Expression and Purification of Calmodulin and its Interaction with the SK peptide

Written by

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Abstract

Calmodulin (CaM) is a highly conserved protein that has been observed to regulate ion channels. Calcium (Ca^{2+}) binds to Calmodulin in order to activate Ca^{2+} activated Potassium channels (SK Channels). Calmodulin is known to responsible for gating the SK channel; however, the mechanism in which it does this is unclear. In order to study this interaction, wild-type CaM, single lobe or half CaM, double lobe CaM, and a SK channel peptide (SKp) were all purified. Purification of proteins was done through high performance liquid chromatography (HPLC) and verified with SDS gels. The CaM-SKp complex was analyzed through CG-MALS (composition gradient multi-angle light scattering). CG-MALS can be used to determine the molar mass of complexes formed. Stoichiometry can be determined if molar mass of the proteins complexing are known. Results from CG-MALS show the formation of stoichiometries that have not been observed before. The introduction of these new stoichiometries adds complexity to the SKp-CaM interaction and require more testing in order to fully elucidate the mechanism of SK channel gating.

1. Introduction

1.1 Calcium Signaling

Calcium (Ca^{2+}) signaling is responsible for a variety functions in the cell including cell excitability, exocytosis, motility, and transcription. One specific function of Ca^{2+} is its ability to activate ion channels, of which includes the Ca^{2+} activated Potassium channels (KCa or SK Channels). Ca^{2+} is able to activate these SK Channels through the protein Calmodulin (Xie et al. 1998).

1.2 Calmodulin

Calmodulin is a highly conserved protein that is expressed by all Eukaryotes and it is known to regulate many processes in the cell. These include inflammation, apoptosis, metabolism, intracellular movement, smooth muscle contraction, immune response, and memory (Hoeflich et al. 2002). Calmodulin is able to accomplish this through the Ca^{2+} signal transduction pathway. One aspect of Calmodulin that makes it very interesting is its flexibility. Calmodulin is only 148 amino acids long, putting it at approximately 16.7 KDa which is pretty small for a protein (Cheung, 1980). However, it is still able to bind and modulate hundreds of other proteins (Hoeflich et al. 2002). The flexibility of Calmodulin can partly be explained by its structure. Calmodulin is composed of two distinct globular domains commonly referred to as the N- and C-Lobe (Schumacher et al. 2001). Both lobes have two Ca^{2+} binding sites and all of these binding sites are distinctly different (Halling et al. 2016). The lobes are connected to each other by a region known as the flexible linker (Schumacher et al. 2001). Along with these parts, there are also hydrophobic clefts that play a huge role in the flexibility of Calmodulin and its ability to bind to a variety of target proteins. A schematic showing the residues and EF hands of

Calmodulin is shown in supplemental figure 1 (S1).One group of proteins Calmodulin is known to interact with is ion channels. Specifically, through Ca^{2+}, it is known to activate SK channels as mentioned before, and it is this interaction that our lab has been focusing on.

1.3 Calcium – Activated Potassium Channels (SK Channels)

SK channels are activated by Ca^{2+} through the mediating protein Calmodulin. Once activated, they release potassium ions down their gradient, exiting the cell. This plays a major role in cells as it helps them return back to their resting state after being excited during the depolarization stage. The mechanism in which these channels are activated is unclear. The structure of these channels is similar to that of the voltage gated potassium channels. There are subunits that span the membrane, with four subunits associating to make a tetramer which serves as the "channel" (Xia et al. 1998). Each of the subunits contains six transmembrane hydrophobic alpha helical domains which are denoted as S1-S6. These alpha helical domains of the channel are connected to each other by linkers, which are named based on the domains they are linking i.e. 3-4 linker. The 1-2 and 3-4 linkers are extracellular whereas the 2-3 and 4-5 linkers are intracellular. Between S5 and S6, there is a loop known as the P-loop which serves as the pore-forming region of the channel (Xia et al. 1998). The loop also contains the selectivity filter of the channel. The selectivity filter is responsible for removing the hydration shell from the potassium ion when it enters the channel (Xia et al. 1998). S3 and S4 contain the voltage sensor of the channel, which is responsible for initiating action potentials and changing the membrane potential based on the stimuli the channel comes across. Both the N-terminus and C-terminus of the channel are intracellular and it is near the C-terminus region that Calmodulin binding has been observed. The region near the C-terminus where Calmodulin binds is notated as the

Calmodulin binding domain (CaMBD) (Schumacher et al. 2001). Calmodulin functions as a Ca^{2+} sensor for the channel and is assumed to be responsible for gating it.

