# Change in the conformation of Troponin with the absence of Ca2+

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## Abstract

The troponin complex is made up of three proteins, troponin I (TnI), troponin C (TnC) and troponin T (TnT). It has been alleged in an unpublished poster of Vinogradova & Fletterick (sk) of the existence of a 'switch peptide'. The switch peptide is a small portion of TnI that seems to move, upon the addition of calcium. This experiment was designed to assess the validity of said poster. This project within the experiment focused mainly on the growing, expression and purification of the troponin complex. Three possible EPR techniques will be used to determine the distance of the calcium absent complex's switch peptide from the coiled-coil of TnI and TnT as compared to the peptide's position distance from the same place in calcium laden troponin. The importance of this movement is that troponin controls tropomyosin which controls myosin and actin interactions, which in turn directly impacts muscle relaxation and contraction. Therefore any change to conformation of troponin I is fundamental to understanding muscle movement. The results of the experiment are incomplete at this time.[1]


## Introduction

Muscle movement is a very intricate albeit amazingly immediate process. Different proteins take on different roles in creating the contraction and relaxation effect of muscles.

[1] This experiment was performed at the Institute for Molecular Biophysics (IMB) at Florida State University in the lab of Dr. Piotr Fajer. The graduate student responsible for creating and completing the experiment was Mr. Jean Chamoun.

Muscles, (cardiac, skeletal and otherwise) are composed of many stacks of myofilaments. Myofilaments are made of molecules and proteins including but not limited to, myosin, actin, tropomyosin and troponin. Myosin and actin bind and create movement because myosin scales along the thin actin filament and by doing so creates movement of muscle. Troponin acts in an inhibitory method by sitting in the crevices designated for myosin. Until the troponin complex moves, myosin is unable to insert itself and therefore the muscle remains inactive or relaxed. The three proteins of the troponin complex act in different ways to accomplish troponin's inhibitory role. TnI binds to the molecule actin, TnC binds to the calcium and TnT binds to the myosin. The function of troponin's calcium altered states has yet to be realized.

Troponin is a complex made of three proteins and is therefore made up of peptides, which are themselves made up of amino acids. TnI's normal chain of amino acids includes many cysteines. In order to have some way of measuring the distance of the switch peptide from the coiled-coil at its alternating positions, Electronic Paramagnetic Resonance (EPR) techniques will be employed. The EPR techniques read labeled cysteines, which are essentially regular cysteines with an extra unattached electron from a molecule with an unbonded oxygen. The project requires only two points on the TnI to measure between. To accomplish this all the cysteines are removed and thus the protein, TnI, becomes cysless. Then two cysteines are inserted in to the amino acid chain in specific places. The 'spin labeling' of those particular cysteines inserted via mutagenesis assists in

receiving a signal from the EPR techniques. The EPR methods will give the distance that the switch peptide is from the coiled-coil in the calcium absent TnI to compare to the original distance of the calcium containing switch peptide in TnI. The words TnI and protein will be used interchangeably because the methods for TnT and TnC are similar unless specifically designated differently.

**Methods and Materials**

*Mutagenesis.* This acquisition of the double mutant of TnI is the beginning step of the entire experiment. It is necessary that the distance between the two cysteine mutations be greater than 50 Angstroms (A) because it needs to be greater than the measurement capabilities of the EPR. It needs to be that large so any signal from the EPR for the calcium deficient TnI will mean an appreciable difference. A primer of around 150 nucleotides of DNA is designed in the lab by using the codes for specific amino acids. The new TnI is coded with only one mutation at one of the following three site; 129, 133, 115. Although the TnI needed in this experiment is a double mutant, we only code for one mutation because the DNA plasmid used already has a cysteine mutation at $160^{th}$ amino acid. Different prep kits are used to amplify the DNA including a Qiagen® polymerase chain reaction (PCR) kit. In the PCR reaction, the DNA is replicated by unzipping the DNA using certain enzymes and then reforming additional strands of DNA.

*Expression.* There are three steps within this step. The first step is to have the protein cloned by E-coli. This is done using media in which the E-coli will flourish. E-coli is used because it will reproduce and clone itself while creating the TnI within it. The protein is then

expressed onto Agar® plates. This does not produce enough TnI to use but it does create a very concentrated sample of TnI. This sample is then frozen at -80°C to prevent it from further expressing or oxygen degradation.

