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# Investigation of the Binding Domain Interfaces of the C-terminus of the Albino3 Insertase and the 43kDa Chloroplast Signal Recognition Particle Subunit via Single Molecule Förster Resonance Energy Transfer

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# Investigation of the Binding Domain Interfaces of the C-terminus of the Albino3 Insertase and the 43kDa Chloroplast Signal Recognition Particle Subunit via Single Molecule Förster Resonance Energy Transfer

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**Abstract**

Fluorescent labeling is a technique used for visualizing functional groups contained in biomolecules by fluorescence imaging. This technique was used in this project to analyze post-translational targeting of light-harvesting chlorophyll-binding proteins (LHCP), which are the core complexes that harvest sunlight to drive photosynthetic electron transfer. This protein is synthesized in the cytosol and post-translationally targeted to the stroma of chloroplasts. CpSRP43 is a signal recognition particle (SRP) subunit unique to chloroplasts, which has been shown to interact with the stroma-soluble C-terminus of the thylakoid-bound Albino3 insertase (Alb3-Cterm). In the chloroplast stroma, targeting to thylakoids is performed via the cpSRP pathway via sequential interaction with cpSRP43/cpSRP54, cpFtsY and Alb3. Although Alb3-Cterm is mostly disordered, molecular dynamics simulations predict a transient helical secondary structure in certain regions. To analyze this interaction, various pairs of labeling sites are chosen to determine the structural changes between cpSRP43 and Alb3-Cterm. Single molecule fluorescence resonance energy transfer (smFRET) was then used to determine structural differences. This project aimed to determine what conformational changes occurs during the cpSRP43 and Alb3-Cterm interaction during post-translational targeting of LHCPs. By introducing a proline mutant to prevent helix formation in various regions, it was determined whether the secondary structure plays a role in the binding. It was also found that the smFRET mutants tested display a high-FRET population in the presence of cpSRP43 which would indicate a helical propensity in the probed region.

# I. Introduction

## i. Overview:

Plant photosynthesis is a very efficient solar energy conversion process which relies on the precise organization of light harvesting chlorophyll binding proteins in thylakoid membranes to capture light. Although the components of LHCP targeting and insertion are known, the timing and binding sites from the stroma to the thylakoids have not yet been elucidated in detail.[6] It is known to involve a specific interaction between cpSRP43 and Alb3 which is shown in figure 1.

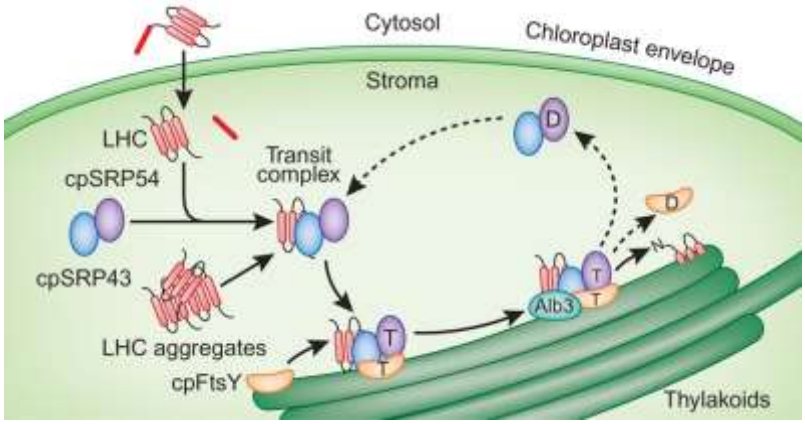


**Figure 1**- Model for post-translational targeting of LHC proteins to thylakoid membrane by cpSRP.[3]

Understanding this interaction in detail would allow for the development of renewable energy by replicating the post-translational targeting occurring in the thylakoid membrane. Additionally, the findings can be used to understand the targeting processes in mitochondrial inner membranes and bacteria due to their similar structures containing densely packed proteins to perform metabolic functions. In mitochondria, Oxa 1 is homologous to Alb3 in chloroplasts and functions in a similar manner, explaining the significance of understanding the unique properties of the targeting system of cpSRP43.[6] Therefore, understanding this pathway will allow for better understanding of other pathways.

CpSRP43 is a signal recognition particle (SRP) subunit unique to chloroplasts, which has been shown to interact with the stroma-soluble C-terminus of the thylakoid-bound Albino3 insertase (Alb3-Cterm). This interaction is detailed in figure 2.

