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

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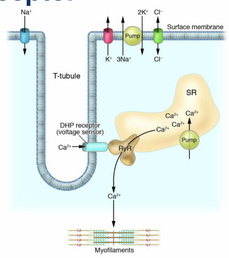
Abstract

Calmodulin (CaM), a two-lobe protein found in all eukaryotes and primarily involved in regulating the levels of calcium within the cell. In muscle, calmodulin regulates the opening and closing of the calcium channel ryanodine receptor, providing the necessary feedback in maintaining appropriate calcium levels during muscle contraction. A single point mutation (V91G) in the C-terminal lobe of CaM causes excessive calcium levels in muscle due to altered interaction with the ryanodine receptor, leading to hyper contraction and muscle failure. The aim of this project was to understand at the molecular level how the V91G mutation affects the structure and/or dynamics of CaM at both high and low calcium levels, by using NMR spectroscopy.

The proton-amide resonances of calcium-free V91G CaM were assigned using several three-dimensional NMR experiments, including HNCACB, CACB(CO)HN, HN(CA)CO, and HNCO. Interestingly, a large subset of the resonances in the C-lobe of CaM was missing; on the other hand, we were able to completely assign the N-lobe resonances. Comparison with the chemical shifts of wild-type CaM published in the literature indicates that the structure of the N-lobe is not affected by the mutation, while the C-lobe undergoes more significant chemical shift changes that are spread throughout the sequence. Future work will be aimed at further investigating the structure and dynamics of the C-lobe of V91G CaM at low calcium concentrations.

Calmodulin modulates calcium flow through the ryanodine receptor

- CaM influences Ca^{2+} release from the SR by directly interacting with RyR1 in the presence or absence of Ca^{2+}.
- At nM Ca^{2+} concentrations, CaM is a weak activator of RyR1, while Ca^{2+}CaM inhibits the channel.
- This forms a biofeedback mechanism that helps to regulate appropriate levels of calcium in the muscle at any given time.



Observations of V91G defect in fruit flies

- The importance of CaM in the regulation of calcium levels can be seen in fruit flies (*Drosophila melanogaster*). 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.
- 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.

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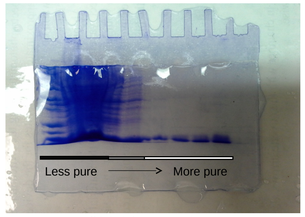
Hydrophobic interaction and gel electrophoresis used to purify and confirm protein expression

- Before NMR analysis of the protein sample began the sample was purified to a very high degree to remove noise from other biomolecules naturally occurring in bacteria cells.
- In a calcium rich environment CaM folds in such a way that its hydrophobic side chains are exposed to the surroundings (i.e. various buffers). This property means that CaM will be soluble in hydrophobic substances, such as a resin.
- In the purification process CaM was first allowed to bond with the calcium and dissolved in a resin, the resin was then washed to remove impurities, finally a Ca^{2+} chelator was used to remove the Ca^{2+} from the protein.
- After the process was repeated several times the protein sample was left in a highly purified state.

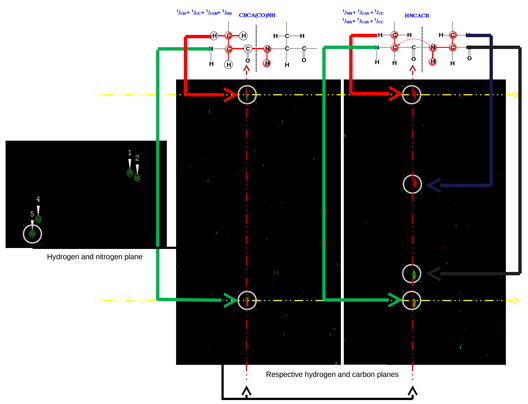


Note: the first column was a reference

- Gel Electrophoresis gives a visual representation of various molecules in the sample based on the size. Allowed for confirmation of protein expression
- The figure illustrates the purity of the sample. Samples were removed periodically; the left side represents samples takes early in the purification process.

