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Structural Dynamics within Escherichia coli's F_1F_o ATP Synthase Complex

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Abstract

The F_1F_o adenosine triphosphate (ATP) synthase complex is a membrane-bound protein found in all domains of life that facilitates energy transduction through a fascinating rotary mechanism. ATP synthase has two motors, the F_1 head, in which rotation of the central stalk catalyzes ATP synthesis, and the membrane-bound F_o motor complex, in which the electrochemical proton gradient drives *c*-ring rotation by proton movement through the subunit *a stator*. ATP hydrolysis can also drive proton pumping in the reverse direction. Recently, cryo-electron microscopy images have revealed the structure of the proton pathway and captured aspects of rotation and elasticity within the complex, but the mechanism of proton-driven rotation of the *c*-ring is not fully understood. Site-directed spin labeling coupled with electron paramagnetic resonance spectroscopy can reveal conformational changes within proteins. To observe structural dynamics of the subunit *a* stator in F_o, and the potential effect of the lipid environment, ATP synthase was purified in native-like environments using amphipathic polymer nanodiscs or reconstitution into proteoliposomes. Mobility of spin labels at several positions and under various biochemical conditions reveal differences in backbone dynamics in the two lipid environments.

1. Introduction

The F_1F_o Adenosine Triphosphate (ATP) synthase complex is an essential protein system found in all domains such as eukaryotic, bacterial, and archaea cells.[1] ATP synthase uses the electrochemical gradient as a source of protons to produce chemical energy for all cells. It can also use chemical energy, ATP, through hydrolysis to pump H^+ which only occurs in bacterial cells.[1] This fundamental protein is a critical biological process that has been studied for decades throughout the fields of biology, chemistry, and biochemistry, however there are mechanistic proprieties to understand of ATP synthase. Therefore, understanding the basics of ATP Synthases' structure, mechanism, and synthesis will be a huge attribute to the expansion of the fundamental knowledge of living cells and will facilitate drug discovery since the protein is associated with cell death.

ATP synthase can be found embedded in the cell membrane of bacteria and the inner mitochondrial membrane of eukaryotic cells. The protein consists of two major structural components--the F_1 head and F_o complex, where the F_o complex is the component that is membrane bound within the cells.[1,2] Both components are found in eukaryotic and bacterial cells, and these components are made of multiple subunits, but these subunits differ in mitochondrial and bacterial cells.[1] Throughout this paper, the focus will be mainly on the bacterial F_1F_o ATP synthase complex. Furthermore, for simplicity and consistency, a general overview of the F_1 head and F_o complex will be discussed first, then bacterial ATP synthase's subunits, and finally what the subunits functions are.

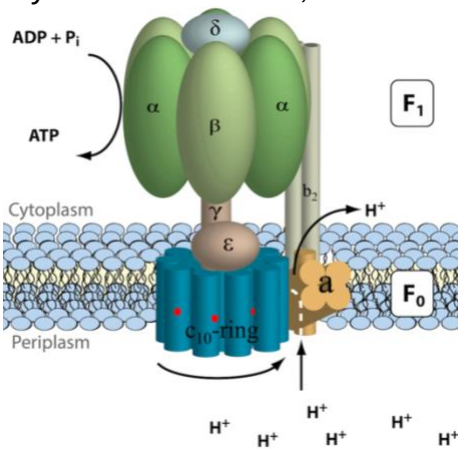

Figure 1. Schematic representation of subunits of ATP synthase.
Courtesy of Steed Lab.

The F_1 head is where the catalysis of ATP synthesis/hydrolysis occurs, and F_o is two proton half-channels which is a H^+-driven reaction that drives the synthesis of ATP. In *Escherichia coli*'s (*E*. c*oli*) ATP synthase complex F_1 head, which is seen in all other bacterial and prokaryotic cells, has three α (alpha) subunits, three β (beta) subunits, one ε (epsilon) subunit, and one γ (gamma) subunit.[2] The F_o complex of *E*. *coli* consists of one *a* subunit, two b_2 subunits, and one δ (delta) subunit. Note that *E*. *coli*'s ATP synthase lacks some of the subunits in mitochondrial ATP synthase where some of these subunits are the same, but only with a different label. For example, the oligomycin sensitivity conferral protein (OSCP) subunit in yeast's F_o complex is the same as the δ subunit in *E*. *coli*, and the δ subunit in yeast's F_1 head is the same as the ε subunit in *E*. *coli*.[2]

Main Components	Yeast / Mitochondrial	*E*. *coli* / Bacterial
F_o complex	• *a* subunit • two *b* subunits • *c*-ring, 10 *c* subunits	1. one *a* subunit 2. two b_2 subunits 3. *c*-ring, 10-15 *c* subunits

	• one *d*, *h*, and OSCP subunit	
F_1 head	• three α subunits • three β subunits • one δ, ε, and γ subunit	4. three α subunits 5. three β subunits 6. one ε and γ subunit 7. one δ subunit

Table 1. F_1F_o ATP synthase complex's subunit in yeast and *E*. *coli*

Now that we have established what all subunits are in bacterial and mitochondrial ATP synthase complexes, we are switching the focus solely on the ATP synthase complex of *E*. *coli* as we discuss the functions of what they can do. The subunits of the protein have an important role to play, but it is easier to sum up their three major components: F_1 head, the stator, and the rotor.[1,2] One of the three components is the F_1 head is made up the hexameric $\alpha_3\beta_3$ unit and this headpiece is where the catalysis of ATP occurs from the reaction of adenosine diphosphate (ADP) and a free inorganic phosphate (P_i). The second component is the rotor which is made up of the *c*-ring and central stalk (gamma, γ), and it rotates as protons are flowing in or out from the concentration gradient. The last component is the stator which helps stabilize the protein from rotation of the F_1 head from the rotor of ATP synthase.