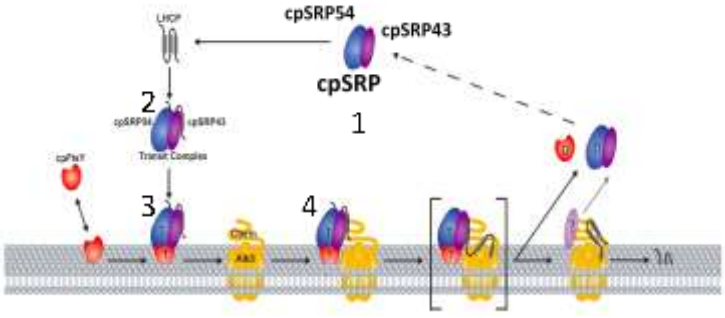


***Figure 2*** *– LHCP targeting pathway using cpSRP involves an interaction at step 4 between cpSRP43 (magenta) and the C-term of Alb3 (yellow) before integration of LHCP into the membrane. (From Heyes Lab)*

CpSRP43 contains 7 domains, a 4-domain ankyrin repeat region flanked by 3 chromodomain regions in the following order: CD1-Ank1-Ank2-Ank3-Ank4-CD2-CD3. It has been identified that the ankyrin repeat region of cpSRP43 is primarily responsible for the interaction with Alb3-Cterm.[5,10]

It was previously found in the Heyes' lab that upon binding to cpSRP43, Alb3-Cterm adopts a more compact structure compared to the unbound structure, suggesting that transient changes in the secondary structure of Alb3-Cterm plays a vital role in the post-translational LHCP targeting mechanism.[1]

### ii. Fluorescent Labeling

Fluorescent-based assays are widely used and a highly sensitive method for observing biological processes. While proteins have intrinsic fluorescence due to resides such as tryptophan that are aromatic, adding extrinsic fluorescence allows for flexibility with location of labeling and enhanced fluorescence. In this experiment, the dyes Alexa488 maleimide and Alexa594

maleimide are used together. Alexa488 is used to observe the excited molecule or the donor while Alexa594 allows the observation of the acceptor. In this experiment, double cysteine mutants are prepared with the cysteine residues at known locations. When the dyes are added to the protein, the goal is to statistically label FRET pairs with one donor and one receptor. To separate the labeled protein from the free dye gel filtration chromatography is implemented. This method uses size exclusions in which the pores are very small only allowing small molecules to spend extended times in the gel such as the free dye. The protein will elute out first because of its larger size that avoids the pores This process is detailed in figure 3.

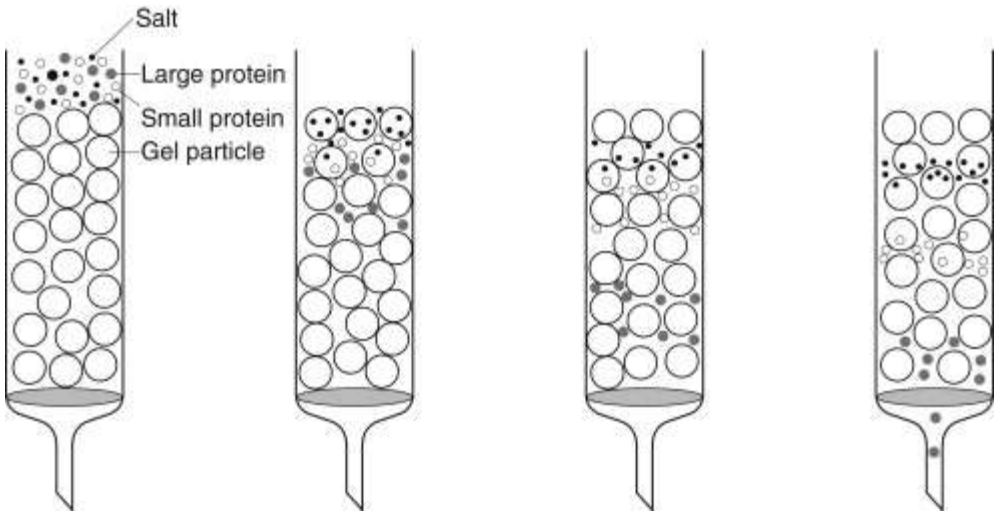


**Figure 3**- Diagram depicting gel-filtration chromatography or size-exclusion chromatography. The separation is shown to be based on the molecules ability to enter the pores of the gel medium. The larger molecules, in this experiment the labeled protein, elute first followed by the free dye.[7]

