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Development of a Reconstitution Protocol for ATP Synthase with Various Lipids

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Abstract

H^+ transporting ATP synthase is a remarkable molecular machine that catalyzes the synthesis of ATP by use of two coupled motors, F_0 and F_1. The use of the H^+ electrochemical gradient drives rotation of the *c* ring, which couples with the F_1 domain to induce conformational changes that catalyze the formation of ATP form ADP and inorganic phosphate. The F_O section of ATP synthase is primarily embedded in the membrane, so it is possible that lipid contacts and composition could play a role in proton translocation and function. Cardiolipin, a phospholipid that comprises a significant portion of the inner mitochondrial membrane, has been observed using cryo-electron microscopy.[8] These lipid effects could be essential for normal function. A protocol for reconstitution of *E. coli ATP* synthase has been developed, with emphasis on different techniques for detergent removal and liposome recollection.

1. Introduction

ATP synthase is a molecular motor that is able to utilize an electrochemical gradient, usually consisting of H^+ ions, to generate ATP molecules from ADP and phosphate. ATP is a ubiquitous energy molecule used by every living cell and is important in providing energy for unfavorable reactions that cells require like synthesis or translocation of resources. ATP synthase is comprised of two coupled motors, one of which is found in the F_0 sector, and another found in the F_1 sector of the enzyme. The F_0 section is comprised of subunits *a*, *b*, and the *c* ring. The F_1 sector contains subunits $\alpha, \beta, \gamma, \delta,$ and ε. This enzyme can utilize a proton electrochemical gradient to drive the rotation of the *c* ring within F_0, which then couples exquisitely with rotation in the F_1 domain. The torque from this rotation is then used by the F_1 part of ATP synthase to generate ATP by causing conformational changes that catalyze ATP's formation[5]. This conversion of chemical and electrical potential to physical motion and back to chemical energy in the form of ATP makes ATP synthase very important in providing our cells with the energy necessary for daily functions as well as having a distinct molecular machine mechanism. The function of ATP synthase is so highly conserved that it is the primary source of ATP, and therefore metabolic energy, in all organisms.

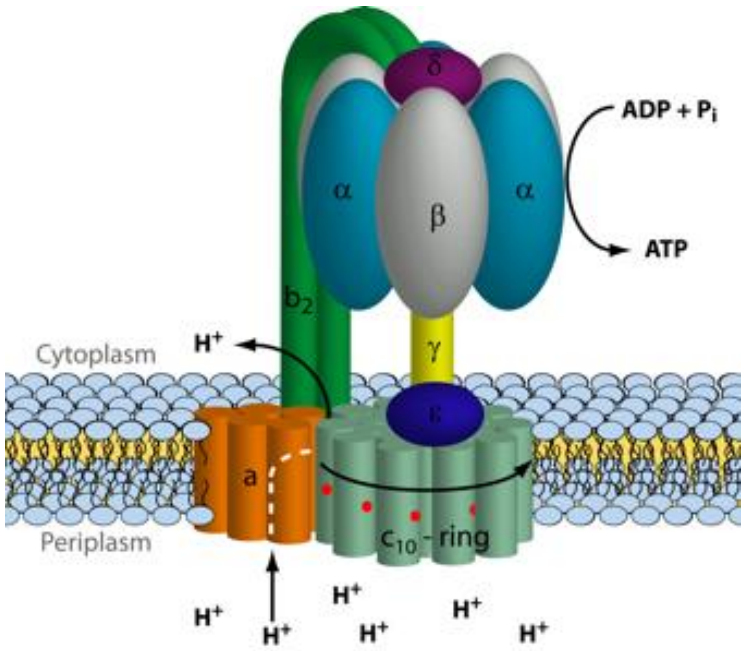

Figure 1 ATP Synthase structure and subunit labeling

The F_0 portion of ATP synthase is comprised of subunits *a*, *b* and *c* with one *a* subunit, 2 *b* subunits, and a varying number of *c* subunits (8-15) depending on the organism the ATP synthase originated from. The channel where protons are passed through the enzyme is found within the *a* subunit, and this is where protons are moved from the periplasmic side of the membrane to the cytoplasmic side down their electrochemical gradient. Four of the five helices of subunit a form a bundle, which assist in half channeling the proton to the *c* ring.[7] This proton is then transferred to a *c* subunit and is rotated fully around the *c* ring before being released via a half channel to the cytoplasmic side. The residues of Arg-210 and Asp-61, which normally form a salt bridge, break and reform to allow movement of the proton and the subsequent rotation of the *c* ring.

