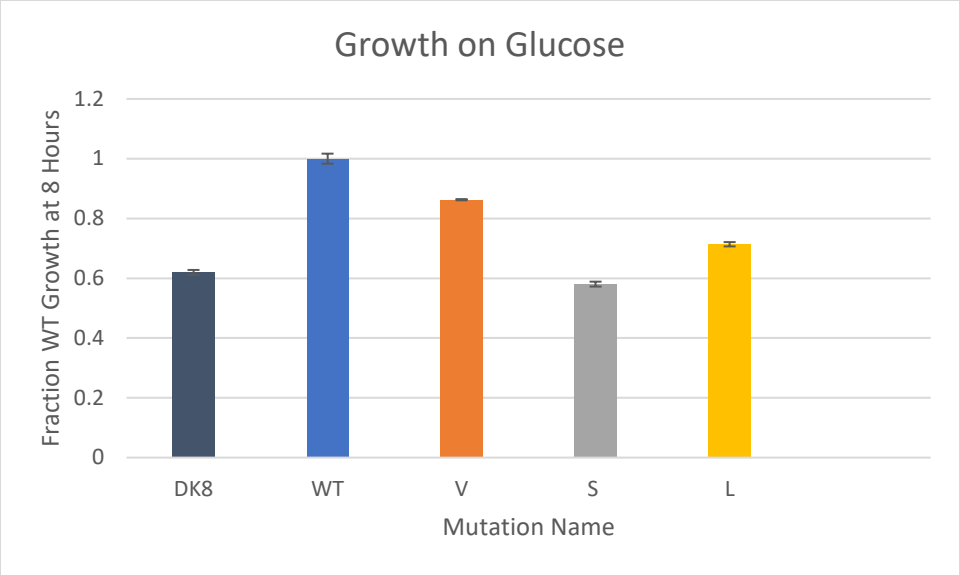

Figure 12: Growth of the three tested mutations on glucose based media as compared to wild type and DK8 cells.

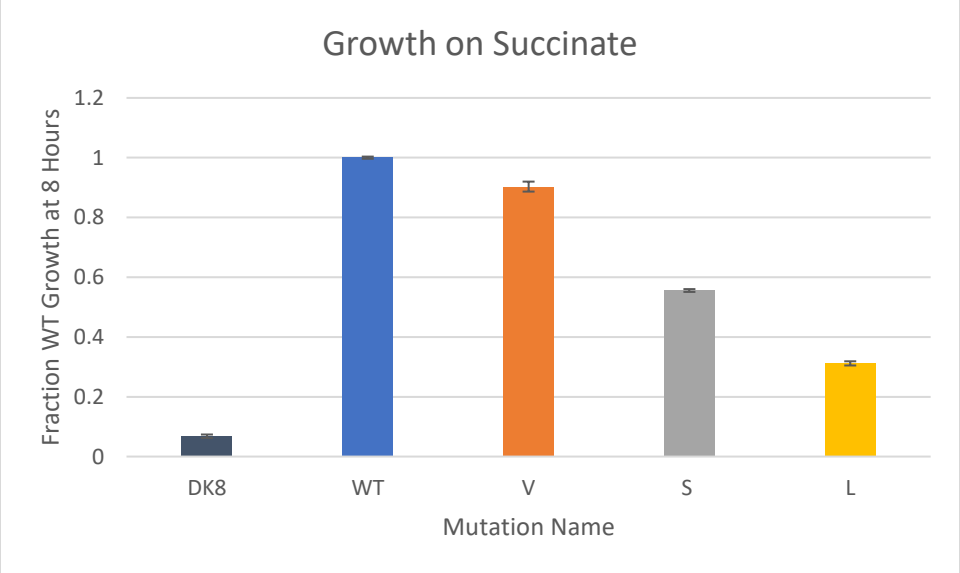

Figure 13: Growth of the three tested mutations on succinate-based media as compared to wild type and DK8 cells.

