Production, Purification, and X-Ray Crystallography of the POTRA Domains of PsToc75

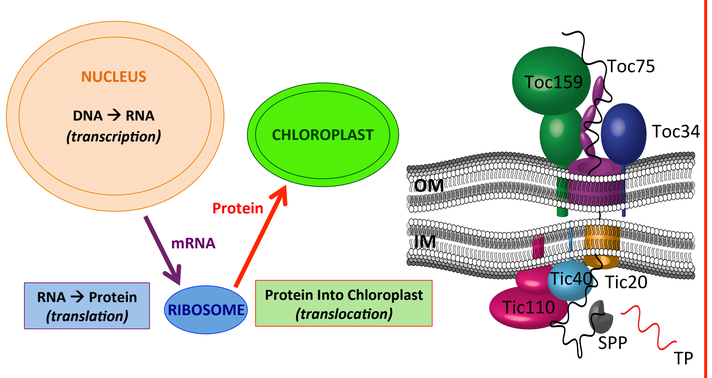
Gabriela Little[1], Richard Simmerman[1,2], Barry Bruce[1,2]

1. Department of Biochemistry and Cellular and Molecular Biology, University of Tennessee, Knoxville TN 37996
2. Tennessee Solar Conversion and Storage Using Outreach, Research, and Education, TN State EPSCoR

Abstract

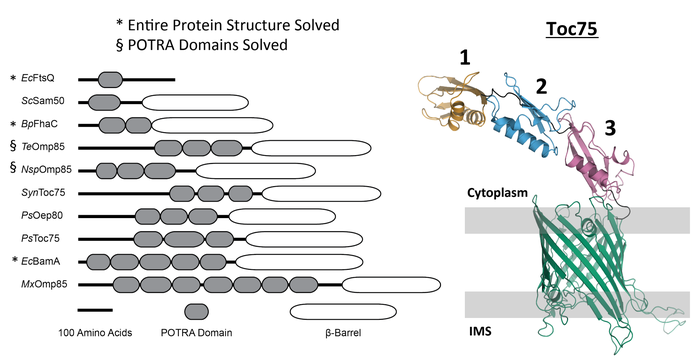
Plastids arose via endosymbiosis when a cyanobacterium was engulfed by a primitive eukaryote. The cyanobacterium was enslaved by the eukaryote, eventually giving rise to a new organelle, termed the plastid. The transition from a free-living cyanobacterium to a cell-dependent organelle demonstrates how the chloroplast ancestor underwent many changes in its physiology and biological processes. The majority of the DNA belonging to the cyanobacterium was scavenged by the nucleus of the host cell. This led to higher fidelity of genetic duplication, due to the proofreading abilities of the DNA polymerase of the host cell. This left the cell with the problem of how to get the now cytosolically transcribed proteins back into the proto-plastid. Eukaryotic photosynthetic cells use the Translocon(s) of the Outer/Inner envelope of the Chloroplast (TOC/TIC) to import proteins necessary for the survival of the plastid. Although much has been uncovered about the machinery necessary for protein import, the mechanism(s) used to accomplish this import remains unclear. Each translocon comprises a pore, through which precursor proteins are translocated along with accessory proteins that assist in translocation. Toc75 forms the pore in the outer envelope of chloroplasts. Toc75 is a member of the Outer Membrane Protein of 85-kilodalton/ Two Partner Secretion (OMP85/TPS) superfamily, which all share an architecture composed of a central membrane channel and cytosolic POTRA (Polypeptide Transport Associated) domains. Structures of POTRA domains from other members of OMP85/TPS have been solved, but the structure of the POTRA domains of Toc75 have not. These POTRA domains are of particular interest because Toc75 interacts with thousands of proteins, far more than other members of the OMP85/TPS. In our project, we have recombinantly expressed the N-terminal POTRA domains of Toc75 insolubly. The POTRA domains were purified via IMAC and have been refolded. Their purity has been accessed by SDS-PAGE. Their secondary structure has been confirmed with circular dichroism, and the peptides are ready to be shipped to our collaborator Dr. Susan Buchanan.

