Crystallization of ASB9-containing Subunit of Cullin-RING E3 Ligase

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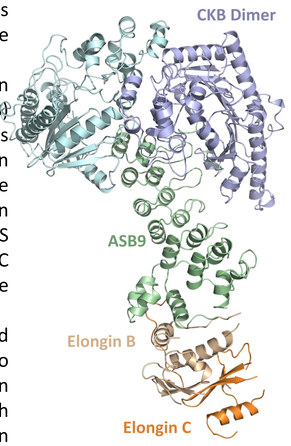
Abstract

Ubiquitin functions by marking proteins for degradation by a proteasome (1). The E3 ubiquitin ligase family of protein complexes catalyzes the transfer of ubiquitin to the protein to be degraded (2). Cullin-RING E3 ligases are the largest class of E3 ligases (3), and the complex of interest is the substrate recognition subunit of one such ligase.

This complex has five individual parts: ankyrin repeat and SOCS box 9 (ASB9), creatine kinase B (CKB) dimer, and Elongins BC; for simplicity this is abbreviated to ACE. CKB, which plays a major part in maintaining metabolic homeostasis of ATP, is the target for ubiquitin-mediated degradation. The ankyrin repeat section of ASB9 binds to CKB while the SOCS box region interacts with Elongins BC (2). Elongins BC in turn bind to Cullin-RING 5 to form the E3 ligase responsible for the transfer of ubiquitin onto CKB (2).

Computational docking models have predicted the structure of the ACE complex, which we hope to verify by obtaining a crystal structure of the 5-protein complex. X-ray crystallography is a technique in which the diffraction of X-rays through a crystallized protein provides data for the reconstruction of a 3D image of the protein. Due to the instability of ASB9 on its own (3) ASB9 has so far only been crystallized with the first 35 residues missing (1). Our goal is to crystallize the entire ACE complex in order to gain a better understanding of the protein-protein interactions involved in the ubiquitination of CKB.



Purification Methods

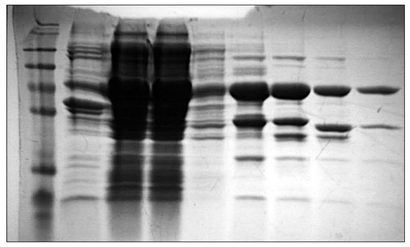
ASB9 was engineered into a vector for expression with a 6xHis tag and a TEV cleavage site, while CKB and Elongins B and C were expressed without tags to allow for the copurification of the substrate-recognition complex. The transformed E. coli were grown to OD_{600}=0.6 at 37˚ C to increase population size then for 16 hours at 18˚ C for protein production after induction by 1mM IPTG. The cells were lysed by sonication and centrifuged to separate the protein from other cell matter. The six-histidine tag engineered onto the complex has a high affinity for nickel, allowing for the specific isolation of ASB9 and any associated proteins. Imidazole-containing buffers were used to elute the ACE complex through the column. TEV protease was added to cleave the six-histidine tag from the ACE complex. Finally, the protein solution was concentrated to 1.5-3.5 mg/mL for our crystallization experiments.

BCA assay and UV spectroscopy were used to verify protein concentration. We did Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis (SDS-PAGE) with samples from the Ni-NTA purification in order to verify that the ACE complex had selectively eluted from the column. SDS- PAGE allows for the analytical separation of protein samples into bands based on size. Next, Size Exclusion Chromatography (SEC) was used to fractionate our protein solution according to size. This separated extraneous proteins and aggregate from the complete 5-protein complex of interest.