### iii. UV-Vis Absorption Spectroscopy

UV-Vis absorption spectroscopy records a UV or visible spectrum as a plot of wavelengths of absorbed radiations versus the intensity of absorption in molar absorptivity as defined by the Lambert-Beer Law.[8] The absorption can be used to determine the concentration of the sample. Beer's Law is expressed as

$$A = \epsilon bC$$

where A is the absorbance, $\epsilon$ is the molar absorptivity, b is the length of the light path in cm, and C is the concentration in mole/liter. UV-Vis spectroscopy if often used for quantitative determination of different analytes.[11]

### iv. smFRET

Single molecule Förster resonance energy transfer (smFRET) is used to identify structural conformation changes by exciting donor and acceptor FRET pairs and measuring their intermolecular distances via quantifying the energy transfer efficiency.[9] To determine if multiple sub-populations of structural conformations are present a single-molecule approach can be implemented which can shed light on the roles of the multiple binding sites that have been reported. This approach allows the distance between two adjacent fluorophores to be determined by determining the absorption spectrum of an acceptor and a donor. FRET determines the efficiency of energy transfer and is favorable withing a 3-10 nm range, which allows it to serve as a ruler. The ratio of acceptor fluorescence relative to total fluorescence is then determined to analyze changes in conformational change.[1]

To measure conformational change using smFRET, the molecules of interest are labeled with specific donor and acceptor fluorophores that are stable under high photon flux and undergo minimal blinking. The positions of the dyes are carefully selected to ensure the distance between to donor and acceptor are within the Förster distance for FRET to occur. The labeled protein is then used with a confocal microscope which utilizes a dichroic mirror to separate the donor and acceptor signals that are then directed onto detectors to record the signal separately. High-quality fluorescence filters were placed in front of each single-photon avalanche diode detector, to collect the Alexa488 and Alexa594 fluorescence, respectively. Figure 4 is a diagram of the diluted labeled protein with respect to the laser sed to excite the donor.

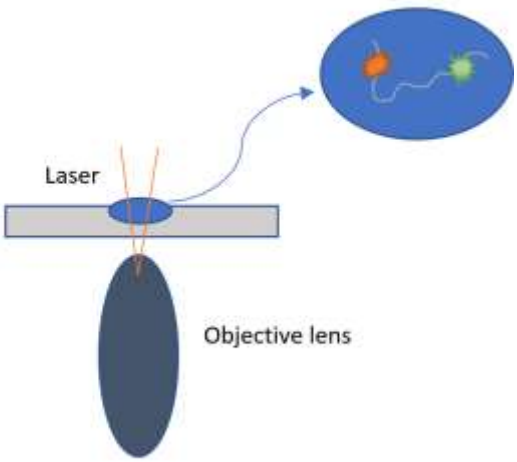


**Figure 4**- Model for smFRET data collection with a confocal microscope and diluted labeled protein (From Heyes Lab).

While analyzing the raw data, the donor and acceptor signal are compared to observe changes in conformations. An abrupt increase in FRET which is demonstrated by a decrease in donor and an increase in acceptor signal indicates a folding of the labeled molecule. FRET occurs when a donor fluorophore in the excited state transfers energy to an acceptor fluorophore. FRET efficiency is the proportion of the donor molecules that are transferred to the excited state

to the acceptor molecules indicating a decrease in intermolecular distance.[1,9] This process is demonstrated in figure 5.