1.4 Current Mechanism for SK Channel Gating

Crystal structures have shown that Calmodulin binds to a SK2 fragment in a rather interesting way. The structures show antiparallel Calmodulins binding to antiparallel SK peptides in a dimeric fashion (Schumacher et al. 2001). This structure has a stoichiometry of 2 SK peptides to 2 Calmodulins (2:2). In this crystal structure, the C-lobe of Calmodulin stays constitutively bound to SKp and Ca^{2+} is only observed binding to the N-Lobe. Even under saturating conditions of Ca^{2+}, the C-Lobe EF hands remain vacant (Schumacher et al. 2001). This structure suggests that channel gating occurs when two CaM molecules bridge two SK peptides resulting in an open channel conformation. This occurs when Ca^{2+} binds to the N-Lobe of Calmodulin (Schumacher et al. 2001). According to this model, only the C-Lobe is involved in binding to the channel, the N-Lobe solely binds to Ca^{2+}, and that it requires a 2:2 stoichiometry. Other published crystals structures show support for a 2:2 stoichiometry between SKp and Calmodulin (Zhang et al. 2012).

1.5 Relevance in Studying Calmodulin

There is a lot of benefit in studying Calmodulin. Mutations in Calmodulin have been observed to cause multiple types of Cardiac arrhythmias which include Catecholaminergic polymorphic ventricular tachycardia (CPVT) and Long QT syndrome (Nyegaard et al. 2012). CPVT is characterized by irregular heart rhythm known as arrhythmia, specifically abnormally fast and irregular heartbeat known as ventricular tachycardia. CPVT is an inherited disorder and is known to cause sudden cardiac arrest that has been responsible for the deaths of many young

individuals. It has been found that CPVT is a result of mutations in Calmodulin, one in the 53rd residue and another in the 97th residue. Both mutations were observed to disrupt Ca^{2+} binding and the mutation in the 97th residue was observed to affect the Calmodulin's ability to bind RYR2, a cardiac sarcoplasmic calcium channel (Nyegaard et al. 2012). This makes sense, as RYR2 channels are responsible for cardiac muscle contraction. RYR2 channels transition from a closed to open state when it experiences an increase in local intracellular Ca^{2+} levels. When it opens, it allows for an influx of Ca^{2+} which causes muscle contraction. It is known that Calmodulin binds to the RYR2 causing the channel to adopt its closed conformation, however, if that interaction is disrupted it will lead to a prolonged opening of the channel resulting in excessive muscle contraction characterized by tachycardia. Long QT syndrome is similar to CPVT and is characterized by fast, chaotic heartbeats typically referred to as tachycardia. The disorder is named long QT due to the abnormal length of the QT interval when taking electrocardiograms (ECG) of people who were known to have the disease. Mutations in Calmodulin that affected the 132nd residue were determined to be responsible for this disorder (Pipilas et al. 2016). It was observed that this mutation also affected calcium binding, specifically in the C-domain and this resulted in impaired calcium dependent inactivation of voltage gated Ca^{2+} channels (Pipilas et al. 2016). Studying Calmodulin and specifically its interactions with ion channels can perhaps unearth possibilities in treating diseases caused by mutated Calmodulins.

1.6 Purpose

Calmodulin and a peptide of the SK channel (SKp) were made in house and were analyzed through various techniques including Composition Gradient Multi Angle Light Scattering (CG-MALS), Spectrofluorometer (PTI), and patch clamp. The CG-MALS provided light scattering

data on the Calmodulin-SKp complexes that formed and gave insight into the molar mass of the complexes that formed and binding affinities between the proteins. My main contribution to the lab has been through expressing and purifying the Calmodulin needed for the experiments carried out by the analytical instruments listed above. I was also involved with carrying out some of the experimental runs with the CG-MALS studying the interaction between Calmodulin and SKp.