The second step uses the frozen stock. That step is to perform a small scale grow up. In this grow up, a small amount of TnI is placed into 4 flasks, each with five milliliters (mL) of media, Terrific Broth (TB), in which TnI will flourish. Then the flasks are incubated at 37°C, human body temperature, and the bacteria with the proteins inside grows at an exponential rate. This lasts for four to five hours.

The third step in the expression process is the large scale grow up. In this step, the volume of media, TB, is increased to one liter (L). The small flasks are poured into very large flasks, two small ones per one large one. Then 500mL of TB is added to each large flask. These flasks are then incubated at 37°C for the bacteria to grow.

*Lysing the cells.* Between *Expression* and this step, we centrifuge the sample to get a pellet of cells. In this step we open the cells within this pellet to expose all the insides of the cell, including the mutated TnI. In this step we use a microfluidizer, which deftly opens the cell wall without leaving any contamination like other methods, such as lysosyme, would leave. Lysosyme is an enzyme that can be added to a sample to open cell walls but the microfluidizer machine works better and is cleaner because it simply uses pressure. Within the microfluidizer, there is a tiny hole, only slightly smaller than a protein cell. The machine uses different gasses to push the cells through there by breaking their walls.

*Harvest.* The next step is to harvest the TnI from the cellular supernatant the microfluidizer creates. This is done with a centrifuge. A centrifuge is a machine with a large spinning inside that applies a great rotating force on a sample that separates the heavy from light weight. TnI is heavy and therefore after centrifugation, the pellet is saved. This differs for each protein in the complex, TnC is light and is in the resulting supernatant whereas TnT is heavy and is in the pellet.

*Dialysis.* This phase is where the TnI is saturated with a buffer to achieve a specific pH. A protein has a specific pH where it is neutral, called the pI. If a protein's pH is lower than its pI, the protein will become positively charged. If a protein's pH is above its pI, the protein will become negatively charged. This is important because the next step is purification and the purification column that the protein will be run through has a certain charge and to be attracted to the column, the protein needs to have the opposite charge of the column.

The pI for TnI is 9.5. The pH that is specified and achieved in lab is 8.0. This is lower than the pI and the TnI thus becomes positively charged. This is important because our column is negatively charged.

*Column.* This stage is the purification step for the protein. A column is mass of a certain resin used to attract only a specific protein. It can be an affinity column, which means that a column is engineered to have antigens specific for a protein. Another type of column is the size-exclusion column, where only the protein is let through because it is of specific size. A column can also be ion exchanging. This is the kind of column used in this phase. The column is a phenol speharose column, which is a

cation-exchanging column. The column is charged oppositely from the protein and thus the protein is attracted to the column.

The column is attached to a Bio-Rad® chromatography machine that pressurizes the column and speeds up the running of the column. It also collects the purified proteins, called fractions. The rest of the substance (contaminants) is flushed through and is called the 'void.' While the void is flushed through, the protein is securely attached to the column. After the void is successfully flushed, the sodium chloride content is sharply increased and the column releases the protein and a purified TnI is collected by the Bio-Rad® system. The extent of purification of the protein is of great interest.

*Gel.* This segment is where it is determined how pure and how concentrated the fractions from the column are. This is begun by analysis of the chromatography graph to determine probable fractions of concentrated TnI. The peaks on the graph signal this. Then a sample is taken from the probable fractions and transformed into a negatively charged sample. Then each sample is inserted in a separate lane of a gel. TnI standards are inserted in two lanes as well. A standard is a known and very clean sample of TnI with which comparisons of newly purified samples of TnI are made. Once in the gel a voltage is applied and the samples travel away from the negative side to the positive side. If the sample is TnI, it will travel to the same position as the TnI standards. The cleaner the sample is the sharper the band will be. This determines how clean the protein is and assures that the sample is actually troponin I.

*Labeling.* At this phase the last preparation for the EPR is to label the two cysteines. The two labels used will be MSL or MTSSL. This adds an unpaired electron to the cysteines via an unattached oxygen in the labels' structures. This is done by inundating the protein with a buffer containing the specified label and urea, called the labeling buffer. This must be done at 4°C or lower.

*EPR.* This segment of the project is just in its infancy. The plan is for EPR to determine a distance of the switch peptide in calcium absent TnI. The distance in calcium added TnI is so large that it is undetectable by EPR techniques. Therefore any signal received by the EPR will represent a decrease in length and therefore confirm the hypothesis that the switch peptide moves with the quantity of calcium.