Proton flow arrangement and structure define the function of the ATP synthase complex. ATP synthase's two modes of mechanism are hydrolysis and synthesis. The synthesis of ATP occurs as protons come flowing in from the higher concentration of H^+ outside of the cellular membrane to the lower concertation inside of the phospholipid bilayer, so these protons are carried through the *c*-ring and are inside a c subunit that binds to one proton.[1] For every ~3.3 H^+ pumped for a *c*-ring with ten c subunits, the ring will turn counterclockwise which builds up tension in the central stalk. As the central stalk builds up this tension, it then makes the F_1 head turn about 120° and because of this ADP binds with P_i creating ATP.[2] Therefore, for an ATP synthase complex with a c_{10}-ring, the complex is able to create about three ATPs with a full 360° rotation in the counterclockwise direction of the F_1 head. While hydrolysis of the protein is done by the use of ATP as its driving force. Hydrolysis only occurs in bacterial cells such as *E*. *coli* and uses water molecules to break off one of ATP's phosphate groups where chemical energy is created from the group breaking off and producing ADP. This energy is used to pump protons out from the higher concentration of H^+ that is inside the cell by turning the *c*-ring clockwise.

Currently, there is a large amount of information known about the mechanism of the ATP synthase complex, however, there are some discoveries the biochemistry field has yet to make. In Steed Lab, there are currently three main projects being done. These projects are: mutational analysis, structural dynamics, and a collaboration with Wolfe Lab on *Pseudomonas aeruginosa*, and the main focus will be on the structural dynamics of

ATP synthase. The main tools used in the structural dynamics project is the powerful biophysical tool, cryo-electron microscopy (cryo-EM), spin-labeling and electron paramagnetic resonance (EPR) spectroscopy. Cryo-EM is a technique widely known in the biochemical field as it generates high resolution images of biological macromolecules and proteins.[3] EPR spectroscopy and spin-labeling provides valuable structural and dynamic information about a wide variety of biological systems by the electron spin of excited electrons from microwaves.[4]

Cryo-EM has been used on ATP synthase to analyze the mechanism, structural dynamics, and conformational changes occurring within the protein. We are actively researching the structural dynamics that may be occurring in the *c*-ring and subunit *a* stator as well as its possible conformational changes. The structural dynamics and conformational changes will be confirmed in the subunit *a* stator via EPR spectroscopy. Although, it is important to note that there are other projects, experiments, approaches/methods, and research that have already been published about the structural dynamics of ATP synthase. This literature is vital as more research will be conducted on understanding the structural dynamics of ATP synthase, now we will discuss the most recent findings that are relevant to the structural dynamics project.

Recently, there have been images and analyses of the protein from the use of cryo-EM and EPR. The most recent high-resolution images of *E*. *coli*'s ATP synthase from cryo-EM have been able to confirm that there are some sort of rotational aspects and elasticity occurring in which the asymmetrical protein complex is able to accommodate such mismatch within its asymmetry of the F_1 headpiece and *c*-ring as the number of protons being pumped through synthesis does not match with the same rotational value of the hexameric $\alpha_3\beta_3$ unit.[3] Another study that was done about a decade ago came to the conclusion that "The inhibition is consistent with the proposed requirement for TMH [transmembrane helical] movements during the gating of periplasmic H^+ access to *c*Asp61 [a Cysteine (Cys) group in the subunit *a* stator]…subunit *a* stator have been implicated in gating H^+ release to the cytoplasm, and previous cross-linking experiments suggest that the chemically reactive regions of the loops may pack as a single domain…" and that there is motion between the TMH and is vital to the function of ATP synthase.[5] These results are promising and show us that there is another reason to continue research on such an essential protein.

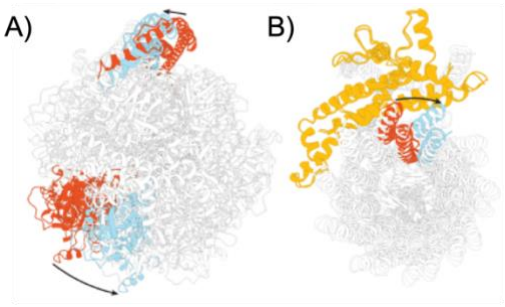

Figure 2. (A) Superposition on the subunit *a* stator from above rotational movement of F_1 headpiece (B) Superposition on the subunit *a* stator from below rotational movement of F_1 headpiece[4]

The research of proton flow, mechanism, and conformational changes throughout the complex will be continued through the structural dynamics project. We ask the questions: Does the subunit *a* stator have structural dynamics? Are there any conformational changes occurring during the function of subunit *a* stator? Or are there any changes in ATP synthase between during its synthesis and proton pumping? These pressing questions have been of pique interest in the biochemistry/chemistry field as they will explain more of how and why the mechanism of ATP synthase occurs and more information on such a fundamental protein system for all provinces. With the use of the latest cryo-EM literature findings of ATP synthase protein in and practicing with EPR spectroscopy, progress will be made on the structural dynamics project and especially since this project hasn't been worked on in a while. Spin-labeling and EPR spectroscopy will be used to analyze extract ATP synthase in its most native environment by using an amphipathic polymer.[4] This amphipathic polymer is made up of hydrophobic and hydrophilic components, which can create nanodiscs of the membrane-bound ATP synthase, and these nanodiscs will have the phospholipid bilayer still intact due to the hydrophobic and hydrophilic components of the polymer. This polymer has been created by another member of Steed Lab, and when analyzing the extract nanodiscs, the results are promising because the protein can stay intact and not dissociate as it would with former methods of extraction--detergents.[6] Without the use of such detergents and instead using an amphipathic polymer, prevailing biophysical tools such as cryo-EM and EPR spectroscopy, the Steed Lab is continuing the structural dynamics and conformational changes project of *E.coli*'s F_1F_o ATP synthase complex.

