# Structural studies of a calmodulin mutant with defective regulation of muscle contraction

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## Background

Calmodulin (CaM), a protein found in all eukaryotes, is primarily involved in regulating the levels of calcium within the cell. The scope of this research project limits the study of calmodulin to its function in muscle fibers. In muscle, calmodulin binds to an ion channel, the ryanodine receptor (RYR), which is responsible for the release of calcium from intracellular storage compartments called the sarcoplasmic reticulum. Calmodulin regulates the opening and closing of the ryanodine receptor: at low levels of calcium CaM primes the channel for opening, while at high calcium levels it closes the channel (Ref. 1). CaM thus acts as a calcium switch, providing the necessary feedback for maintaining appropriate calcium levels during muscle contraction. This role is essential in preventing damage to muscle tissue; excessive calcium exposure is harmful and can lead to hyper-contraction and muscle failure.

The functions CaM performs in cells are linked to its unique structure: the protein has a dumbbell shape, with two globular domains (N-terminal and C-terminal) connected by a flexible linker which allows conformational flexibility (Ref. 2, Figure 1). Moreover, CaM can bind up to four calcium ions, and interacts with many target proteins in a calcium-dependent manner. The structure of calmodulin is different at low versus high calcium concentrations, and as a result CaM can bind to targets in different ways depending on the concentrations of calcium in cells.

The process of excitation-contraction coupling (EC coupling) is an essential part of both skeletal and cardiac muscle contraction. In muscle cells, electrical impulses from neurons result in membrane depolarization; this signal initiates a complex process which ends in the release of calcium into the cell, resulting in contraction (Ref. 3). Although many aspects of EC coupling are well understood, there are still many unanswered questions about the role CaM plays in this process. In particular, the molecular details of how CaM acts as a calcium switch to regulate the flow of calcium ions are not known.

Functional studies have shown that if there is a defect in CaM that allows for excess calcium to be released, muscle fibers may be significantly damaged. The importance of CaM in the regulation of calcium levels can be seen in fruit flies (*Drosophila melanogaster*) that have undergone a single point mutation, specifically a replacement of valine with a glycine at position 91 in the CaM protein (V91G CaM). In genetic studies by Dr. Beckingham at Rice University, defective insects experienced hyper-contraction of muscle fibers and eventual muscle failure due to excessive calcium leakage in cells (Figure 2), indicating that the feed-back function of CaM was compromised (Ref. 4). Biochemical studies have shown that this is due to an altered interaction between the mutant CaM and the ryanodine receptor at both low and high calcium concentrations (Ref. 5). However, no atomic-level structural details of the V91G mutant CaM and its altered interaction with RYR are available.

**Procedure**

In order to observe any structural changes in the V91G mutant it was first necessary to produce a sample for study with nuclear magnetic resonance (NMR). Therefore, the first weeks of research were spent growing, extracting, purifying, and preparing the sample for NMR. The first part, protein growth, involves a culture of bacteria in which the target protein, calmodulin, will be grown. Using basic techniques in biology host bacteria were grown in a solution of LB, glucose, and potassium phosphate overnight at 37°C. The following morning the optical density of the bacteria in the solution was monitored until red, 600nm wavelength light, was reflected at a ratio of 0.4 to 0.6. Once the culture was of sufficient density a small amount, 1mL of .4 molar to be exact, of IPTG was added to the bacterial solution to induce the production of the target protein in the bacteria. The bacteria were then allowed to grow the target protein for four hours before being moved into a storage container for the next step.

The target protein was now present; however, before analysis of the protein could be performed it had to first be removed from its bacterial host. Using a buffer to resuspend the bacterial cells, the organelles, proteins, and other components of the bacteria were now free floating in the solution which could then be separated. Using a very powerful centrifuge the different parts of the bacteria formed a

slimly pellet of matter at the bottom of the centrifuge bottle. Once removed the pellet was then suspended in a solution of EDTA, a substance that strips calmodulin of its calcium. Then using a technique called sonication ultra-high frequency sound waves were allowed to bombard the sample and lyse open the bacteria. At this point the target protein, calmodulin, was removed from the bacteria, but still in solution with all other protein and organelles of the host bacteria.