2. Materials and Methodology

2.1 PCR Mutagenesis

PCR mutagenesis was done in order to make all the new constructs for Calmodulin including various mutant Calmodulins, double lobe Calmodulins, and single lobe Calmodulins. Primers were ordered from NextGen Sequencing Inc. PCR mutagenesis was run with a blank, then primers were inserted and the constructs were formed. Once it has finished, the constructs were then stored for transformation. In order to confirm our construct is valid, samples would be sent to Genewiz for DNA Sequencing to validate whether the construct was synthesized correctly.

2.2 Bacterial Transformation

Bacterial transformations were required for protein expression. These were carried with BL21 cells. First, BL21 cells were removed from -80^{o}C freezer and were thawed slowly on ice as the cells are temperature sensitive. Approximately 100 ng of DNA of the construct being expressed was added to 25µL of competent BL21 cells. The mixture was left alone on ice for 15 minutes and then heat shocked at 40^{o}C for approximately a minute. Samples were immediately removed from the heat and placed on ice for two minutes. Afterward, 175µL of SOC broth is

added to the sample and incubated in the shaker at 37^{o}C for approximately an hour. The samples were then removed and 100µL were spread onto an agar plate with either Carbenicillin or Kanamycin in order to select for BL21 cells with the plasmid. The plate was incubated with the lid side down at 37^{oC} for 16-18 hours and afterward stored at 4^{o}C with again lid slide down to prevent condensation.

2.3 Protein Expression

Protein expression was conducted by first autoclaving two 50ml conical tubes with 20 ml of LB Medium and two 2L Erlenmeyer Flasks with 750 ml of LB medium. These mediums were required per construct. Once medium had been cooled down, Carbenicillin (50-100 µg/ml final) was added to all the mediums at a 1:1000 molar ratio. Next, a sterilized toothpick was used to poke a single colony from the transformed BL21 cells with the desired construct and dropped into the 50 ml conical tube with the 20 ml of LB medium. This was known as the starter culture. This step was done twice and both 50 ml conical tubes were put in a 37^{o}C shaker and left for approximately 7 hours. After the allotted time had passed, the starter cultures were removed from the shaker and placed in a 4^{o}C fridge to stop growth and left overnight. On the next day, starter cultures were each transferred to the 2L Erlenmeyer flasks containing the 750 ml LB medium. The LB cultures were mixed in order to normalize the optical density readings taken later during the protocol so that we would only have to measure one of the flasks. Both inoculated cultures were put into the 37^{o}C shaker. Absorbance was measured at 600nm and was taken every half hour until the absorbance has reached 0.6-0.7. Once it has reached this optical density, it is induced with IPTG and added to the culture at a 1:1000 molar ratio. The induced cultures are kept in the 37^{o}C shaker for an additional 2-3 hours. Afterwards, the cells were harvested through centrifugation. This was carried out by transferring the induced cultures into 500 ml centrifuge

bottles and spinning them down at 6250g for 10 minutes. The pellets were saved and the supernatants were disposed however, 50 ml of the supernatant was kept in order to suspend the pellets. The suspended pellets were then collected in 50 ml conical tubes and centrifuged at 4000g for 10 min. The supernatants were then tossed out and the pellets were labelled and stored at -80^{o}C until purification.

2.4 Protein Purification

Protein purification was performed by first suspending the pellet of the desired construct with a B-Per (Bacterial Protein Extraction Reagent) buffer. The B-Per reagents was made by combining various concentrations of B-Per buffer, protein inhibitors, EDTA, EGTA, $MgCl_2$, DTT, and Benzonase (Universal nuclease). 15mls of the B-Per solution was used to resuspend each frozen pellet. The suspended pellets were then centrifuged at 20000g for 20 minutes at 4^{o}C. The resulting supernatant was removed and stored on ice and the pellet was again resuspended with the B-Per solution and spun down at 20000g. This step was performed twice to maximize protein yield. The supernatants were then combined and the volume was recorded.

The next part of the purification protocol involved using ammonium sulfate precipitation. First, dry ammonium sulfate was added to the supernatants collected to 60% saturation (13.58g/40 ml). The mixture was stirred for one hour in a cold room kept at 4^{o}C. This enabled complete the salt to completely dissolve. The solution was then centrifuged at 30000g for 30 minutes at 4^{o}C. The supernatant was saved and dry ammonium sulfate was added to 80% saturation (8.04g/ml). The pH was measured and adjusted to 4.2 with glacial acetic acid. The mixture was stirred for an hour or until the salt had completely dissolved, and then spun own at 30000g for 30 minutes at 4^{o}C. The pellet was saved and then suspended with 15 ml of 100mM

Tris pH 7.4 plus protease inhibitors. pH was measured and adjusted to 7.0 with 1 M NaOH if necessary. The solution was then centrifuged at 50000g for 30 minutes and the supernatant was collected and stored.