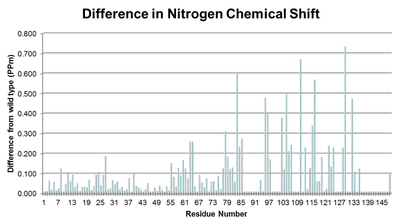
Structure of the mutant is ascertained primarily through four NMR experiments

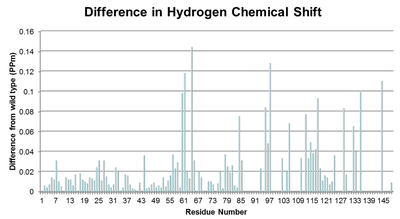
- The two lobes of CaM have very similar chemical environments; consequently, there are several redundant sequences of amino acids making CaM especially hard to assign with NMR.
- In order to properly assign the protein several different data sets, in addition to the standard HNCACB and CACB(CO)HN experiments, had to be used in parallel to ensure that sequences of amino acids were being placed in the correct spot in the chain.
- The HNCACB and CACB(CO)HN NMR experiments gave information useful in assignment of the residue type making them effective in placing sequences in the chain rather than identifying the order of a given sequence.
- The HN(CA)CO and HNCO NMR experiments gave accurate information about the order in which residues occur rather than information about the residue type, making it superior in identifying order.
- Only by using these two sets of NMR experiments was the assignment of CaM possible.



Differences in chemical shifts in the C-lobe substantiate claims that conformational changes are occurring

- The conformational shift in the V91G mutant protein (graphs below), shows the difference in chemical shifts between the wild type control and the data taken on the V91G mutant. The data shows large changes in environment for the C-lobe, where the mutation is located, and minimal changes for the N-lobe.

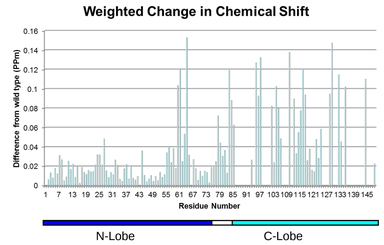




- From this it is possible to assert that the mutation is having an effect on the conformation of at least the C-lobe, as was predicted based on the V91G mutant's inability to regulate calcium.

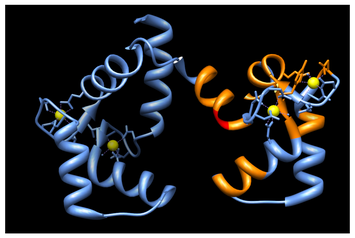
Missing data may indicate large disruption in and around the alpha helix near the mutation

- The weighted chemical shift, a combination of nitrogen and hydrogen chemical shifts, shows that residue near the V91G mutation, especially in the C-lobe, experienced a large change in their chemical environment
- However, only basic conclusion about the conformation of the protein can be made due to the amount of missing information.



- The missing information can be explained by a variety of theories; however, only our intuition suggest that either the sample was contaminated or the mutation is causing a disruption in the alpha-helix where it is located.
- In order to rule out the possibility of simply a bad sample the protein will have to be re-expressed and retested.
- If, however, the missing data is a result of disruption in the alpha helix new methods of data collection will have to be investigated.

Conclusion/Recapitulation



- . 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. The diagram to the left shows the location of the missing data peaks, orange, in relation to the mutation, red.
- This missing data could be the result of a contaminated protein sample for NMR collection. However, 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.
- Our other explanation for the missing data is that the V91G mutation destabilized 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 claim.

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Future Research Opportunities

Further Investigation of V91G Mutant

- Taking additional NMR experiments of the V91G mutant must be done in order to come to a conclusion regarding the missing data.
- Given the protein proves to be highly dynamic, as hypothesized from the result of these previous experiments, new techniques for assignment would have to be investigated.
- Examination of the alpha-helix, where the mutation presents itself, could help to explain our previous results.

Automated NMR Data Assignment

- Data collection by means of traditional NMR data analysis software (e.g. sparky) could benefit greatly from automation of data peak and/or residue assignment.
- Using techniques from experts in NMR data collection in combination with theories from computer science, namely artificial intelligence, the development of fast and accurate assignment appear promising.
- Currently a suitable heuristic is being developed to assist in assignment.