The next step in preparing a sample for NMR was to purify the bacterial pellet so that calmodulin was in very high concentration. Calcium was added to the solution and calmodulin was allowed to bond. When calmodulin is bonded to calcium the protein exposes its hydrophobic side chains making it more lipid soluble than water soluble. Therefore, a technique called hydrophobic interaction was used to capitalize on this fact and allow for a high degree of purification. A hydrophobic resin was added to the solution and suspended so that the hydrophobic calmodulin attached itself to the resin. Then, through a series of washes in hydrophilic buffers, all of the other parts of the bacteria were removed leaving behind a highly concentrated form of the target, calmodulin.

While the sample should contain purified protein at this point in the procedure the target protein has yet to be observed directly by any mean. Sending a sample to be tested using NMR would be risky since it is impossible to know whether the protein was actually expressed by the bacteria, so verification of expression was the next and final step before sending the sample to be tested. Using gel electrophoresis the presence of the target protein in the sample was confirmed (Figure 3). The protein band that was seen after the washes corresponds to a protein of similar size as calmodulin which provided strong enough evidence to continue with NMR.

Nuclear magnetic resonance is a technique used in structural biology that is similar to MRI which in used in diagnostic medicine. The nuclei of the protein atoms are aligned in an intense magnetic field and are then bombarded with radio waves at specific frequencies to resonate only certain type of nuclei. Using information about the resonant frequencies of three types of atoms (hydrogen, nitrogen, and carbon) an accurate depiction of conformational changes can be made about the protein. NMR data were

acquired at the NMR facility at the University of Iowa Medical School. After taking data on the sample made in the lab it was necessary to process the data into a form that could be interpreted.

The final and most complicated part of this research project was data processing and analysis using two software programs, NMRPipe (processing), and SPARKY (data analysis). After processing was completed with NMRPipe, the next step was to begin analysis of the data. In this particular NMR project, four different experiments were used to get information about the positions of different residues in the protein: HNCACB, CBCA(CO)HN, HN(CA)CO and HNCO. Each experiment gives information about the same residues and the redundancy of the data is what allows for the data peaks to be assigned to corresponding residues on the protein chain (Figures 4 -7). Once the data peaks were assigned, a comparison to data about the wild type protein was made concluding the research project.

**Results**

The experiments done on calmodulin were successful in acquiring some data; however, the dataset was incomplete. After assignment was finished, it was clear that much of the C-lobe of the protein was "missing" data peaks while the N-lobe was almost completely assigned (Figure 8). The missing information could be attributed to many different sources; however there are only two that appear plausible. The first is that the protein sample that was sent to be tested using NMR was bad or contaminated in a way that disturbed the data collection. This is not consistent with the data, since the missing data appears to be localized to the C-lobe and the N-lobe is fully visible. The second justification for the missing data may be that the V91G mutation destabilizes the protein making it highly dynamic in a calcium-free environment. This is consistent with the data that was collected; however, there is not enough information at this time to confidently assert this to a major publication.

Despite a large portion of the data being missing it is still possible to draw a few basic conclusions about the conformational shift in the V91G mutant protein. Figures 9-11 shows the difference in chemical shifts from the wild type control and the data taken on the V91G mutant. The data shows large changes in chemical environment in the C-lobe, where the mutation is located, and minimal shifts in

the N-lobe. From this it is possible to assert that the mutation is having an effect of the conformation of at least the C-lobe, as was to be expected.

Finally, in order to clear up any doubts about the nature of the missing data it is advisable to check the protein sequence of the sample run during this summer and conduct another set of experiments with both the wild type and V91G mutant to ensure identical conditions in the protein. These new experiments should provide enough information to either answer the questions that remain after work this summer or merit the continued study of the temperamental target protein.