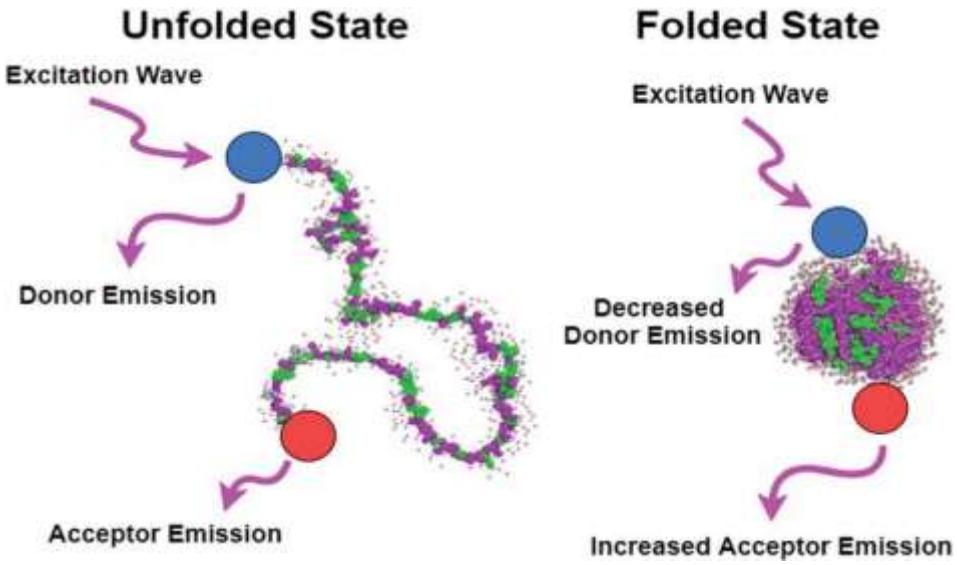


**Figure 5**- Diagram depicting the conformation changes of a labeled molecule and the effect on FRET efficiency. It is shown that when the molecule becomes folded there is an increase in acceptor emission indicating a smaller distance between the donor and acceptor dyes.[4]

## II. Materials and Methods

### i. Preparation of Mutants

The Alb3-Cterm and cpSRP43 mutants used in this project were provided by a collaboration with the Kumar Group at the University of Arkansas, specifically by the graduate student Patience Okoto. The preparations of these mutants are part of a separate project and therefore are not explained in detail in this paper. Briefly, these mutations change specific residues on each of the proteins to cysteine residues to enable fluorescent labeling of the proteins at known positions using cysteine-reactive dyes.

### ii. Fluorescent Labeling

The double-cysteine Alb3-Cterm mutants were labeled at a concentration of 1mg/ml, using Alexa488 maleimide and Alexa594 maleimide with a dye to protein ratio of 10 to minimize the nonspecific fluorescence labeling. The labeled proteins were placed in a dark drawer at room temperature for approximately thirty minutes to incubate. The excess free dye was removed from the labeled proteins using size elution chromatography. Gel filtration was implemented with Bio -Gel P6 polyacrylamide gel and gravity gel filtration. While running the proteins through the column, a UV lamp was used to determine where the labeled protein had filtrated to and if separation occurred. This process is shown in figure 6. The protein should elute first due to its higher mass and was collected in an Eppendorf tube. The buffer used in all these experiments was 10mM HEPES, 10mM MgCl2, and 100mM KCl with 10% glycerol at pH 7.5.

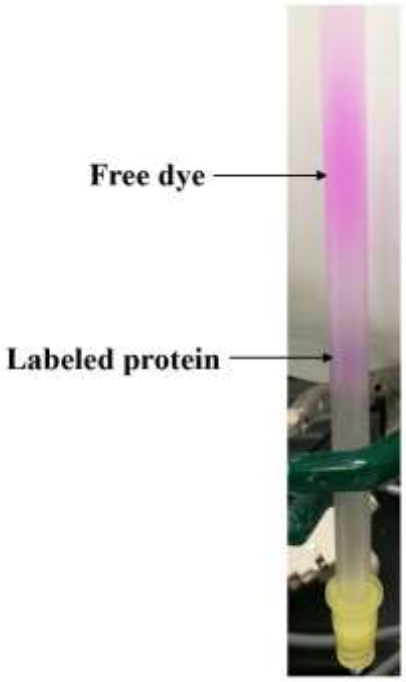


**Figure 6** - Separation of FRET-labeled Alb3-Cterm and unreacted, free dye (From Heyes Lab)

### iii. UV-Vis Absorption Spectroscopy

After the protein has been labeled and filtered out of the column, UV-Vis absorption spectroscopy was used to measure the absorbance of the diluted protein to ensure it successfully labeled, and to quantify the labeling efficiency. To do this, the UV-Vis spectrometer was calibrated using the buffer as a blank to get a baseline measurement. Once a good baseline was established, the labeled protein was diluted with a ratio of 10µL of protein into 140µL of water

to make a 15x dilution. The absorbance spectrum was then collected for the diluted protein, allowing the determination of the relative concentrations of the diluted protein and the attached dye.[8]

### iv. smFRET

To begin data collection, smFRET was performed to measure conformational changes when Alb3-Cterm binds to cpSRP43, using the labeled molecules described above. smFRET was carried out using a MicroTime 200 microscope controlled by SymPhoTime software that implements a laser tuned to the sample excitation wavelength. It collects fluorescence photons on a photon-by-photon basis. The sample was diluted to pM concentrations to enable single molecule detection.[1] The femtoliter confocal laser focus was placed ~30 $\mu$m above a No. 1 glass coverslip surface and ~20-min-long traces showing bursts of diffusing single cpSRP54 proteins were collected. Each dilution was repeated for ten trials. To compare the FRET efficiency to the baseline of the labeled proteins, ten trials were also run of only cpSRP43, only cterm, and of buffer. The donor and acceptor fluorescence traces were binned at 1-ms time-resolution and were analyzed using a homemade MATLAB program to generate smFRET histograms. Single protein bursts were selected from the traces only when the total burst intensity met a threshold of 10 counts/ms.[2]