2. Methods

Caution! *Biological agent Escherichia coli was handled following BSL 1 protocol where containers with liquid media are bleached (10% of total volume) for minimum 20 minutes.*

2.1. Growth

For optimum yield, three liters of LB (100 µg/mL ampicillin) in Fernbach flasks inoculated with three LB small cultures of desired strain (wild-type or modified Cys residues in the subunit *a* stator) are made under sterile conditions. Large cultures were incubated at 37°C with shaking (240 rpm) for 7-8 hours. Cells were pelleted by centrifugation for 15 min at 4,000 x g at 4°C in a swinging bucket rotor. Cells were stored at 4°C overnight or proceed with next steps.

2.2. High-pressure lysis

Inside-out membrane vesicles (ISO vesicles) were prepared from resuspended cells in TMDG buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 10% v/v glycerol, pH 7.5, 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 µg/mL DNase). Cells were lysed through an Avestin B-15 homogenizer at ≥15000 psi (1 pass). After high-pressure lysis, lysate was centrifuged for 10 min at 9,000 x g at 4°C. Collected supernatant from lysate and centrifuge for 60 min at 193,000 x g at 4°C. Supernatant was discarded and membrane pellets were stored on ice at 4°C (short term) or -80°C (long term).

2.3. Extraction and Purification of F_1F_o ATP synthase Complex

2.3.1. Extraction, Purification, and Reconstitution of F1Fo ATP synthase in Cholate/Deoxycholate Mixed Detergent Micelles

2.3.1.1. Micelle Extraction and Purification

To extract the ATP synthase, membrane pellet was resuspended in extraction buffer (30 mM imidazole, 5 mM $MgCl_2$, 0.1 mM K_2EDTA, 40 mM 6-aminocaproic acid, 15 mM *p*-aminobenzamidine, 0.2 mM DTT, 0.8% soybean phosphatidylcholine [Sigma, Type IV-S], 1% w/v dodecyl-β-d-maltopyranoside (DDM), 2.5% w/v glycerol, pH 7.5) then incubated extraction mixture at 4°C with rocking for 30 min. Centrifuged extraction mixture for 30 min at 100,000 x g at 4°C and collected supernatant. For purification, Ni-affinity chromatography was done with a pre-packed Ni resin His-Tag purification column on a peristaltic pump. To do this, the column containing extraction mixture was washed with wash buffer (extraction buffer with 0.05% w/v DDM) and the protein was eluted with elution buffer (wash buffer with no phosphatidylcholine and 400 mM imidazole) while fractions were collected. After extraction, run gel electrophoresis on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 150 V with 2x Laemmli sample buffer and after SDS-PAGE, stained gel with Coomassie blue dye and Precision Plus Protein Dual Xtra Ladder for fraction analysis, then collected and stored fractions containing protein on ice at 4°C overnight.

2.3.1.2. Micelle Reconstitution

ATP synthase was reconstituted into unilamellar lipids, which formed proteoliposomes (PLs). To create the PLs, 30 µL of lipids were extruded (10-13 passes) through 100 nm membranes and were put into solution with 10% cholate/lipid. Before reconstitution of protein into the PL, ATP synthase were spin-labeled with 2,5-dihydro-2,2,5,5-tetramethyl-3-[[(methylsulfonyl)thio]methyl]-1H-pyrrol-1-yloxy MTSSL (spin-label 100mM in DMF) (20-fold of MTSSL per Cys) then sat at room temperature for one hour, and added MTSSL again then stored on ice at 4°C. Then the 5 µL spin-labeled protein was reconstituted into the PLs (can store on ice at 4°C). Before purification, the spin-labeled protein is filtered through gel spin-columns for size exclusion chromatography at 4,000 x g for 1-3 min. Filtrate was collected and store on ice at 4°C for purification.

2.3.2. Extraction, Purification, and Reconstitution of F_1F_o ATP synthase with lauryl maltose neopentyl glycol (LMNG)[7]

2.3.2.1. LMNG Extraction and Purification

To extract the ATP synthase, membrane pellet was resuspended in Ni-A/DDM extraction buffer (50mM Tris, 5 mM MgCl2, 20 mM Imidazole, 100mM NaCl, 30 mM 6-Aminohexanoic acid, 10% glycerol, 1% DDM, pH 8) then incubated extraction mixture at 4°C with rocking for 30 min. Centrifuged extraction mixture for 30 min at 100,000 x g at 4°C and collected supernatant. For purification, Ni-affinity chromatography was done with a pre-packed Ni resin His-Tag purification column on a peristaltic pump. To do this, the

column containing extraction mixture was washed with wash buffer (Ni-A with 0.005% LMNG) and eluted the protein with elution buffer (Ni-A with 200mM Imidazole and 0.005% LMNG) while fractions were collected. After extraction, run gel electrophoresis on SDS-PAGE at 150 V with 2x Laemmli sample buffer and after SDS-PAGE, stained gel with Coomassie blue dye and Precision Plus Protein Dual Xtra Ladder for fraction analysis, then collected and stored fractions containing protein on ice at 4°C overnight.

