The structural characteristics of PKA

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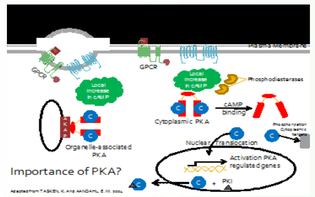
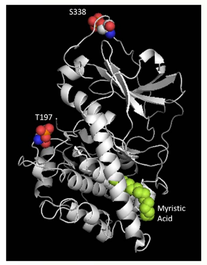
Protein kinase A (PKA) is a protein regulated by a secondary messenger; cyclic adenosine monophosphate (cAMP) . cAMP responds to an extracellular message and turns it into an intercellular response by activating PKA. There are different subunits of PKA including the regulatory subunits and the catalytic subunit. Together they form a holoenzyme which goes through a conformational change which releases the C subunit when cAMP is present.

cAMP regulates PKA intercellular activity



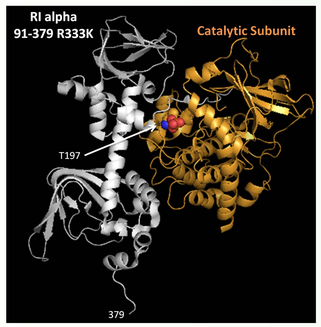
PKA is activated after an extracellular signal from a hormone sends a signal to a protein on the double membrane, which sends the signal to the G proteins; alpha, beta and gamma. These then send cAMP to the regulatory subunit of protein kinase A (PKA) when the α splits from the β and γ. This releases the Catalytic subunit and allows it to go phosphorylate different parts in the cell that need to be phosphorylated. To turn this off, cAMP leaves the R subunit and this allows the R subunit to make a conformational change that allows the C subunit to reattach to it.

PKA Myrstylated Catalytic Subunit (MCat)

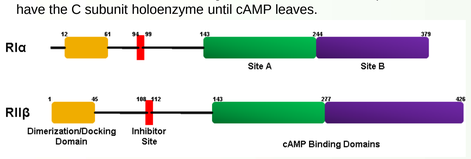


This crystal is of the PKA Catalytic subunit with myristic acid added to it. What we are doing is to try and find out more about what the myristic acid changes in the catalytic subunit. The Catalytic subunit is made up of two lobes; a small lobe and a large lobe. The myristic acid is added to the n-terminal of the C subunit. The above picture is of the C subunit alpha.

Structure of PKA holoenzyme



This is a picture of the PKA alpha holoenzyme which is missing the first 90 amino acids because it is difficult to obtain crystals of the domain and the unorganized area with the inhibitor site. The GFP protein will connect to the N-terminus of the protein to show its exact orientation which has not been figured out yet. The T197 amino acid is the docking part of the R subunit which binds to the C subunit. In the below picture is a chain that shows the difference between the RIα and the RIIβ subunits. The Site B is the first part that cAMP must bind to in order to bind to Site A on the cAMP binding domains, this makes it impossible to have the C subunit holoenzyme until cAMP leaves.



Transforming E.coli to obtain the protein

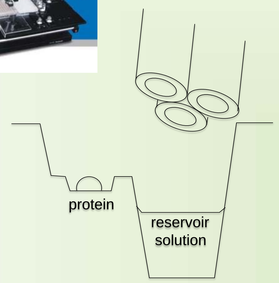
The first thing we did was to create the E.coli transformation in which the wanted DNA was added to the E.coli cells and then they went into an ice bath, then a quick heat shock, then back on ice. This was then put on an agar plate in which the E.coli grew in 37 C. Later, it is put into a starter culture where ampicillin is added in order to see if the E.coli still grows. This occurs because ampicillin is also added to the E.coli plasmid in order to make sure the wanted DNA is also made. Later, these starter cultures are added to large beakers to make more cells. Finally, the cells grow large enough to be induced by IPTG, and after a few hours, they are spun down in the centrifuge into cells.

Purification of Proteins

Next the cells is lysed by a micro fluidizer to open the cell up and let all the inside out. It is then spun down again and this allows all the proteins to stay in the spin supernatant. This is then added to a nickel resin in order to put it in a vacuum filter to press out all the other proteins not held to the nickel resin. Later elution is added to the vacuum filter in order to obtain the protein. This is added to a machine in order to get the protein necessary to crystallize.

Crystallization

3-bore tip

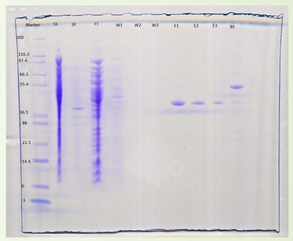


Crystallography is used to see the structures of the proteins. To do this, first the protein has to be almost completely purified and generally ends up small enough to be in an eppendorph tube. Then, it is added to multiple wells with multiple different conditions by a robot. If the solution is not taped down after the robot is done, there is a possibility that the protein could evaporate, therefore it must be taped with clear tape. After a few days, we looked back into the wells with a microscope to look for the crystals.

What is the structure of the PKA holoenzyme?

