Interaction of ASB9 with creatine kinase

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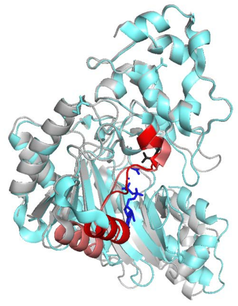
Introduction

Creatine Kinase is an enzyme that works in the mitochondria to utilize ATP, the "energy molecule" of life, and Creatine, an energy molecule used in tissues that require rapid amounts of energy such as the heart and the brain. CK breaks one of the phosphate groups off of ATP, transforming it into ADP, and places the phosphate on Creatine, transforming it into Phosphocreatine, and visa versa. There are five main types of CK in humans. We researched brain type (CKB) and muscle type (CKM). Studies have shown that during heart failure, raising CK levels can help prevent tissue damage. A better understanding of how CK is removed by ASB9 could help develop novel treatments for heart failure and disease. The protein ASB9 has been found to bond with CK, inhibiting its functions and marking it for degradation by proteasomes. Of the 18 types of known human ASB9, only the first two variants are known to interact with CK. The lab has hypothesized that it's ASB9's ankyrin repeats, located at the N-terminus, that bind to CK. Pinpointing the interactions between these two proteins is essential in understanding how CK is regulated throughout the body, and how we might discover ways to alter CK levels.

Structure of Creatine Kinase (closed)

Creatine Kinase is an enzyme that binds to ATP and Creatine, closing in the process. The X-Ray crystal structure of its closed form is pictured here. Creatine Kinase has not been crystalized in its open state, which is the state that binds to ASB9.