### v. Data Analysis

To locate and match the corresponding donor and acceptor signals, the raw data was extracted to be analyzed. The time traces of each donor were determined with its respective acceptor signal and used to construct a FRET efficiency histogram from the time trace for each molecule, which is observed when there is a burst of signal in the raw data. A histogram of

FRET efficiency and several events was used to determine the protein folding and unfolding of smFRET assays.[1,9] To ensure the accuracy of selected bursts caused by unlabeled protein, the unlabeled cpSRP43 FRET values were subtracted from the labeled cpSRP43 FRET values. This is done by subtracting an averaged control value of donor-only emissions that was collected by labeling each mutant with only Alexa488 fluorescent dye.[2]

## III. Results and Discussion

Prior to experimentation with smFRET, it was necessary to identify double-cysteine mutants to probe regions of the protein where a helical structure is expected. The histograms generated using the MATLAB program were used to predict where there could be a helical secondary structure.[2] The protein is not expected to have a structure, but the propensity indicates interaction that suggests helical structure formation. Three pairs of residues to place the cysteine residues to establish an interaction that produces 40-50% FRET. This value of FRET was chosen because it would allow observational changes in conformations and therefore shifts in FRET efficiency. If the FRET efficiency increases, it indicates the formation of helical structure and tightening of the protein creating a decrease in distance between the two residues.[1] These mutant pairs are demonstrated below in figure 7.

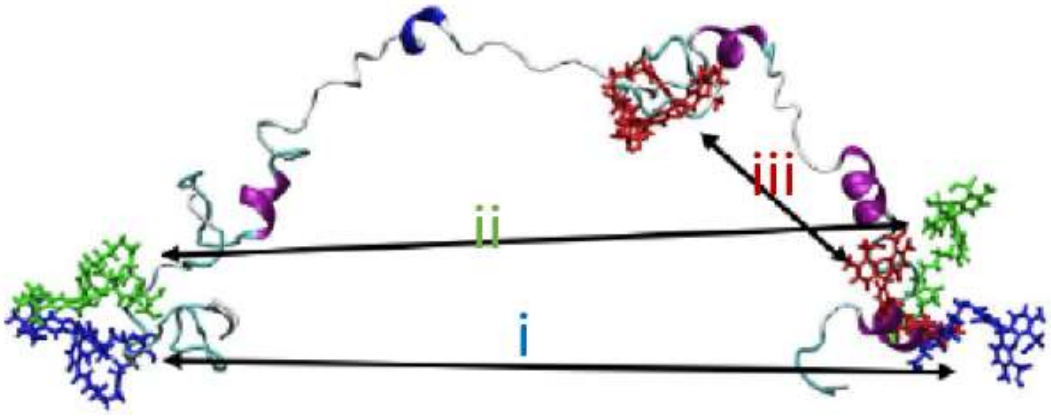


**Figure 7** – Diagram of double-cystein pairs positions that were labled with donor and acceptor fluorescent dyes (From Heyes Lab)

After performing smFRET on the labeled pairs, the raw data was analyzed, and the helical propensity of each region was determined using the previously discussed method. The helical propensity findings were in agreeance with the distance distributions derived from the experimental smFRET data for the double cysteine mutant. Overall, the dye-dye distance distributions and the secondary structural predictions agreed and provide a similar picture where cAlb exits an intrinsically disorder domain and contains one or more helical segments that occur transiently. These helical propensity findings for the specific mutants are visualized in figure 8.