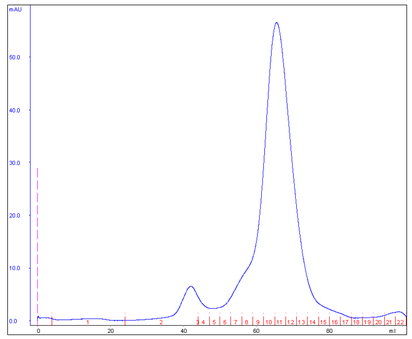
Ni-NTA Purification

Purification Analysis

ACE Purification Visualization by 13% SDS-PAGE

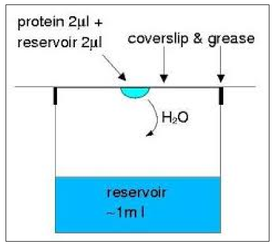


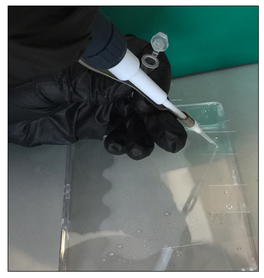
ACE Purification and Analysis by Size Exclusion Chromatography

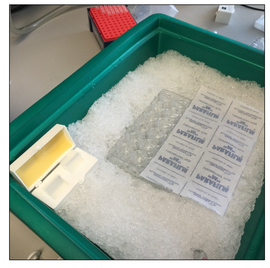


Crystallization

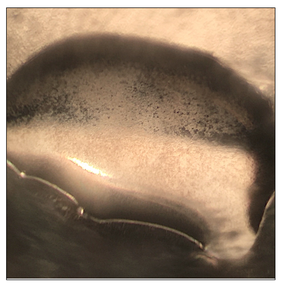
Hanging Drop Model







Isolated protein complex was concentrated to 3.5 mg/mL and screened with a Hampton Crystal Screen I formulation modified to utilize available reagents. We used the hanging drop method in which the protein droplet is suspended over a well. We prepared the screen in 24-well trays at room temperature as well as 4°C. Micro-crystals and granular precipitate show a possibility of larger crystals with the same solution, while brown precipitate shows that the solution is not one we would want to pursue. The plates were left alone for a couple of days before we viewed them under the microscope looking for signs of crystallization. We prepared a second screen with varying concentrations of precipitants and buffers that gave positive results. This included different types of PEG, isopropanol, MPD, ammonium sulfate, and lithium sulfate at different concentrations and pHs. This screen involved six 24-well plates, all to be left at 4˚ C. We doubled the size of the droplets, from 2 µL to 4 µL.



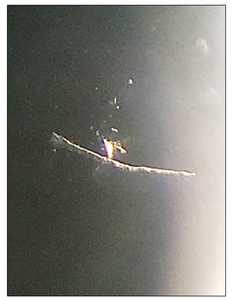
Granular Precipitate

Brown Precipitate

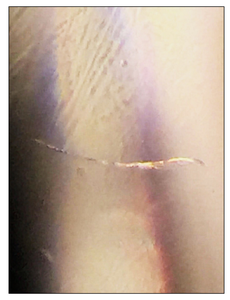
Results

We found many micro-crystals and a few small crystals in a variety of our test solutions. They were all roughly the same size and shape, giving us reason to believe it was truly the ACE complex that was crystallized. The most preferable buffers seemed to be Tris and HEPES, which range from pH 7.5-8.5. Keeping the trays at 4˚ C seemed to give better results, with more micro-crystals and less aggregation. We also found that salts were not very necessary as they were not used in the second screen, which gave better results. We did find a few decent sized crystals that could be used for X-ray crystallography; however, they are not very well ordered, meaning the resolution from them would not be very good.

15% MPD, 0.1M Tris pH 8.5



15% Isopropanol, 0.1M Tris pH 8.5



15% PEG 8000, 0.1M Hepes pH 7.5

Further Directions

Further screens will be run to hopefully acquire a more ordered crystal. Slight changes in pH or concentration of precipitant will allow the ideal range to be found. Increasing the protein concentration used in these screens would also be beneficial as most crystals formed in the higher range. Though the crystallization of this structure will provide structural understanding of the substrate recognition, the final goal is to understand the dynamics of not only the ligase but also the other enzymes involved in ubiquitin activation and conjugation. To achieve this, the entire nine-protein complex will have to be prepared *in vitro*.

Acknowledgements

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