Chloroplasts Depend on the Host Cell for Protein Production



Chloroplasts rely on their host cell for protein production. mRNA is transcribed from DNA in the nucleus. Then, ribosomes translate mRNA into an amino acid sequence. The resulting proteins must then be translocated through the outer membrane of the chloroplast.

POTRA Domains are an Integral Part of OMP85/TPS

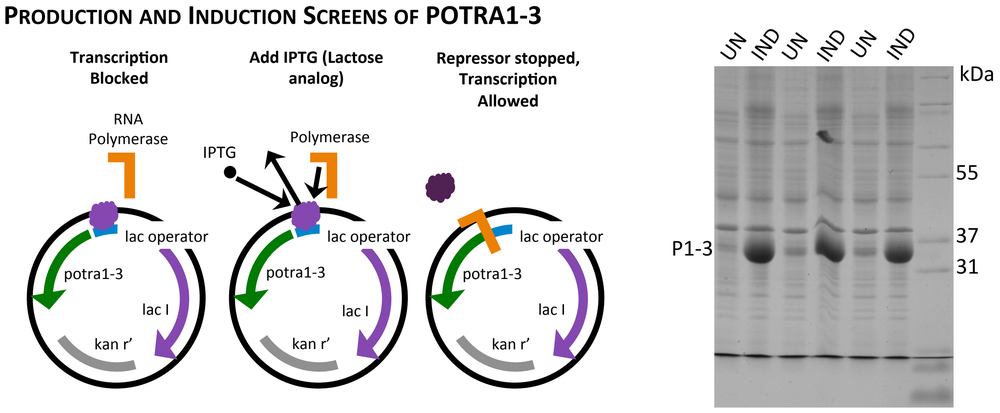


Toc75 belongs to the Omp85-TPS (Outer-Membrane Protein of 85-kilodaltons/ Two Partner Secretion) superfamily whose members possess a common architecture of a pore and POTRA domains. Members of the superfamily are present in gram-negative bacteria, mitochondria, and plastids. Toc75 is of special interest because it interacts with more proteins than other members of the Omp85-TPS superfamily.

Solving the Structure of POTRA1-3 Domains

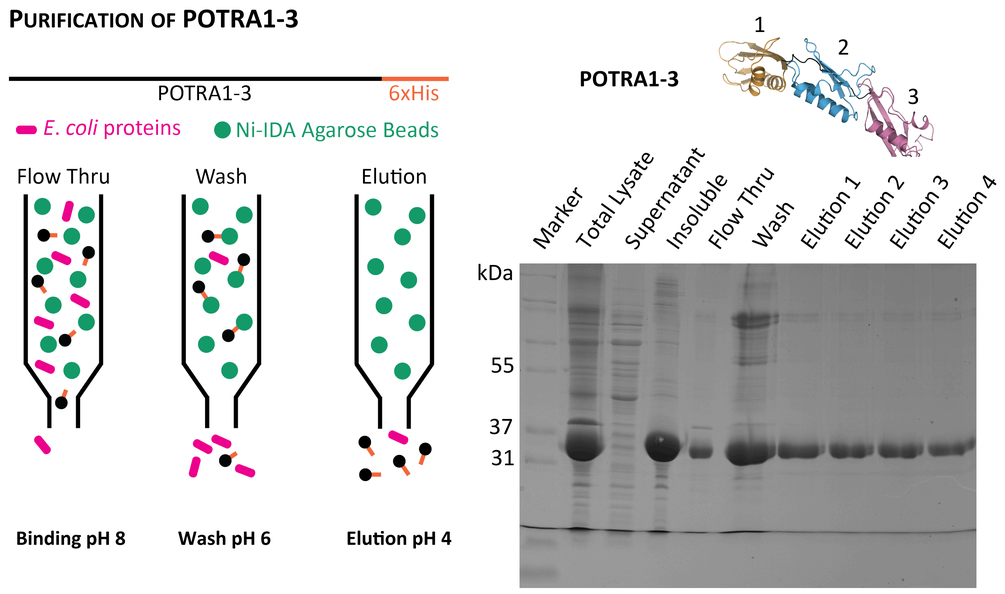
Solving the structures of the POTRA1-3 domains will provide the opportunity to determine their roles in and the mechanism of protein import into chloroplasts. This would potentially allow for more precise manipulation of protein import into chloroplasts. Altering protein import could make photosynthesis more efficient, which may facilitate plant growth to yield more food products or increase biofuel production.