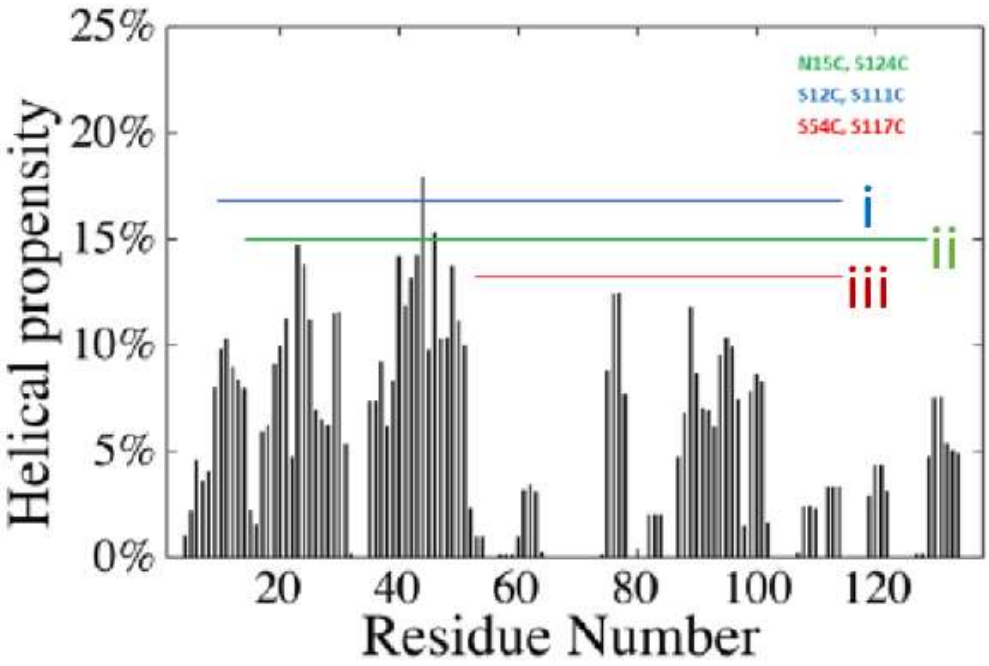


**Figure 8** – Diagram showing the helical propensity of probed regions shown in figure 7 as a function of the residue number for Alb3-Cterm. The labeled regions correspond with those shown previously show a peak at roughly 15% around residue 45. The upper right corner of the diagram details the mutants present. (From Heyes Lab)

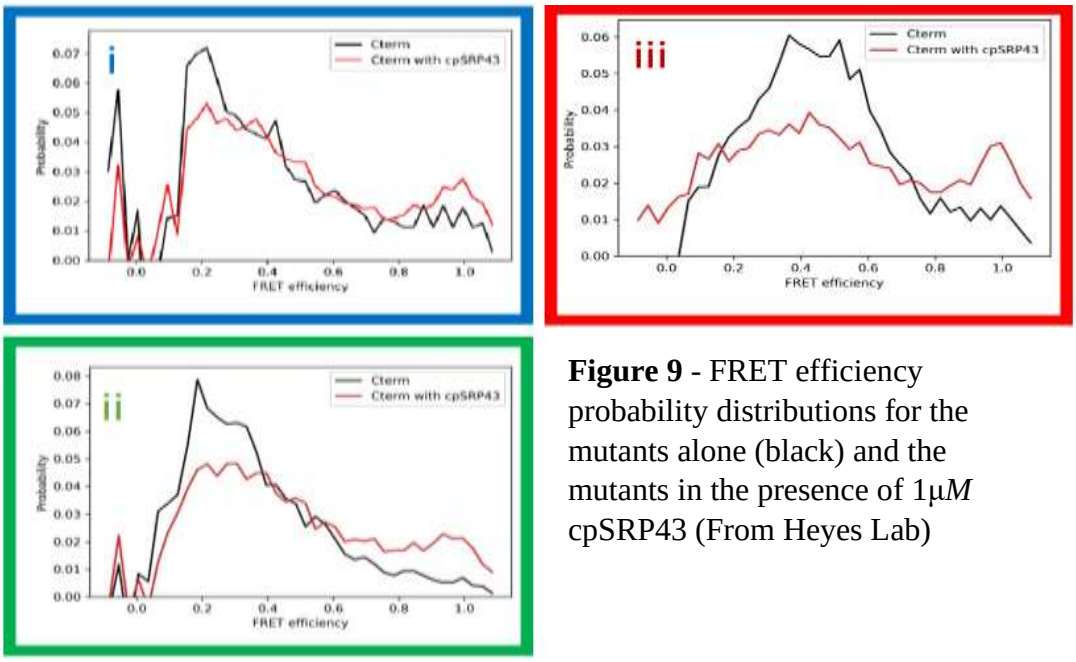


**Figure 9** - FRET efficiency probability distributions for the mutants alone (black) and the mutants in the presence of 1$\mu M$ cpSRP43 (From Heyes Lab)

In figure 9, when the FRET efficiency is high, as indicated by a value of 1.0 on the x-axis, the donor and acceptor fluorescence are closer together indication a compaction of the protein structure and helical propensity. The graph shows this tightening is only present after the addition of cpSRP43 indicating the conformational changes are a result of the C-term and cpSRP43 binding. Mutant iii is shown to have the greatest change in FRET efficiency meaning it is more likely helix formation occurs in that region.