Lipid bilayer composition is thought to alter membrane protein functionality, but more research is required before definitive claims can be made. For example, Ca^{+2} ATPase has two states, E1 and E2, which seem to be favored with different lipid composition and even has differing activities based on the lipid environment it is reconstituted in.[3] Previous research has also concluded that head group structure of lipids when altered can lead to changes in the reconstituted protein of LmrP, a membrane bound transporter.[2] This was mainly seen when comparing head groups that had the ability to hydrogen bond to head groups that had been methylated. They were also able to locate a critical Asp-68 which, when mutated, caused changes to the protein as if it were in a membrane with methylated head groups. These results indicate that the Asp-68 and the hydrogen bonding lipid head groups interact to sense proton gradients that are crucial to this membrane transporters function.

This mechanism for proton sensing could be used by ATP synthase since it is also a membrane bound protein that requires the presence of a proton gradient for its function. Since F_0 is primarily embedded in the membrane it is thought that it could be most susceptible to influences by lipid contacts. Additionally, cardiolipin residues have been reported near subunits *a* and *b*, which could be used to trap protons near the membrane and allow for proton translocation into the half channels. Since cardiolipin is found almost entirely within the mitochondrial and *E. coli* inner membrane and is known to trap protons, the influence it has on the function of ATP synthase could be essential. This research focuses on the reconstitution of the F_1 F_0 complex into artificial membranes with differing lipid composition and concentration to determine possible lipid effects on function. The primarily focus will be on the effects of phosphatidylethanolamine and phosphatidylcholine since phosphatidylethanolamine is found in *E. coli* membrane naturally, and phosphatidylcholine is commonly used to create artificial membranes.[2]

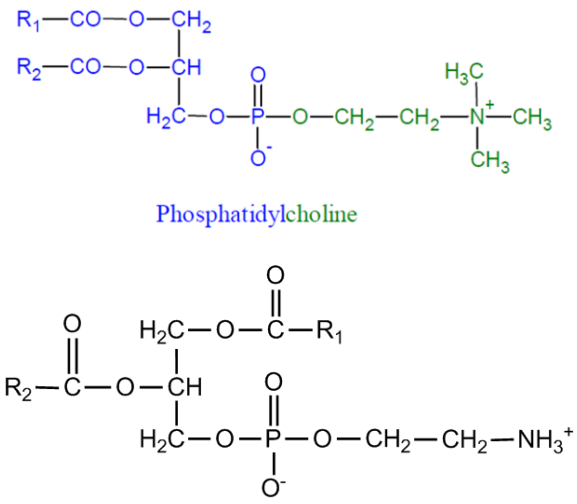

Figure 2 Structure of phosphatidylcholine and phosphatidylethanolamine, respectively

2. Experimental

Wild type *E. coli* cells were grown and harvested by centrifugation at 193,000 x g and made into inside out membranes with six passes through a high-pressure homogenizer at 20,000 psi and stored at -80 °C. F_1 F_0 was then purified from vesicles by affinity chromatography with a nickel column and imidazole wash. Cells were engineered with poly histidine sequence on the β subunit which allows for nickel binding. Samples of all elution fractions were run on SDS-PAGE protein gels and stained with 5:5:1 water: methanol: acetic acid and 0.25% (W/V) Coomassie dye. Gels confirmed collection of F_1 F_0 and allowed screening for purity of the purification process. The most concentrated two samples from both gels were combined and saved at -80 °C. Aliquots of purified F_1 F_0 were tested for protein concentration using a modified Lowry assay.[9] After concentration was established purified aliquots were tested for functionality and completeness of the protein complex with a modified ATPase assay.[10]

Unilamellar vesicles of phosphatidylcholine were prepared by dissolving 30 mg of lipids in minimal amounts of chloroform. The chloroform was then evaporated under nitrogen and lipids were left overnight in a desiccator. Lipids were then resuspended in TMN buffer (50 mM Tris, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.5 pH) with 0.25 mM pyridine to a total volume of 3 mL in a 45° C water bath. The suspension was then homogenized six times at 20,000 psi to produce unilamellar vesicles.