Production and Induction Screens of POTRA1-3



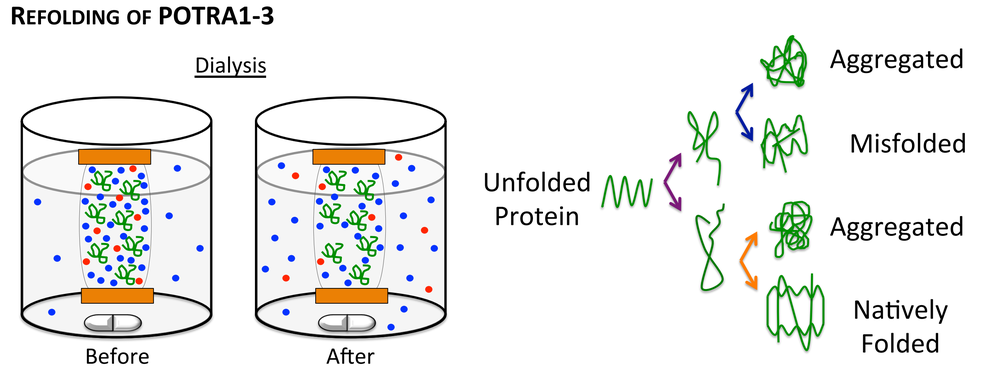
One µl of pET30(a)-POTRA1-3 was added to chemically competent BL21(DE3) cells. The cells were heat shocked, supplemented with SOC media, and screened on LB-agar plate containing 50 mg/ml kanamycin. Induction screening was then performed to select colonies which best expressed POTRA1-3. The addition of IPTG inhibits the lac repressor from blocking the lac operator from RNA Polymerase so RNA Polymerase can bind to produce POTRA1-3. We measured protein production for both uninduced and induced *E. coli* colonies to pick an efficient *E. coli* colony to produce the protein. The colony that produced the most protein after induction was chosen for protein production. ImageJ was utilized to quantitatively compare protein production among the three colonies.

Purification of POTRA1-3



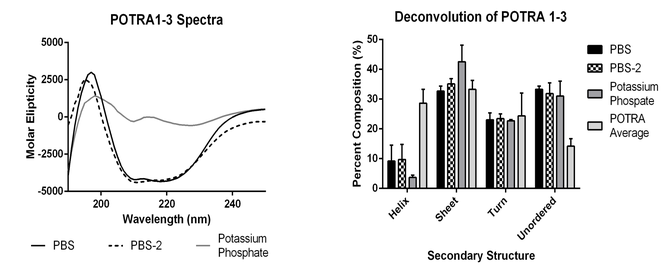
Cells were lysed and POTRA1-3 was solubilized then purified via IMAC (Immobilized Metal Affinity Chromatography). We collected samples during different stages of chromatography for SDS-PAGE (Sodium Dodecyl Sulfide-Polyacrylamide Gel Electrophoresis). The samples include the following: Flow thru after the 6xHis Tag of the POTRA1-3 domains bind to the Nickel beads, Wash 1 containing nonspecifically bound proteins, Wash 2 containing proteins with more specific interaction to Nickel, and Elutions which primarily contain POTRA1-3 because the 6xHis tag is fully protonated at pH 4. In the gel above, Elution 1 was eliminated due to unnecessary protein contaminants.