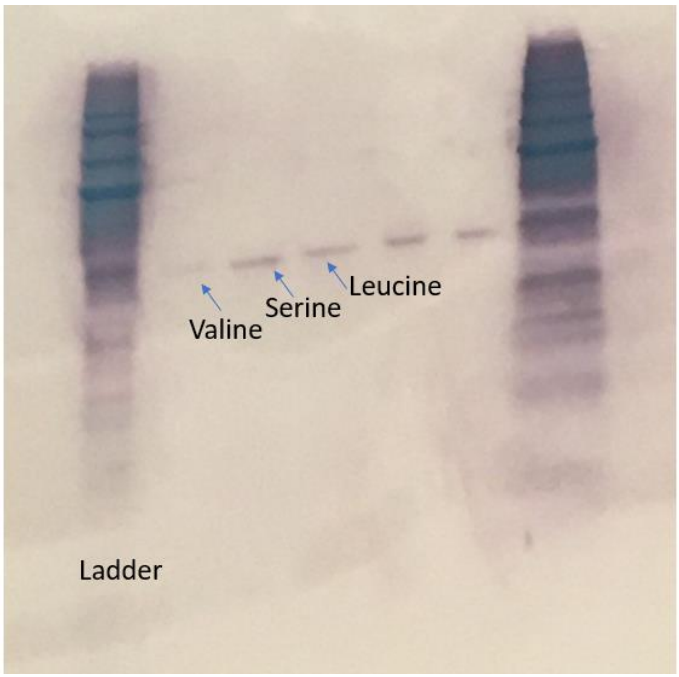

Figure 14: Results of Western Blot assay on the three tested mutants.

The results from figures 12, 13, and 14 show the mutations have been properly inserted into the membrane and still allow growth of the cell. Figures 12 and 13 show the results of the growth assay, with the wild type as the positive control and DK-8 as the negative control on succinate. On both media, the mutated bacteria grew, and while that growth was consistently lessened in comparison to the wild type, it was still to a greater degree than the DK-8. Figure 14 shows the results of the western blot assay, with the marked mutants being compared to wild type. The tagged subunit shows up clearly for all of the mutants as well as the wild type, indicating the desired insertion into the membrane to be present in both.

4. Conclusion

The objective was to test site Y73 for important chemical properties, with the hypothesis that steric bulk, specifically in the form of an aromatic ring, was important to function. Cystine, valine, and leucine were all tested at that location and confirmed that smaller hydrophobic groups could not replace Y73. The serine mutation, on the other hand, maintained a higher level of function than anticipated by size alone in proton pumping and ATP synthesis assays, which suggests hydrogen bonding may be more important at site Y73 than previously expected.

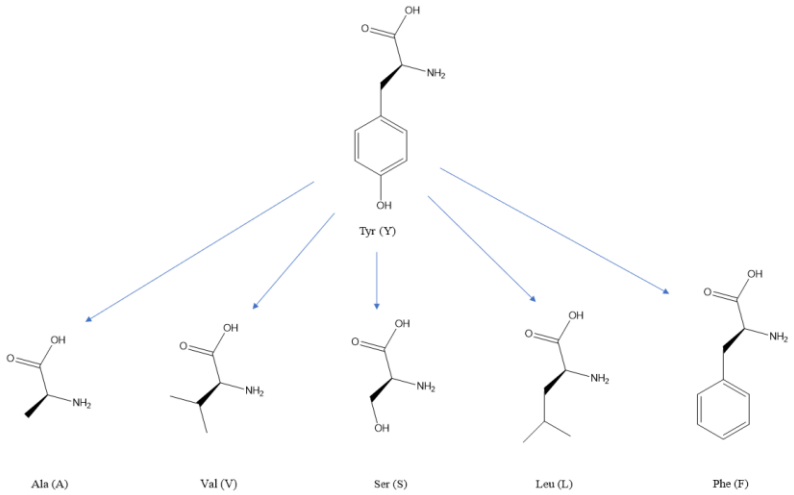

Fig. 12: Y73 and the full range of mutations intended to be tested there. V, S, and L have already been examined, while A and F will be tested in the future.

The next step is to test the same mutants at F76 to see how results are impacted at a site which bears similar steric bult to Y73, but lacks the potential hydrogen bonding capabilities. By carrying this out, it will be easier to understand how these residues function within the enzyme, as well as further general knowledge about the enzyme itself. Since the two residues being considered are periplasmic, they are likely to behave similarly to residues in a similar position on the membrane boundary, which frequently include aromatic side chains, which are thought to identify the placement of the residue in the membrane[5]. Alanine mutants will also be tested at both sites, to further understand how a range of sizes can impact function at the sites. The alanine mutants are expected to perform with less functionality than valine, based on size and the lack of hydrogen bonding capability. Phenylalanine will also be tested at site Y73 in order to see how a mutation with similar bulk, but lacking the hydrogen bonding capabilities will affect functionality at the site. These mutations have begun, with several in the process of preparation currently.

5. Acknowledgements

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6. References

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