2.3.2.2. LMNG Reconstitution

ATP synthase was reconstituted into unilamellar lipids, which formed PLs. Dry polar *E.coli* LMNG lipids were dissolved in $CHCl_3$ and let sit out for $CHCl_3$ to evaporate. Resuspended lipids in reintegration buffer (20 mM MOPS, 30 mM $NaSO_4$, 100 mM KCl, pH 7.4) into solution with a 50-60°C water bath for a final concentration of 10mM. To create the PLs, lipids were extruded (10-13 passes) with a 400mM membrane filter and were put into solution. Before reconstitution of protein into the PL, ATP synthase was spin-labeled with MTSSL then sat at room temperature for one hour, and added MTSSL again then stored on ice at 4°C. Then the spin-labeled protein is reconstituted into the PLs at a protein to lipid ratio of 1:50. Rocked PLs for about 30 min at 4°C then diluted 10-fold with reintegration buffer and centrifuged for 10 min at 456,000 x g at 4°C (store on ice at 4°C until further analysis via EPR spectroscopy).

2.3.3. Extraction, Purification, and Reconstitution of F_1F_o ATP synthase with Digitonin (GDN)[3]

2.3.3.1. GDN Extraction

To extract the ATP synthase, membrane pellet was resuspended in Ni-A/GDN extraction buffer for a final concentration of GDN of 4mM with half an EDTA tablet then incubated extraction mixture at 4°C with rocking for 30-35 min. Centrifuged extraction mixture for 30 min at 100,000 x g at 4°C and collected supernatant. For purification, Cobalt (Co)-affinity chromatography was done with a pre-packed Co resin His-Tag purification column on a peristaltic pump. To do this, the column containing extraction mixture was washed with wash buffer (Ni-A buffer) and eluted the protein with Ni-B buffer while fractions were collected. After extraction, run gel electrophoresis on SDS-PAGE at 150 V with 2x Laemmli sample buffer and after SDS-PAGE, stained gel with Coomassie blue dye and Precision Plus Protein Dual Xtra Ladder for fraction analysis, then collected and stored fractions containing protein on ice at 4°C overnight.

2.3.3.2. Purification via Size Exclusion Chromatography (SEC) of detergent extracted F_1F_o on Fast Protein Liquid Chromatography (FPLC)

Pooled fractions containing ATP synthase were further purified through SEC on FPLC with concentrated F_1F_o. Before concentrating the protein, added MTSSL fractions containing protein which sat at room temperature before MTSSL was added again (can store on ice at 4°C). Using Amicon Ultra-4 filter concentrators (100 kDa cutoff), spin-labeled protein was added into concentrators and centrifuged on a swinging bucket rotor at 4,000 x g at 4°C until concentrated to a volume of 500 µL. Equilibrated and eluted SEC column on FPLC with TMNG/GDN buffer (50 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl, 40 mM 6-Aminohexanoic acid, 10% Glycerol, 1mM GDN, pH 7.5) to prep for further

purification on the FPLC. Fractions containing protein were collected and ran gel electrophoresis on SDS-PAGE (1x SDS) at 150 mV with 2x Laemmli and Coomassie blue dye and Precision Plus Protein Dual Xtra Ladder for fraction analysis, and collected and stored fractions containing protein on ice at 4°C until analysis via EPR spectroscopy.

2.3.4. Detergent-Free Extraction and Purification with Amphipathic Polymers

2.3.4.1. Amphipol 18 Polymer Extraction

To extract the ATP synthase, membrane pellet was resuspended in Ni-A buffer then incubated the extraction mixture at 4°C with rocking for 30 min. Added 5% Amphipol 18 polymer dropwise to cleared extract and incubated at room temperature for 60 min. Centrifuged extraction mixture at 193,000 x g for 10 min at 4°C. For purification, Ni-affinity chromatography was done with a pre-packed Ni resin His-Tag purification column on a peristaltic pump with Ni-A buffer, washed the column with the Ni-A buffer, and eluted the protein with Ni-B buffer and collected fractions. After purification, ran gel electrophoresis on SDS-PAGE (1x SDS) at 150 mV with 2x Laemmli and Coomassie blue dye and Precision Plus Protein Dual Xtra Ladder for fraction analysis, and collected and stored fractions containing protein on ice at 4°C overnight.

2.3.4.2. Purification via SEC of detergent free F_1F_o on FPLC

Pooled fractions containing ATP synthase were further purified through SEC on FPLC with concentrated detergent-free F_1F_o. Before concentrating the protein, added MTSSL to fractions containing protein which sat at room temperature before MTSSL was added again (can store on ice at 4°C). Using Amicon Ultra-4 filter concentrators, spin-labeled protein was added into concentrators and centrifuged on a swinging bucket rotor at 4,000 x g at 4°C until concentrated to a volume of 500 µL. Equilibrated and eluted SEC column on FPLC with TMNG buffer to prep for further purification on the FPLC. Fractions containing protein were collected and ran gel electrophoresis on SDS-PAGE (1x SDS) at 150 mV with 2x Laemmli and Coomassie blue dye and Precision Plus Protein Dual Xtra Ladder for fraction analysis, and collected and stored fractions containing protein on ice at 4°C until analysis via EPR spectroscopy.