Further analysis was done to confirm those results using a proline mutant. Proline acts as a helix breaking amino acid because of its unique cyclic structure that makes it rigid. A proline mutant was used to determine if the change in conformation was a result of a helix since it would prevent that formation and result in the absence of an increase in high-FRET.

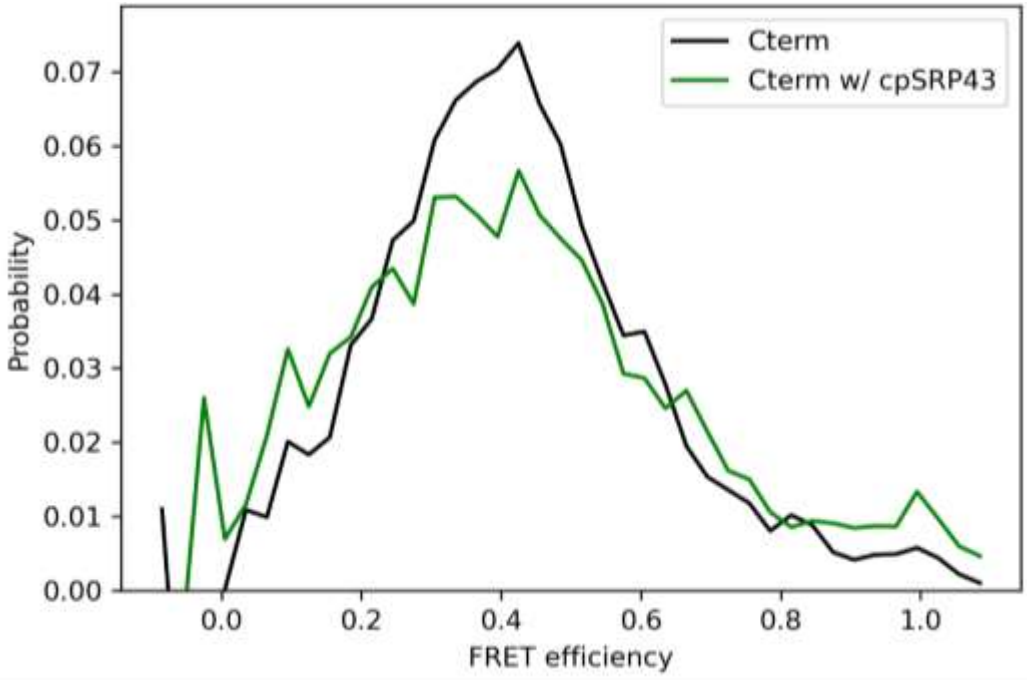


**Figure 10** - FRET efficiency probability distribution for helix-breaking mutant version of mutant iii (From Heyes lab)

The results shown in figure 10 confirm that a helix is forming in the region probed by mutant iii because the FRET efficiency no longer experiences a drastic increase. This means there is little change in the conformation of the proline mutant C-term when cpSRP43 is added. Further testing of double-cysteine mutants in regions near mutant iii enabled the fining of a region with greater change in FRET efficiency indicating a more accurate determination of the region of helix formation. This new mutant has provided the greatest change in FRET efficiency upon binding cpSRP43 that has been tested in the Heyes lab and shows the most likely region of helical formation. The result of smFRET analysis of the new mutant is shown in figure 11.