Refolding of POTRA1-3



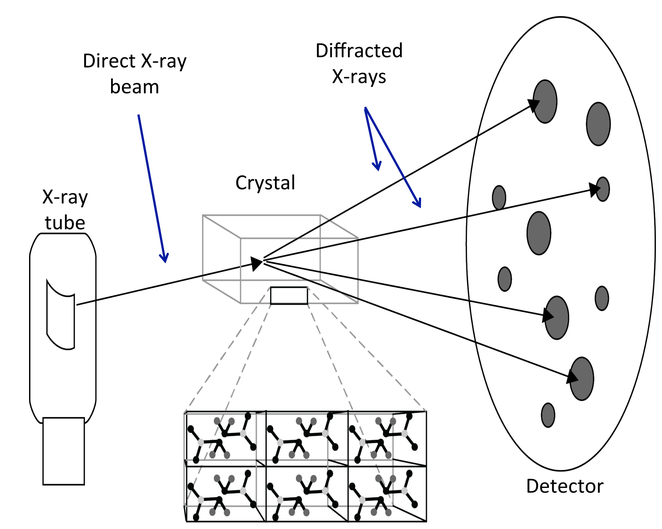
Refolding Method	Steps	Results
(Dialysis) 1x TBS without Urea	Dialyzed protein (pH 4) with 1x TBS without Urea (pH 8)	Unsuccessful, not sure if the pH change or lack of Urea is the cause
(Dialysis) 1x TBS with high Urea	Dialyzed protein (pH 4) with 1x TBS containing Urea (pH 8)	Unsuccessful even with added Urea
(Dialysis) 1x TBS with continuous removal of Urea	Set up system to continuously pump in 1xTBS without Urea and pump out 1x TBS Urea	Unsuccessful
(Dialysis) 1x TBS with 0.1 M Arginine, 0.005 M BME, and Urea	2M Urea (twice), 1M Urea, 0M Urea	Protein did not precipitate and had some secondary structure
(Gradient of TBS to PBS on column) 0.1x PBS, 100 mM imidazole	Elute column with 0.1x PBS, 100 mM imidazole	Protein did not precipitate and had more secondary structure

Circular Dichroism Spectroscopy



Circular dichroism involves the difference in absorption of left and right circularly polarized light of chiral molecules. We deconvoluted our spectra using a set of 37 soluble proteins to determine the secondary structure of purified POTRA domains. We compared our results to structurally solved parts of the 37 proteins.

X-Ray Crystallography to Solve the Structure of POTRA1-3



Crystallography uses ordered lattices of proteins to diffract x-rays producing patterns that can be deconvoluted revealing protein structures. X-ray crystallography allows for the observation of much smaller objects than light microscopy because x-rays have roughly 40,000 times smaller wavelengths than visible light.

Conclusions and Future Directions

This work evaluates the structure of POTRA1-3 in PsToc75 for possible insight about the function of these domains in chloroplast protein import. While we have produced and purified enough protein for crystallization screens, the protein did not contain the desired amount of α-helixes. After revising the experiment, we completed a CD scan immediately after protein purification. There was a higher percent of α-helixes, but still not the desired amount. We also used 0.1x PBS instead of 1x TBS, which has high salinity that could affect the POTRA1-3 domains. Further research regarding the structure and role of POTRA1-3 in PsToc75 would be worthwhile for future implications regarding plant immunity to herbicides and the production of biofuel.

Acknowledgements

I would like thank Dr. Barry D. Bruce for being supportive of my involvement in his laboratory. I am very appreciative of Dr. Bruce, Richard Simmerman, and all the other lab members for advising me during this research project and providing suggestions to my work. Also, I am grateful for the funding provided by the Tennessee Plant Research Center, which helped me participate in the 24th Western Photosynthesis Conference.

References

1. Arnold, T., Zeth, K., & Linke, D. (2010). Omp85 from the thermophilic cyanobacterium Thermosynechococcus elongatus differs from proteobacterial Omp85 in structure and domain composition. *J Biol Chem, 285*(23), 18003-15.
2. Koenig, P., Mirus, O., Haarmann, R., Sommer, M. S., Sinning, I., Schleiff, E., & Tews, I. (2010). Conserved properties of polypeptide transport-associated (POTRA) domains derived from cyanobacterial Omp85. *J Biol Chem, 285*(23), 18016-24.
3. Sanchez-Pulido, L., Devos, D., Genevrois, S., Vicente, M., & Valencia, A. (2003). POTRA: a conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. *Trends Biocheml Sci, 28*(10), 523-6.
4. Simmerman, R. F., Dave, A. M., & Bruce, B. D. (2014). Structure and function of POTRA domains of Omp85/TPS superfamily. *Int Rev Cell Mol Biol, 308*, 1-34.