2.4. Continuous Wave-Electron Paramagnetic Resonance (cw-EPR) Spectroscopy

To analyze ATP synthase using EPR spectroscopy, the protein needed to be concentrated to have optimum results for analysis of the structural dynamics of the subunit *a* stator. For concentration of liposomes, the spin column flowthrough was diluted with about 2 mL and centrifuged for 10 min at 456,000 x g at 4°C. Discarded supernatant and resuspended PLs with minimal volume of reconstitution buffer. For Amphipol 18 polymer extraction, SEC fractions were concentrated to a volume of 50 µL with a spin concentrator and a swinging bucket rotor at 4,000 x g.

Both PLs and Amphipol 18 polymer protein samples were collected into 50 µL capillary tubes and loaded into the EPR spectrometer at ambient temperature. The parameters for collecting spectra were microwave power (10 mW), center field (g=2),

sweep width of (100G), and modulation amplitude (1.2 G). The spectra were normalized using the value of the double integral.

3. Discussion and Results

Spin-labeling and EPR spectroscopy are invaluable biophysical tools used to examine the structural dynamics of purified ATP synthase within PLs or amphipathic polymers.[4] To achieve accurate structural analysis of ATP synthase, obtaining a functional and purified protein in a native-like environment is crucial. Our focus is applying the diverse purification methods, particularly detergent extraction, to isolate *E. coli* ATP synthase from its cellular membrane.

Eowyn Dowdall selected the optimal polymer for detergent-free extraction of ATP synthase, considering the critical criteria to maintain a functional protein such as solubilization efficiency and preservation of the native structure.[8,9] This optimal extraction is a direct reflection of maintaining a native-like environment for ATP synthase to conduct EPR spectroscopy, facilitating structural dynamic analysis. The chosen polymer, Amphipol 18, has the unique ability to create nanodiscs by solubilizing the phospholipids hydrophobic surface of the membrane and stabilizes membrane proteins all in one step.[8] Amphipol 18 precisely extracts ATP synthase from resuspended cell membranes, enabling the formation of nanodiscs around the preserved native phospholipid bilayer in *E. coli*. Overall, Amphipol 18 demonstrates versatility both prior to and during the biophysical analysis of ATP synthase. In contrast to the varied detergent extraction methods explored, the selection of Amphipol 18 for detergent-free extraction is a crucial strategy for maintaining the native environment of ATP synthase, thus essential for subsequent biophysical analyses.

Various detergent extraction methods including Mixed Micelles, LMNG[7], and synthetic digitonin (GDN)[3] were applied, each yielding distinct protein yields and levels of purity for membrane proteins such as ATP synthase. Continuing the protein purification process, the choice between nickel or cobalt for metal affinity chromatography significantly impacts both protein yield and purity. In Figure 3A, Ni-affinity chromatography demonstrated higher concentrations and yields of ATP synthase. However, it still exhibited multiple low-level contaminants observed in both unknown and known bands (e.g. (i) and (iii)) in SDS-PAGE gels, resulting in reduced purity. In contrast, Co-affinity chromatography, despite yielding lower concentrations of ATP synthase, exhibited significantly higher purity compared to Ni-affinity chromatography as seen in Figure 3B. Although the lower concentrations of ATP synthase with cobalt may pose challenges, the increased purity could lead us in the right direction of accurate biophysical analysis of the protein.

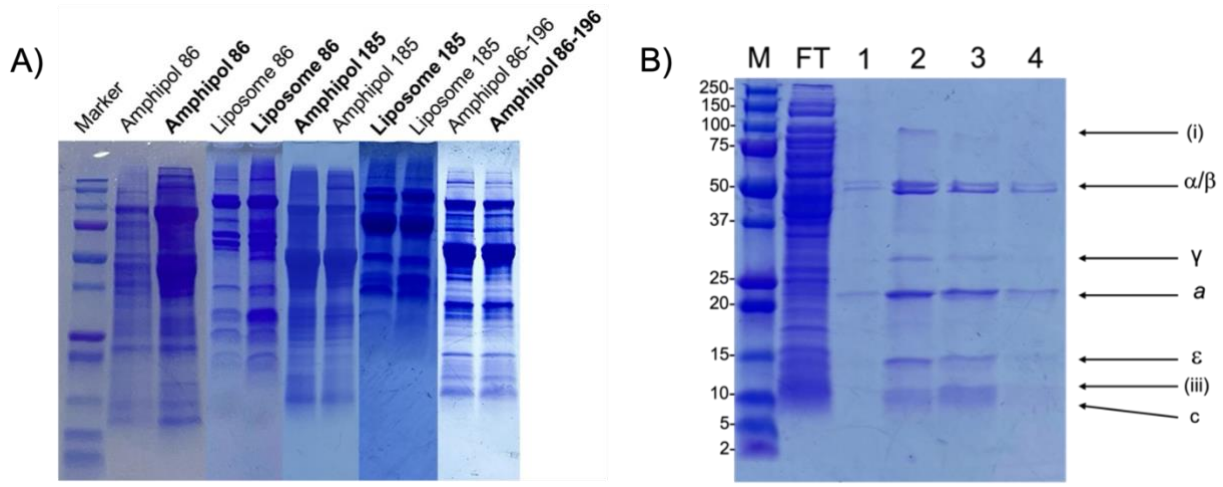

Figure 3. (A) SDS-PAGE completed after either Amphipol 18 extraction or detergent extraction of ATP synthase using mixed micelles and have multiple low levels of contamination. (B) SDS-PAGE of ATP synthase GDN extraction purified with Co-Affinity chromatography, as evidenced by the western blot. *__bolded__ labels have higher concentration of protein. The identification of separated ATP Synthases' subunits are cross referenced with Sobti et. al SDS-PAGE gel of purified E. coli ATP synthase with labeled subunits (Figure 1) and minor contaminations from E. coli proteins in wells 4 and 5: (i) Ribonuclease E iii) ElaB.