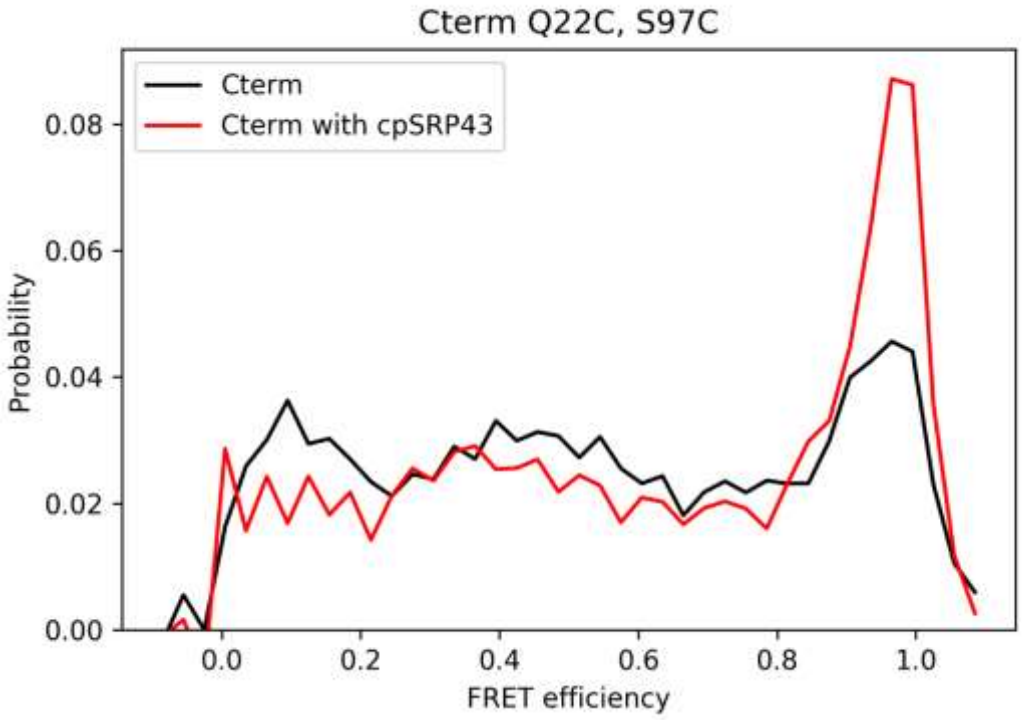


**Figure 11** - FRET efficiency probability distributions for the Q22C, S97C mutant alone (black) and the mutant in the presence of 1μ*M* cpSRP43 (From Heyes Lab)

The results in figure 11 indicate a change from approximately 0.4 to 0.9 in the probability of a helix formation in that region upon binding cpSRP43. These findings confirm a high probability of helix formation in this region.

All the smFRET mutants tested in this experiment showed a broad mid-FRET efficiency peak indicating a high degree of flexibility. They also exhibited a high-FRET population in the presence of cpSRP43 which indicated a tightening in the probed region and confirmed there is an interaction between the two. The largest population of high-FRET molecules came from the mutant probing residues from ~80 to ~100, suggesting that helix formation in this region is particularly prevalent.

## IV. Conclusion

smFRET proved to be an effective and useful method to analyze the conformational changes in Alb3-Cterm occurring upon the binding to cpSRP43, which is a key step during post-translational targeting of LHCPs. These experiments reinforced and expanded upon previous literature findings that found an interaction between the two furthering the understanding of this pathway. The results of the project also suggest the region where helical formation could occur further establishing the interaction present. The conclusions were also supported by introducing a helix breaking mutant to observe the absence of high-FRET efficiency upon binding to cpSRP43. The main obstacle encountered during these experiments was properly labeling the Alb3-Cterm mutants. It was often that proteins were not labeled after performing the procedure and no separation in the gel-filtration was observed. This made it difficult to perform smFRET analysis and to confirm results. In the future, repeat experiments with the proline helix-breaking mutation should be performed to ensure reproducibility as well as additionally experiments with the Q22C, S97C mutant. The mutants should also be tested with the full cpSRP complex (cpSRP43/54) to determine if the same conformational change occurs. Intermolecular smFRET with Alb3-Cterm and cpSRP43 should also be performed to determine the binding region

between the two proteins. Figure 12 shows some proposed labeling sites to perform intermolecular smFRET in the future.

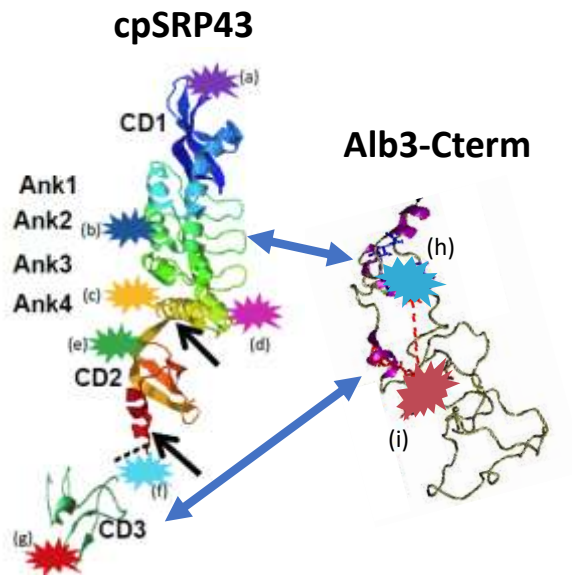


**Figure 11** – Diagram of potential labeling sites on cpSRP43 and Alb3-Cterm, labeled (a) – (i). (From Heyes Lab)

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