**References**


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**Figure 1. Ribbon diagram representation of calcium-loaded CaM.** The crystal structure of CaM indicates that the molecule is formed of two globular domains (lobes) connected by a long central linker that is flexible in the middle. The protein can bind four $Ca^{2+}$ atoms (shown as red spheres). The location of the V91G mutation is indicated by the arrow.

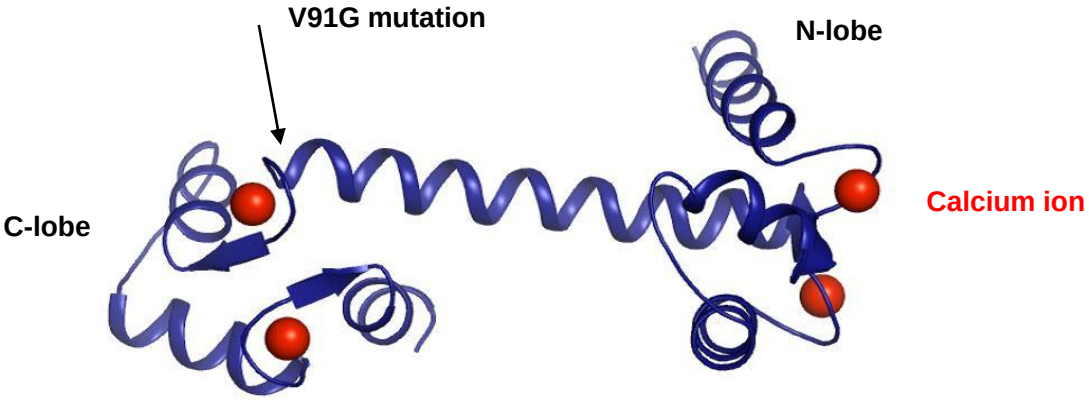
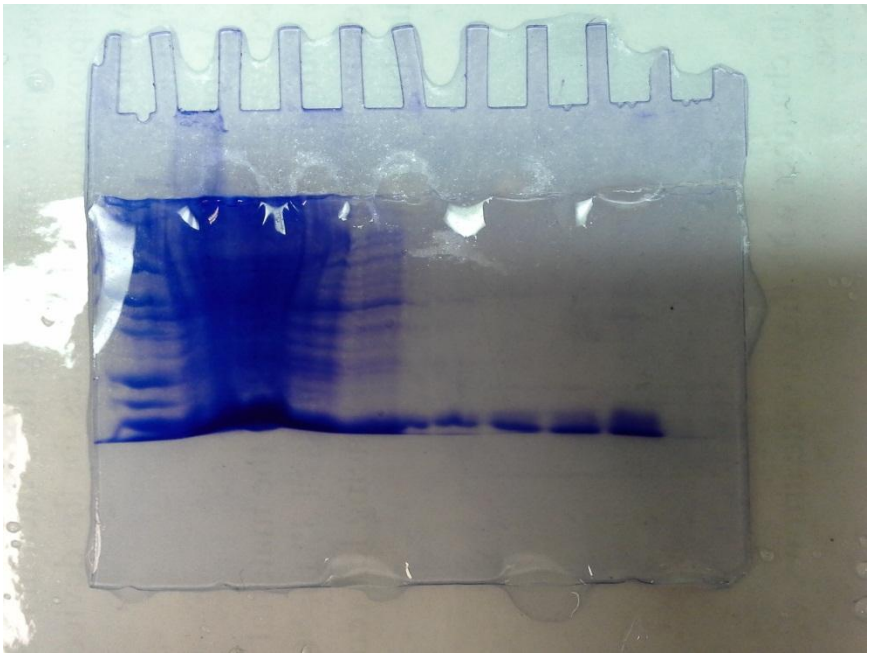



**Figure 2. Wild-type (a) and V91G mutant (b) fruit fly larvae.** The mutant larvae have significant musculature defects due to muscle hyper-contraction. Adapted from Wang et al. (2003).