In Figure 3, the use of GDN detergent extraction and purification by Co-affinity chromatography has shown purity in an SDS-PAGE gel that has not been seen before in Steed Lab (Figure 5D and 3B), but also successful in Sobti et. al GDN detergent extraction of *E. coli* ATP synthase to obtain an accurate cryo-EM structure of the protein. The result from the use of GDN and Talon Resin (Co metal chromatography), continued to show promise with our purification of GDN and Co-affinity chromatography on *E. coli* Wild-Type (WT) by following Sobti et. al protocol.[3]

However, for the detergent extraction of ATP synthase using LMNG and Ni-affinity chromatography was done by following Godoy-Hernandez et. al published protocol and the literature called for a LMNG Auto-insertion Reintegration (LAiR) for the rapid reintegration of integral membrane proteins, such as ATP synthase, into lipid bilayers in the matter of minutes.[7] Also using the LAiR method, we should obtain a low critical micelle concentration during the process of reconstitution and minimizing usage of LMNG and DDM detergents, so during the formation of PLs there isn't interference when diluting the detergent. LAiR of *E. coli* ATP synthase showed, to some extent, a cleaner gel seen in Figure 5A in comparison to the traditional method of extraction and purification via Mixed Micelles Figure 5B.

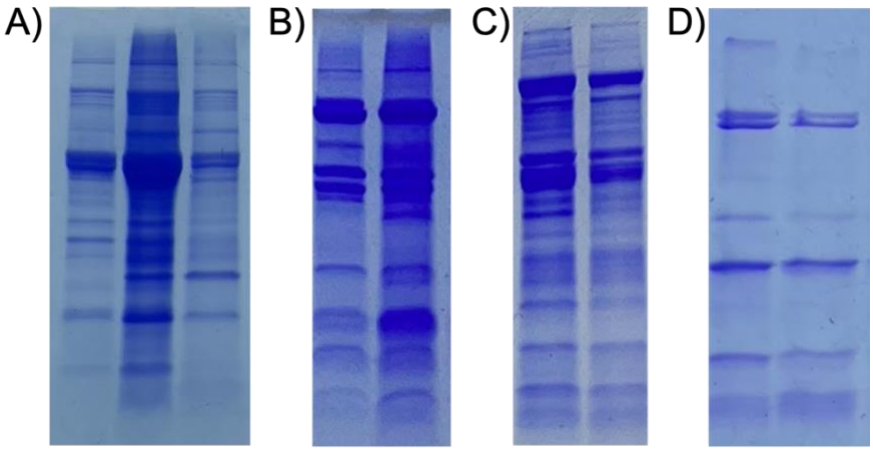

Figure 5. Comparison of LMNG, Mixed Micelles, Polymer, GDN SDS-PAGE Gels of (A) LMNG detergent extraction of WT with Ni-affinity chromatography purification, (B) Mixed Micelles extraction of 86 with Ni-affinity chromatography purification, (C) Amphipol 18 polymer extraction of 86 with Ni-affinity chromatography purification, and (D) GDN detergent extraction of WT with Co-affinity chromatography purification

With GDN detergent extraction and Co-affinity chromatography exhibiting the highest purity of a standard growth batch, the insufficient protein yield became clear. Attempting to replicate the successful purification process of WT for the first time (Figure 3D) with a 6L batch revealed a capacity issue with the 1mL Talon resin cartridge utilized in GDN and cobalt. The capacity of the cartridge is 20mg/mL, and due to exceeding the capacity, the column was overloaded and reduced the amount of protein present in the fractions and instead in the flowthrough seen in SI 1. Despite repurification efforts, the protein remained in the flowthrough and remained absent from any of the fractions seen in SI 1.

However, purification of WT with a 6L batch was reproduced, only that the cleared extract was loaded into the cartridge in two equal volumes. The cleaning protocols of the cartridge were implemented between purification of the two volumes. Regardless of these adjustments, the protein predominately remained in the flowthroughs seen in SI 2.

After purification and extraction, cw-EPR spectroscopy is completed on spin-labeled Cys residues on the subunit *a* stator on positions 185 and 86 in native-like environments using amphipathic polymer nanodiscs (left) or traditional reconstitution with Mixed Micelles into PLs using Ni-affinity chromatography (right) are shown in Figures 6 and 7:

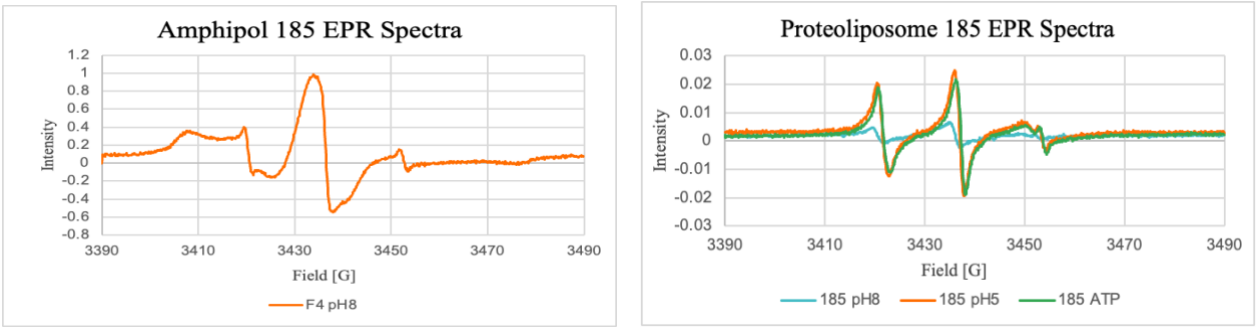

Figure 6. EPR spectra of 185 in Amphipol 18 (left) and in proteoliposomes (right). The paramagnetic MTSSL on position 185 is more mobile in PLs than in the Amphipol 18 polymer nanodiscs and no apparent effect from lowering the pH and addition of ATP

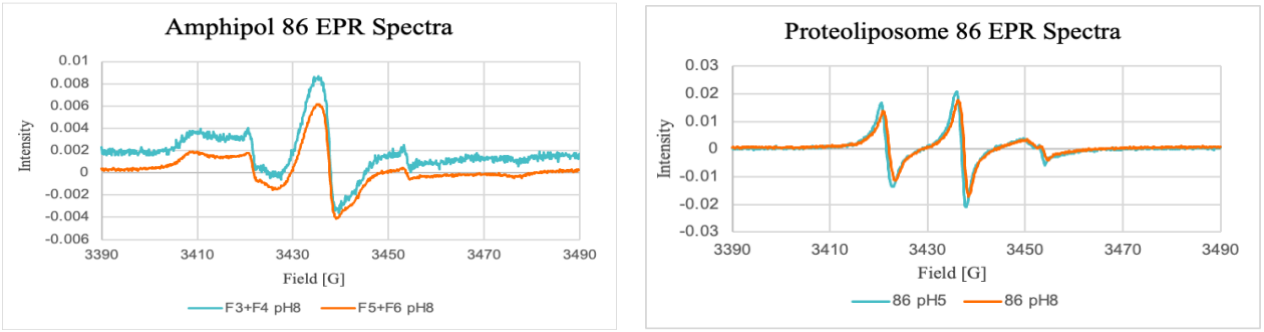

Figure 7. EPR spectra of 86 in Amphipol 18 (left) and in proteoliposomes (right). The paramagnetic MTSSL on position 86 is more mobile in PLs than in the Amphipol 18 polymer nanodiscs and no apparent effect from lowering the pH and addition of ATP

The spin-label, MTSSL, is generally more mobile in the PLs at both positions which has been seen in other protein reconstitution into the PLs.[4] The label is more restricted in the polymer nanodiscs environment when compared that of the PLs, which is currently the only significant differences between the two lipid environments. Also, there are no significant pH shifts when lowering the levels of 185 and 86 from pH 8 to pH 5, as well as the addition of ATP.

4. Conclusions

In summary, the investigation of extracting and purifying ATP synthase with detergents such as Mixed Micelles, LMNG, and GDN as well as Amphipol 18 polymer have shown improvement. Though, with the Amphipol 18 polymer, LMNG and Mixed Micelle detergent extractions and purifications revealed the presence of various persistent low-level contaminants that need further removal to increase purification. Notably, the GDN detergent extraction exhibited higher purity, although increasing the yield has proven difficult during the purification phase via Co-affinity chromatography. The EPR spectra analysis revealed that there are no significant pH shifts when lowering the pH

levels of 185 and 86 from pH 8 to pH 5, but notable differences in environments are taken into account that of the nanodiscs and liposomes, as they have been documented previously. Our future research will focus on refining and optimizing the reconstitution methods before reintroducing ATP synthase into PLs. Additionally, the use of an alternative tag, such as the Strep-tag, is proposed to have higher purity, along with the exploration of cobalt affinity chromatography for detergent and detergent free extractions. The advancements in understanding the structural dynamics within *E. coli* F_1F_o ATP synthase Complex were completed by the improved purification processes enhances the overall efficiency of the biochemical studies in our field.

Acknowledgment

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References

(1) von Ballmoos, C.; Wiedenmann, A.; Dimroth, P. Essentials for ATP Synthesis by F_1F_o ATP Synthases. *Annu. Rev. Biochemistry* **2009**, *78*, 649-672. DOI: 10.1146/annurev.biochem.78.081307.104803

(2) Miesfeld, R. L.; McEvoy, M. M. Biochemistry. In *W.W. Norton & Company, Incorporated*, 1st ed.; Twitchell, 2017; 551-577

(3) Sobti, M.; Walshe, J. L.; Ishmukhaetov, R.; Zeng, Y. C.; Robinson, C. V.; Berry, R. M.; Stewart, Alastair S. G., Cryo-EM structures provide insight into how *E. coli* F_1F_o ATP synthase accommodates symmetry mismatch. *Biol. Chem.* **2020**, *11*. DOI: https://doi.org/10.1038/s41467-020-16387-2

(4) Mchaourab, H. S.; Steed, R. P.; Kazmier, K. Toward the Fourth Dimension of Membrane Protein Structure: Insight into Dynamics from Spin-Labeling EPR Spectroscopy. *Structure* **2011**, *19* (11), 1549-1561. DOI: 10.1016/j.str.2011.10.009