The last part of the purification protocol involved using high performance liquid chromatography (HPLC). First, the collected supernatant was put through a Desalt column and was desalted into 20 mM Tris (pH 7.4). Selected fractions were then injected into a Q-HP Anion exchange column with buffers 20 mM Tris and 20 mM Tris + 1.5 M NaCl. Selected fractions were collected, combined, and injected into a C18 column with degassed H_2O and Acetonitrile. Before the C18 step, all pumps of the HPLC system were washed with water. This was done to avoid freezing the pumps, which would occur if salt were to mix with the acetonitrile. Selected fractions were collected, combined, and injected into another desalt column in order to put our protein in 5 mM HEPES. Concentration of the protein was conducted by taking UV absorbance of the selected fractions from the final desalt and using Beer's Law to determine concentration from measured absorbance.

2.5 SDS PAGE

SDS PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis) was performed in order to determine which fractions contained Calmodulin in cases where the HPLC data was ambiguous. These were also done to reaffirm that Calmodulin was being purified throughout the purification procedure. SDS PAGE was carried out by first making SDS Gels. These gels were made by matching a big and small plate and snapping them together with a plate holder. The resolving gel was made first by combining 9 ml of Acrylamide (40%), 4.5 ml of H_2O, 4.5 ml of Lower Gel Buffer, 90 µL of APS (10%), and 9 µL of TEMED. The solution was gently mixed

and 7.2 ml of the resolving gel was pipetted between the small and large plate. After the resolving gel was added, 1 ml of ethanol (70%) was added and served as a Gel topper to keep it from drying. The gel was left alone and polymerized for an hour. The Stocking gel was then made by combining 0.75 ml of Acrylamide (40%), 4.5 ml of H_2O, 1.5 ml of Upper Gel Buffer, 30 µL of APS (10%), and 6.66 µL of TEMED. The solution was mixed and pipetted in between the plates until the buffer reached the top. A green comb was then inserted into the space between the plates and the gel was allowed to polymerize for an additional 45 minutes. Afterward, SDS gels were removed from the plate holder and stored at 4^oC until needed. The measurements listed were enough to produce two SDS gels. The gels were run by removing the green comb, pipetting samples into the wells of the gel, and running an electric current (usually around 140 mV) through the gel for 45-60 minutes. The gel was then removed, placed in Comatassie Blue stain, and put on a rocker for a few hours or until bands were visible. Gels were put into Destain in order to see bands more clearly.

2.6 CG-MALS Protocol

Required proteins for the experimental run (Calmodulin and SKp) were removed from the -80^oC freezer and thawed on ice. The proteins were then diluted to the preferred concentration for the experiment, labeled, and stored in the fridge until needed for the experimental run. The buffer being used for the experiment was filtered with 0.02 µm Nylon membrane filter, and then transferred into three 50 ml conical tubes. The CG-MALS machine was then turned on, and a buffer exchange with H_2O was done to clean out the system. This was done by attaching three 50 ml conical tubes with water to all three pumps. Afterwards, a buffer exchange was done with the filtered buffer needed for the experimental run in order to

equilibrate the system. Once the system was equilibrated, the protein solutions were then attached to the pumps and the protocol specific to the experimental run was started.

3. Results

3.1 Protein Purification HPLC Spectra

During my time in the lab, I was able to synthesize many types of Calmodulin including Wild-Type, single point mutation Calmodulins, single lobe Calmodulin, and double lobe Calmodulins. For the purposes of this thesis, I will present the data regarding the purification of a double C-Lobe Calmodulin in order to demonstrate how protein purification data is analyzed. The synthesis of double C-Lobe Calmodulin was the first time it has been purified in the lab.