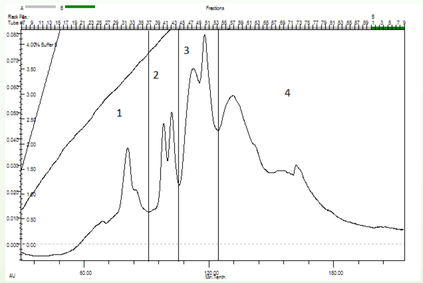
What is the structure of the catalytic enzyme when it is myrstylated?

Prep Gel for the Myrstylated Catalytic Subunit



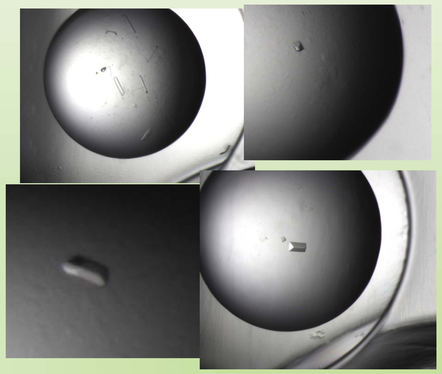
The prep gel first contains a marker, which shows the different sizes of the amino acid chains. The SS stands for spin supernatant and SP is short for spin pellet. The spin supernatant consists of the proteins after the cells are lysed open while the spin pellet be the rest of the lysed cell. Next, there is the FT, or flow through sample; this sample is from the first purification in which the nickel resin was added to the specific MCat protein, and then, was vacuum filtered to get rid of any protein that did not bind to the nickel resin. Then, W1, W2, and W3 were the washes done after to purify the protein further using buffer. Lastly, E1, E2, and E3 are the elutions which contain the MCat protein. This specific gel shows that the elutions contained the correct protein, and; because of the single band; only that protein. The BS stands for bead sample which indicates how much protein was still on the resin after being eluted.

MCat protein purification



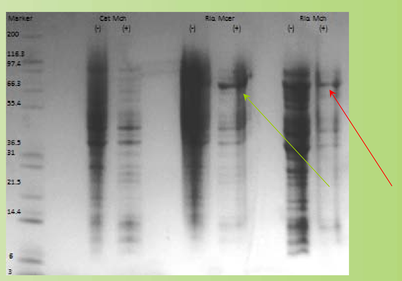
In this graph, the first part (1) contains 4 phosphate (S10, S139, T197, S338) PKA proteins, the second (2) contains 3 phosphates (S10, T197, S338) PKA proteins, the third (3) contains 2 phosphates (T197, S338), and the fourth part contains 2 phosphates (T197, S338) and myristic acid. These assignments are based on mass spectroscopy done previously. The fraction used for crystallization later is part 4.

Crystallized MCat Proteins



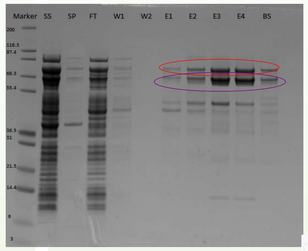
Protein crystals were created under specific conditions after PKI was added to it. On the top left crystal, the condition was 0.1 M MES pH 6.5, 1.6M $MgSO_4$. On the top right, the protein was under the conditions of 0.1M Bis-tris pH 6.5,2.0 M $AmSO_4$ as well as the bottom left conditions lastly, the right bottom picture was 0.2M Ammonium (Am) Acetate, 0.1M HEPES pH 7.5 .

Fluorescent Protein Expression Check



In this gel, 3 proteins are expressed with the GFP protein which is a fluorescent tag. The tag was induced in the (+) samples to produce the GFP with IPTG, while the other lane (-) is not induced. The uninduced have large amounts of proteins because they are all the regular proteins of E.coli before they are transformed to create the new protein. The (+) wells have the protein with an arrow drawn to it allowing it to glow. In the RIα Mch and Mcer, the GFP protein was created by the E.coli while the Cat Mch was not created and have no band of the protein and were therefore, not used in further purification and examination.

Mcherry and Mcerulean purification gels



The gel shows the Mcherry purified protein. Like in the MCat protein, there is a Marker, SS, SP, FT, BS, Washes and Elutions. In this gel, the Mcherry was not well bound to the RIα and that the RIα may have been broken up by proteases, showing this experiment to have a week bond between the tagging protein and the PKA Regulatory subunit.

In this gel, the Mcerulean is being purified with the same regulatory subunit as the above Mcherry histine tag. Here, the proteins did attach to each other showing one big band instead of two medium bands like the above Mcherry purification gel.

Histidine Tagged protein

These proteins were purified and will later go through a purification process and then be crystallized in order to see which orientation the RIα holoenzyme is actually in. They glow because of the GFP tag that binds to the N-terminate of the RIα.

Conclusions and Future Projects

- What we obtained were the beginnings to future projects:
  - We obtained some crystals for the MCat and PKI so we can pinpoint the exact conditions the protein crystallizes in.
  - We were able to obtain the protein of the PKA holoenzyme
- For the myrstylated catalytic subunit; we still need to try the specific conditions on larger amounts of proteins to try and obtain larger crystals.
- From that we still need to look at the conformational changes when myristic acid is added.
- For the fluorescent proteins, we still need to purify them further in the machine and attempt to crystallized them.
- After that, we still need to make larger crystal structures and look at the orientation of the RIα holoenzyme

Acknowledgments

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