To reconstitute F_1 F_0 in vesicles, mixtures of lipids, protein, and detergent were made using the following parameters: 20:1 Lipid: protein(w/w), 4:1 Lipid: Detergent (mol/mol), final detergent concentration of 1.5 mM, with final volume no more than 600 µL. Both DDM and sodium cholate were used as detergents. DDM is commonly used in protein purification procedures and sodium cholate is used for reconstitution. The reconstitution mixtures were rocked at 4° C for at least 30 minutes. Several techniques were attempted to remove or lower detergent concentration.

3. Results

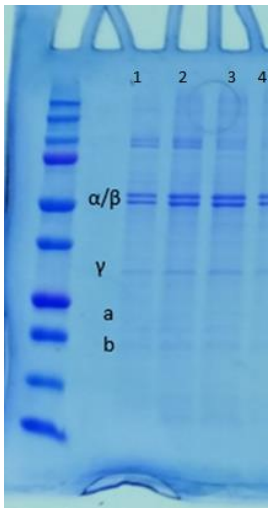

Figure 3 SDS-PAGE gel showing many protein subunits of F_1 F_0, only lanes 2 and 3 were saved to attain highest concentration

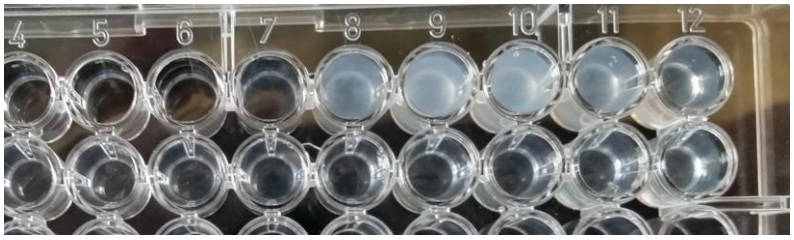

Figure 4 shows elution fractions of G-50 size exclusion column, fractions 9 and 10 collected

Figure 5 example of G-50 spin column used

3.1 G-50 Column

A prepacked Sephadex G-50 column was used to collect liposomes. Turbid fractions, shown in figure 4, were collected and tested for protein on SDS-PAGE pre-cast protein gels and stained with 5:5:1 water: methanol: acetic acid and 0.25% (W/V) Coomassie dye. G-50 was also used in a spin column, as seen in figure 5, with a swinging bucket rotor. G-50 was added and spun at 100 x g for 2 minutes to remove buffer. Liposomes were then added, and the column was spun again, this time collecting the flowthru.

3.2 Adsorbent Beads

Amberlite XAD-2 adsorbent beads were also used to remove detergent from liposomes. The reconstitution mixtures were diluted to 5 mL and then 80 mg of beads per mL of the original reconstitution mixture were added and left rocking at 4° C overnight. Beads were then filtered out and the solution was spun at 100,000 rpm for 30 minutes. Supernatant was poured off and liposomes were resuspended in 100 µL of TMN buffer. Liposomes were also collected by diluting the mixture to 5 mL and then spinning solution at 100,000 rpm for 30 minutes. Liposomes were collected by centrifugation and resuspended in 100 µL of buffer.

According to figure 2, purification was a success, and aliquots were tested for activity and F_1 F_0 coupling. Average protein concentration was found to be 2.6 mg/mL. Recollected liposomes were run on protein gels to confirm protein insertion, despite the usage of a variety of techniques described in the literature, protein was not seen in the liposomes.

4. Conclusion

Reconstitution of F_1 F_0 in vesicles was unsuccessful. The protein could be coming off the column separately, which means it could be found in other fractions. However, insertion into the liposomes is not occurring. This could be due to low levels of F_0, or the type of detergent used in the reconstitution mixture. The latter is unlikely since both detergent choices did not yield proteoliposomes. The former, however, could be investigated by improving the purification procedure to increase F_0 concentration. These conclusions will be further investigated to better understand the effects of lipid environment on *E. coli* ATP synthase.

5. Acknowledgements

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6. References

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