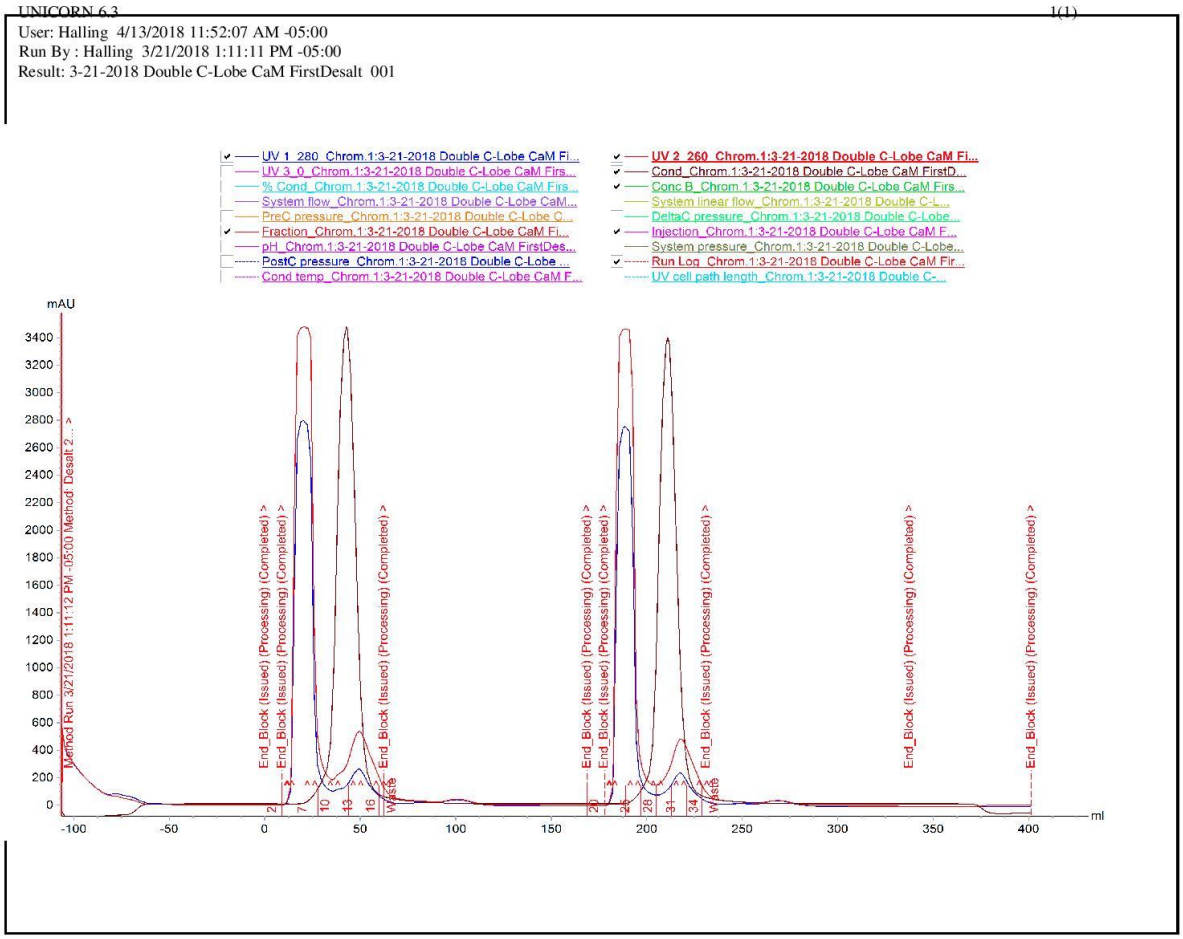

Figure 1: Double C-Lobe Calmodulin Initial Desalt

As mentioned before, the sample is first injected into the desalt column in the HPLC. Figure 1 shows the typical spectral data collected by the HPLC. The blue and red lines represent the absorbance of the sample at 280 nm and 260 nm respectfully, and the brown line represents conductance. In this spectra there are red and blue peaks that overlay each other and this corresponds to the protein in the sample coming off the column. After the red and blue peaks there is a brown peak, which marks an increase in conductivity. This peak corresponds to the salt coming off the column. The salt is a result from the previous ammonium sulfate precipitate protocol done earlier during the purification process. As intended, the column effectively removes the salt from protein sample, and also puts the protein in 20 mM Tris. In this specific desalt, a double injection was performed the previous purification steps. That is why there are two sets of peaks in the spectra. Fractions that contained the red and blue peaks were collected, combined, and stored for the anion exchange.