A control will be used to be sure that the EPR is not incorrectly reading the cysteines of two different troponin complexes as opposed to correctly reading two cysteines within a complex.

**Results**

As yet, there are no results for the entire experiment, in terms of EPR distances. There are results for the protein expression. The results of the TnI expression and purification are positive. The gel from then TnC is clear and succinct, as a gel should be. The graph from the chromatography system shows the appropriate peaks on the absorption line (the lower line). The gels and graphs from the TnI purification are just as concrete. The TnT results are less glowing. TnT is temperamental and it basically has yet to grow well enough to purified.

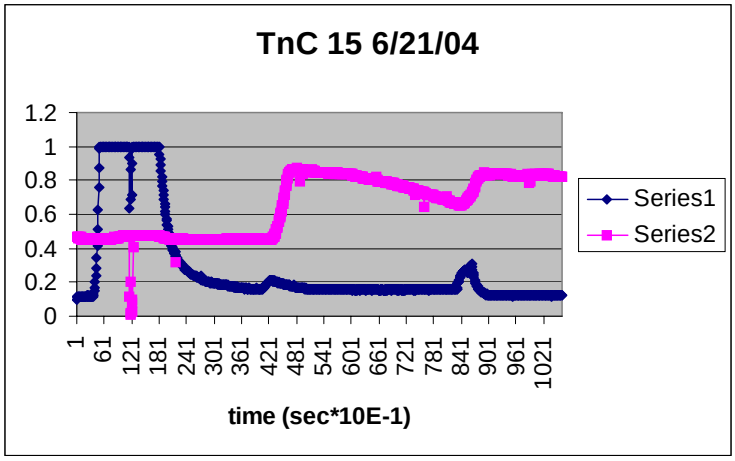
**Discussion**

Other methods beside EPR could be utilized but EPR will give us the most exact results. The three types of EPR to be used are electronic coupling, dipolar coupling and DEER. They have measurement sensitivity of 8A, 10-20A and 50 A respectively. The movement of the switch peptide is a widely debated topic among biophysists. Another closely related project is being worked on that measures the distance of the switch peptide from the N-terminus of TnC with and without calcium. The results of these two experiments should be conclusive as to whether the switch peptide actually switches.

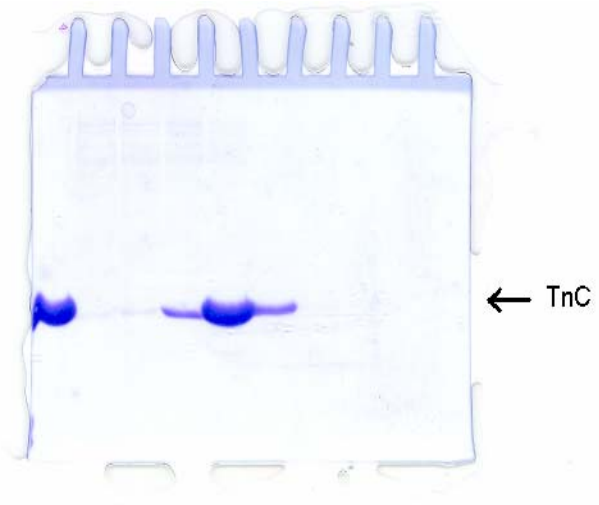
**Conclusion**

It has been alleged in an unpublished poster of Vinogradova & Fletterick (sk) of the existence of a 'switch peptide'. Through microbiology, molecular biophysics, protein chemistry and EPR techniques, this experiment attempt to verify the theory. The bulk of the specific project was protein purification, especially of TnI. This was done in order to determine the distance of the calcium absent troponin complex's switch peptide from the coiled-coil of TnI and TnT as compared to the peptide's position distance from the N-terminus of TnC in calcium laden troponin. The importance of this movement is that troponin controls tropomyosin which controls myosin and actin interactions, which in turn directly impacts muscle relaxation and contraction. Therefore any change to conformation of troponin I is fundamental to understanding muscle movement.

## Graphs and Pictures




[1]Bio-Rad® chromatography graph. The lower (blue) line is the one whose peaks determine concentration of fractions. The top line (pink) is the salt concentration.




[2]TnC gel. TnC samples in lanes four through six. TnC standard in lane one. The thicker middle one is more concentrated but slightly les pure. The thinner ones are more pure but less concentrated.

**References**

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