**Figure 3. Gel Electrophoresis used to confirm expression of target protein.** The protein band on the far-right represents the most highly purified protein sample of the target protein, calmodulin. The far-left is a marker protein which is used as a guide for measuring the size of the protein in the sample. All samples in-between represent intermediate steps in the purification process.



**Figures 4-7. NMR experiments.** The four experiments here give varying information about both the type of residue represented and the sequence in which the residue occurs. The top two, HNCACB and CBCA(CO)HN, are primarily used in assigning residue type, and the bottom two, HN(CA)CO and HNCO, are primarily used to confirm sequence of the residues in assignment.

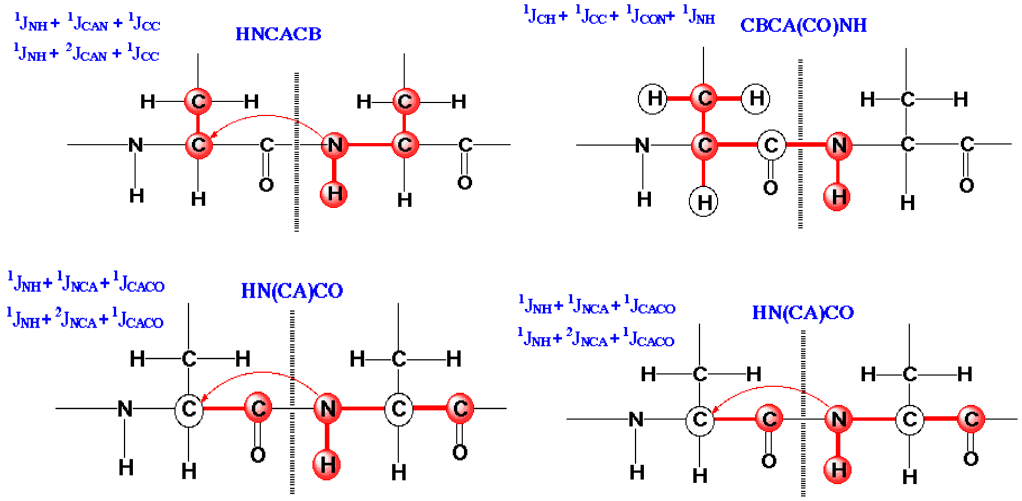
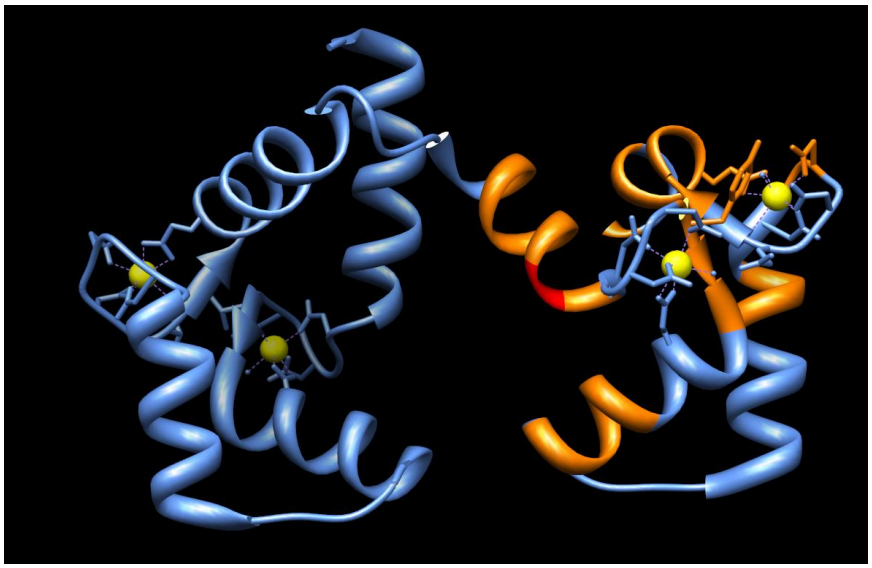
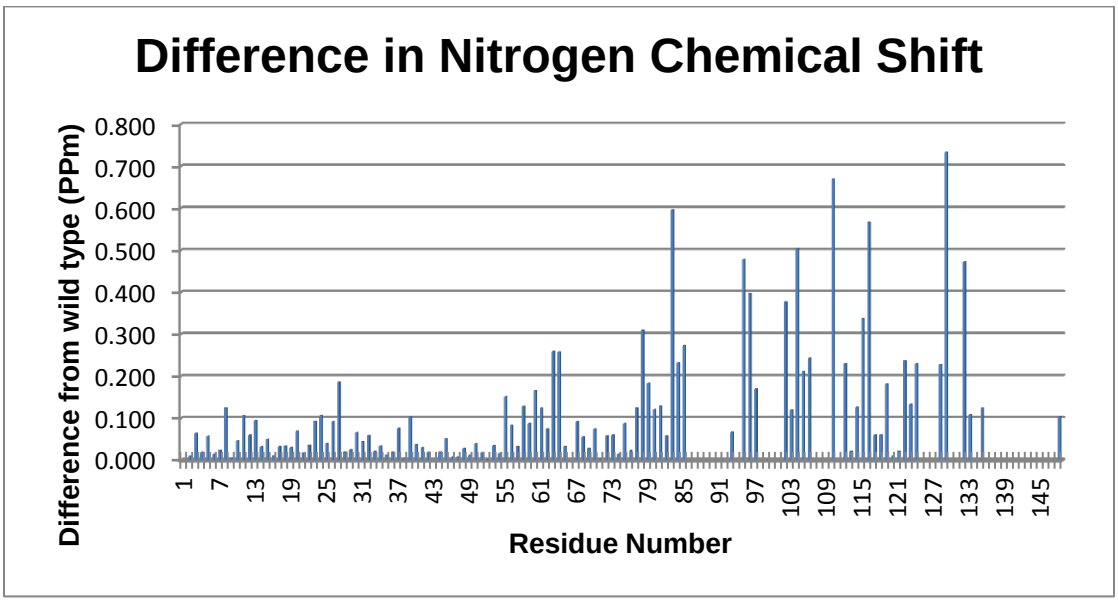
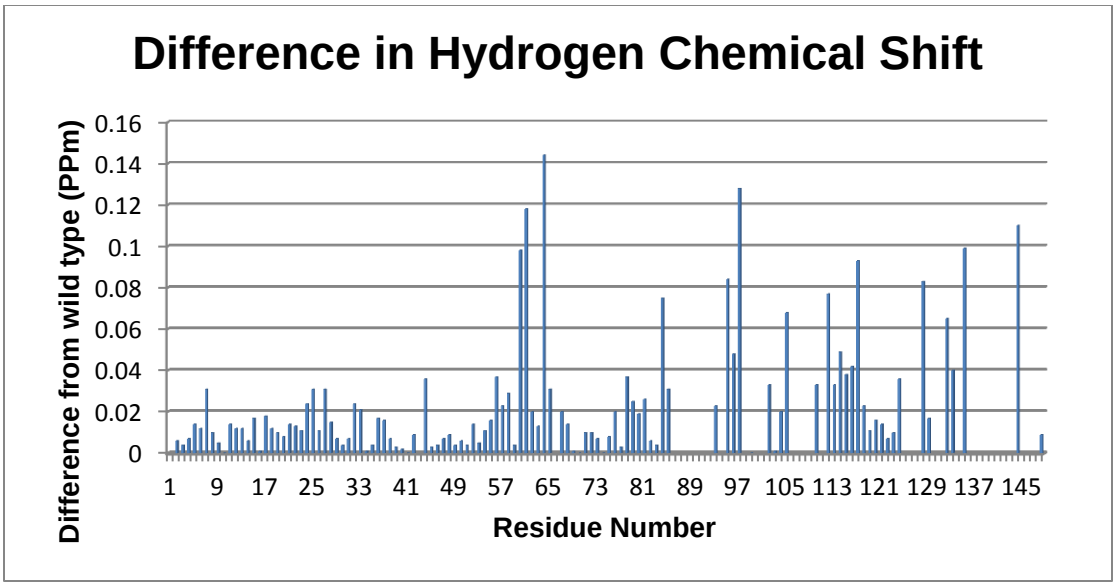