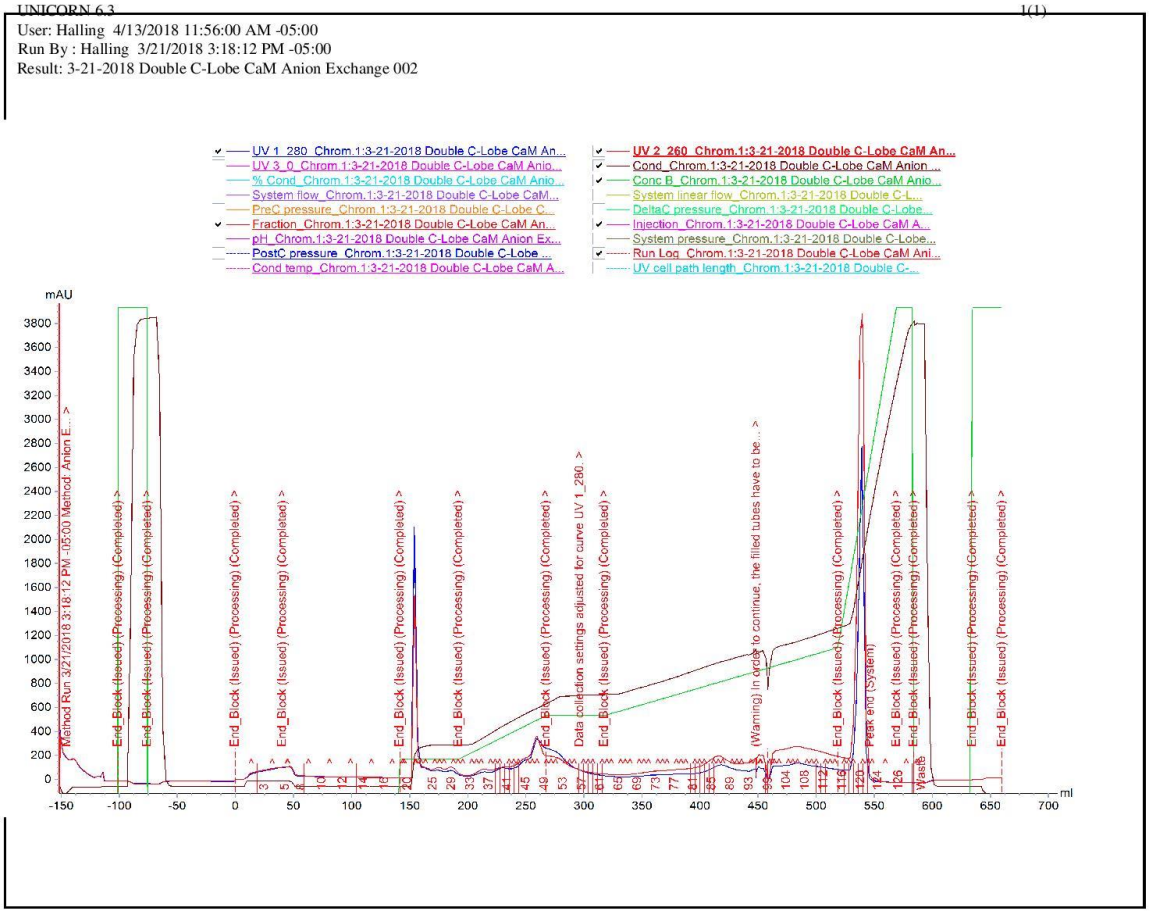

Figure 2: Double C-Lobe Calmodulin Anion Exchange

Figure 2 provides the HPLC data from running the double C-Lobe Calmodulin sample through the anion exchange column. The lines are the same as that of the desalt spectra, however, the new green line represent the concentration of the buffer in pump B, which in this column 20 mM Tris + 1.5 M NaCl is used. From the data there are multiple red and blue peaks present, however, based on previous runs Calmodulin typically comes off the column 240 mM NaCl. This is shown at the small peak approximately around the 260 ml mark. Presence of appropriate bands corresponding to Calmodulin was observed on a SDS Page gel, which ensures the protein is contained in those fractions. Notice the large red and blue peak at the end of the diagram. That peak corresponds to the bacterial proteins and other biomolecules separated from the Double C-Lobe Calmodulin. The fractions containing the blue and red peak at the 260 ml marker were collected, and stored for the next column.