(5) Moore, K. J.; Fillingame, R. H. Obstruction of Transmembrane Helical movements in Subunit *a* Blocks Proton pumping by F_1F_o ATP synthase. *J. Biol. Chem.* **2013**, *288* (35), 25535-25541. DOI: 10.1074/jbc.M113.496794

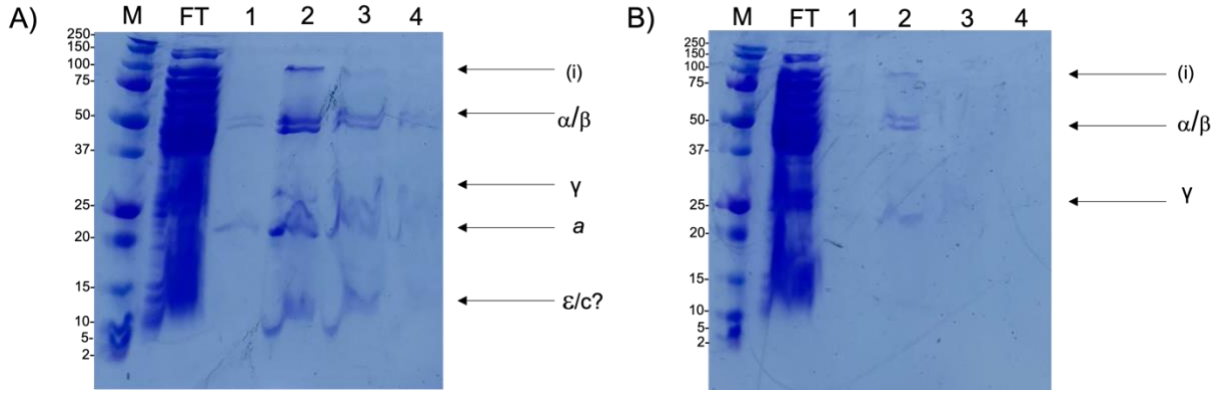
(6) Pocanschi, C. L.; Dahmane, T.; Gohon, Y., Rappaport, H.-J . A.; Kleinschmidt, J. H., Popot, J.-L. Amphipathic Polymers: Tools To Fold Integral Membrane Proteins to Their Active Form. *Biochem*. **2006**, *45* (47), 13954-13961. DOI: 10.1021/bi0616706

(7) Godoy-Hernandez, A.; Asseri, A. H.; Purugganan, A. J.; Jiko, C.; de Ram, C.; Lill, H.; Pabst, M.; Mitsuoka, K.; Gerle, C.; Bald, D.; McMillan, D. G. G. Rapid and Highly Stable Membrane Reconstitution by LAiR Enables the Study of Physiological Integral Membrane Protein Functions. *ACS Cent. Sci.* **2023**. DOI: 10.1021/acscentsci.2c01170

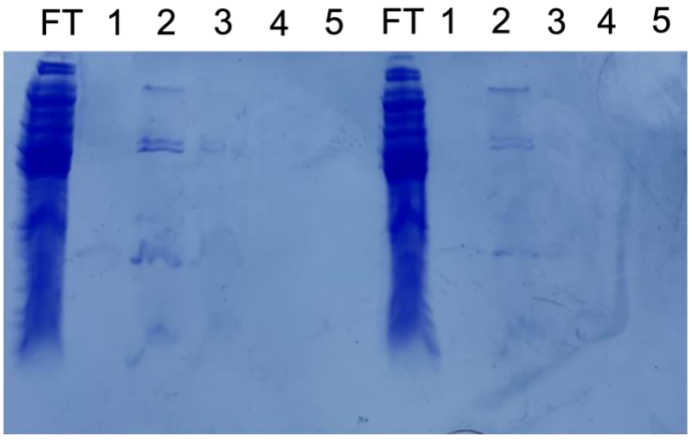
(8) Knowles, T.J.; Finka, R.; Smith, C.; Lin, Y.P.; Dafforn, T. and Overduin, M. Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. *J. Am. Chem. Soc.* **2009**, *131* (22), 7484-7485 DOI: 10.1021/ja810046q

(9) Pollock, N.L., Lee, S.C., Patel, J.H., Gulamhussein, A.A. and Rothnie, A.J. Structure and function of membrane proteins encapsulated in a polymer-bound lipid bilayer. *Biochim. Biophys. Acta.* **2018**, *1860* (4), 809–817 DOI: 10.1016/j.bbamem.2017.08.012

Supplemental Information



Supplemental Information 1: SDS-PAGE gels of WT trial 1 to reproduce the results of first purification of ATP synthase with GDN detergent extraction and Co-affinity chromatography. Wells labeled M, FT, 1, 2, 3, and 4 are marker, flowthrough, fraction 1, fraction 2, fraction 3, and fraction 4, respectively. (A) is the gel after the purification of cleared extract and (B) is the gel on the right is purification of the flowthrough after completing purification via Co metal affinity chromatography using a pre-packed Talon resin cartridge.



Supplemental Information 2: SDS-PAGE gels of WT trial 2 to correct the outcome of protein being present in flowthrough. The two equal volumes of purified cleared extract via GDN detergent extraction and Co-affinity chromatography/ Gel wells labeled FT, 1, 2, 3, 4, and 5 are flowthrough, fraction 1, fraction 2, fraction 3, fraction 4, and fraction 5, respectively.