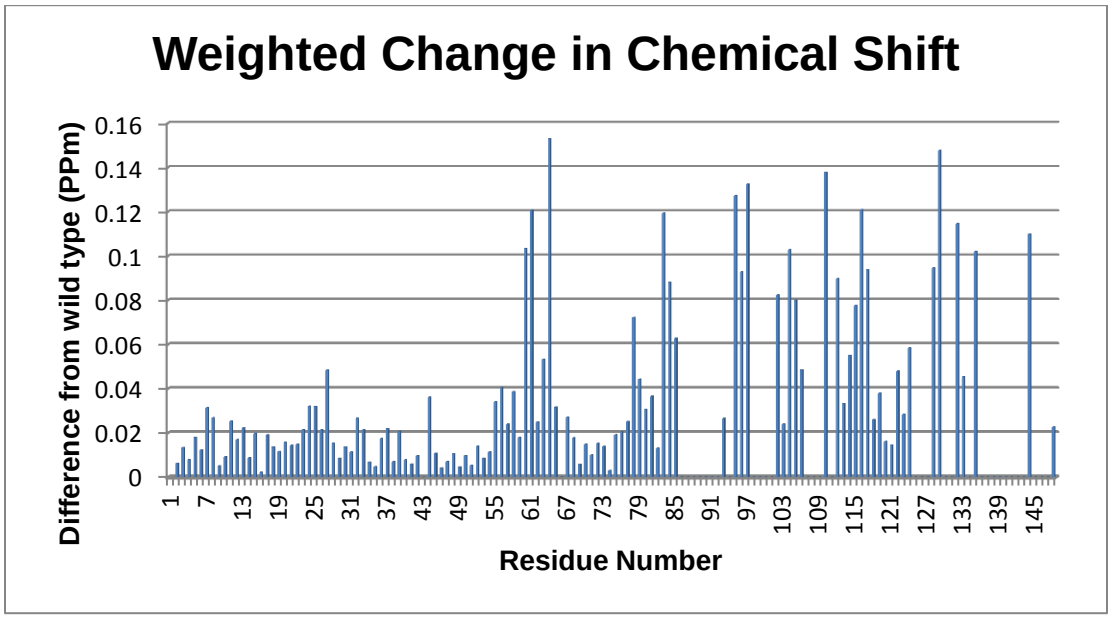
**Figure 8. Missing data peak information.** The NMR experiments taken this summer are missing information about many of the residue in the C-lobe of the protein. The section of the protein in the diagram highlighted in red represents the mutation and the sections highlighted in orange represents all locations on the protein where data is missing.



**Firgures 9-11. Differences in chemical shifts.** The value represented by the bar graph show the difference in chemical shift between the wildtype and V91G mutant for each individual residue. The larger the value the greater the change in the environment around the specific residue; a greater change chemcial shift implies a greaters conformational change.




Difference in Hydrogen Chemical Shift
Difference from wild type (PPm)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1 9 17 25 33 41 49 57 65 73 81 89 97 105 113 121 129 137 145
Residue Number


Weighted Change in Chemical Shift
Difference from wild type (PPm)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1 7 13 19 25 31 37 43 49 55 61 67 73 79 85 91 97 103 109 115 121 127 133 139 145
Residue Number