ASB9 Ankyrin Repeat Domain Sequence and Structure

```
         10        20
TPLHLAAXXGHLEIVEVLLKZGADVN
.::: :   :::  :..:::.:.::
SPLHEACLGGHLSCVKILLKHGAQVN
         80        90

         10        20
TPLHLAAXXGHLEIVEVLLKZGADVN
.:.: ::  ::.: :. :.  :....
SPIHEAARRGHVECVNSLIAYGGNID
  140       150       160

         10        20
TPLHLAAXXGHLEIVEVLLKZGADVN
:::.::    .   :. ::..::::::
TPLYLACENQQRACVKKLLESGADVN
 170       180       190

         10        20
TPLHLAAXXGHLEIVEVLLKZGADVN
.:.: ::  ::    .. :...:  ::
SPMHEAAIHGHQLSLRNLISQGWAVN
 40        50        60

         10        20
TPLHLAAXXGHLEIVEVLLKZGADV
:::  :   :  . :..::..:.:.:
TPLFNACVSGSWDCVNLLLQHGASV
    110       120

         10        20
TPLHLAAXXGHLEIVEVLLKZGAD
.:::.:.:  :.. .:.  :::
SPLHAVARTASEELACLLLMDFGAD
        210       220
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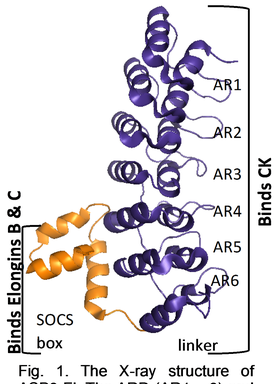


Fig. 1. The X-ray structure of ASB9-FI. The ARD (AR1 – 6) and linker are shown in violet the SOCS box domain is colored orange.

Isolating the Gene Fragment by PCR

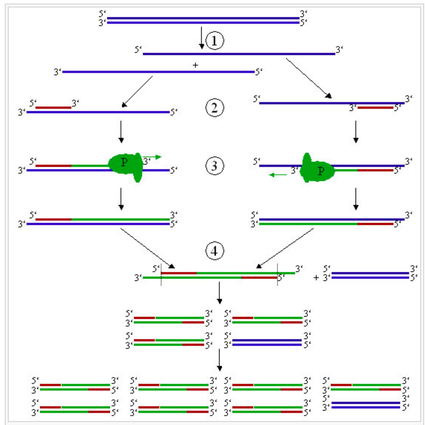
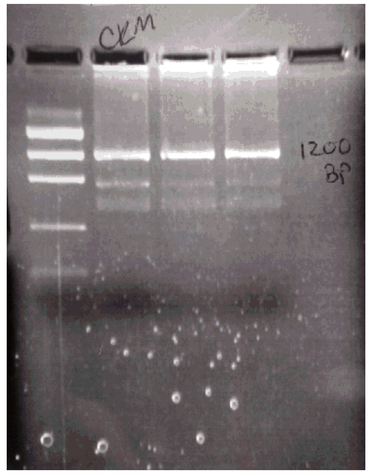
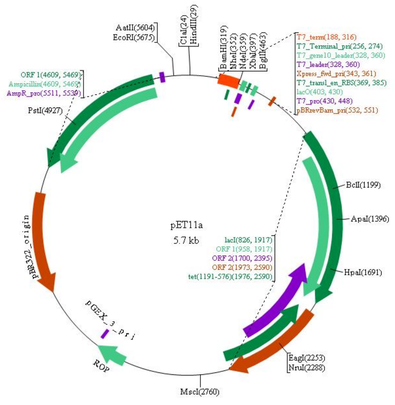


Figure 2: Schematic drawing of the PCR cycle. (1) Denaturing at 94-96°C. (2) Annealing at (eg) 68°C. (3) Elongation at 72°C (P=Polymerase). (4) The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle.



Creating an Expression Plasmid



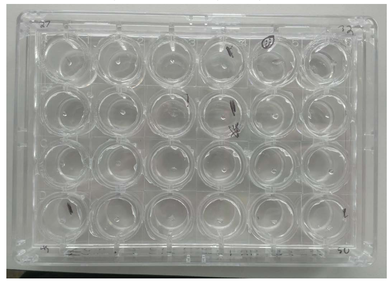
The PCR worked, and the amplified gene formed a very visible band in the gel. This band was cut out and purified. The amplified gene was then digested with restriction enzymes (in our case BAMH1 and NDE1) that gave it "sticky ends", or cuts that leave some nucleotides unbonded. The pET11a vector was maxiprepped and digested with the same enzymes so the sticky ends will overlap with the ones on the gene. The gene was then ligated into the newly created hole in the vector plasmid. The vector was placed in solution with "max-efficiency competent" E. coli bacteria, which were heatshocked into absorbing the plasmids. The bacteria were placed on plates laced with the antibiotic ampicillin (the pET11a plasmid, carries an ampicillin resistance gene) so any bacteria not containing the modified plasmid are killed off while the survivors might grow into colonies). After a growth period, no colonies were observed. We will have to try this again in the future.

Purification of ASB9

ASB9 was purified by size exclusion chromatography. In this method, the protein is run through a column of small, porous particles. Smaller molecules run through these particles and are slowed down, while the larger proteins are able to bypass the particles and run through more quickly.

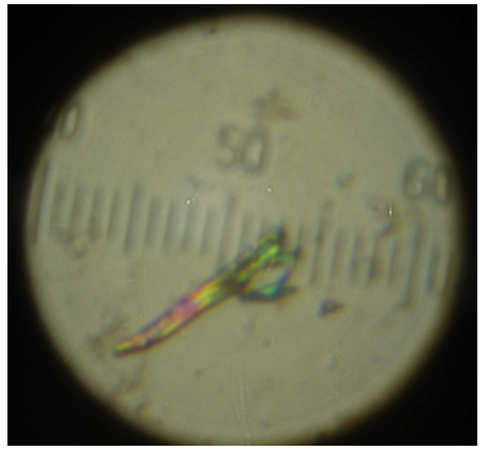
Protein Crystallization

Proteins, like many other macromolecules, can form into crystal lattice structures if placed in ideal conditions. These protein crystals can then be analyzed through X-ray Crystallography, so that the tertiary structure of the protein might be determined. A beam of X-rays is scattered through the crystal at different angles, and the diffraction patterns are recorded. These patterns are compiled to find the 3D locations of atoms in a single protein molecule. Protein crystals used in this process are difficult to make and are inherently fragile, as such complex molecules have trouble forming into a crystal lattice. To form such crystals, a drop of the protein is combined with an equal volume of a buffer containing a precipitant and the mixture is suspended in an airtight system above a well of that same buffer and precipitant. As water from the drop evaporates, the concentration of protein and precipitant in the drop rises slowly until it reaches the idea concentration for crystallization. Because the system is in equilibrium, the concentration will hover around an ideal value. Factors such as the pH of the buffer, the precipitant used, and the concentration of the protein can affect the chances of a crystal forming, so many different buffer/precipitant combinations must be tested.



Crystallization of Creatine Kinase (open state)

CK crystallized when placed in a buffer containing the precipitant Ammonium Sulfate ((NH4)2SO4), which is interesting, because sulfate has similar structure and chemical properties to phosphate, the ionic compound that CK normally interacts with. Although it might be a coincidence, CK probably had an easier time interacting and forming a regular pattern with such a familiar molecule in the solution.



The crystallized protein molecules have chiral properties (they rotate light passing through them). So if a protein crystal is viewed through a polarizer, it will stand out from the background sharply with a change in color, as it does in the picture shown (insert pic), even though it might be clear in nonpolarized light.

Now that we have found conditions in which CK crystallizes in the open state, we can use these to crystallize the complex of ASB9 with CK.

References

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- M. A. Debrincat *et al.*, Ankyrin repeat and suppressors of cytokine signaling box protein Asb-9 targets creatine kinase B for degradation. *J Biol Chem* 282, 4728 (2007).
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