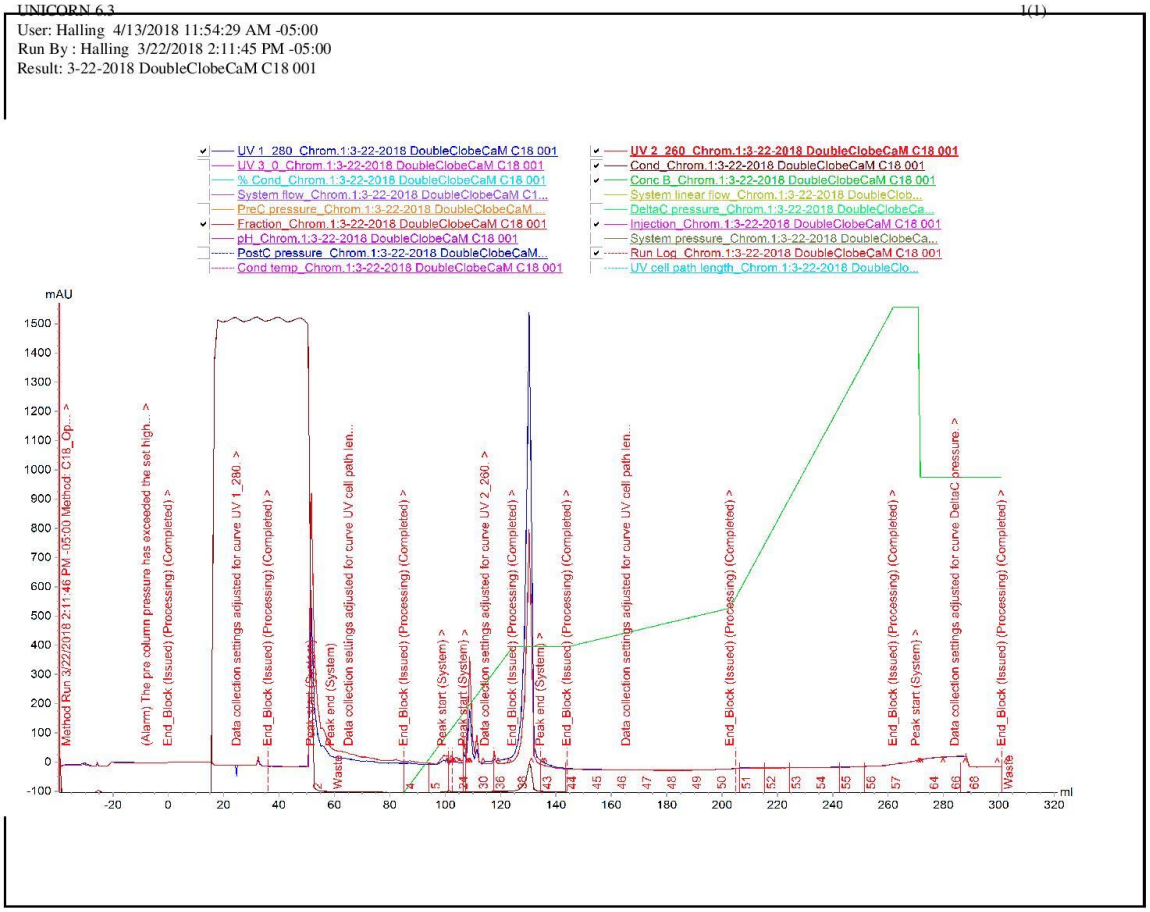

Figure 3: Double C-Lobe Calmodulin C18

Figure 3 provides HPLC data reflecting double C-Lobe Calmodulin's run through the C18 column. The lines represent the same measurement; however, the green line corresponds to the concentration of Acetonitrile as different buffers were used for the C18 column. There was a large red and blue peak present at approximately the 130 ml mark. At this point, the column is in 30% Acetonitrile, which is typically where Calmodulin has been seen to fall off the column. The blue peak is also shown to be much larger compared to the first two spectra, and this can be attributed to the protein sample becoming more concentrated. The fractions containing the red and blue peak were collected and stored for the final desalt run.

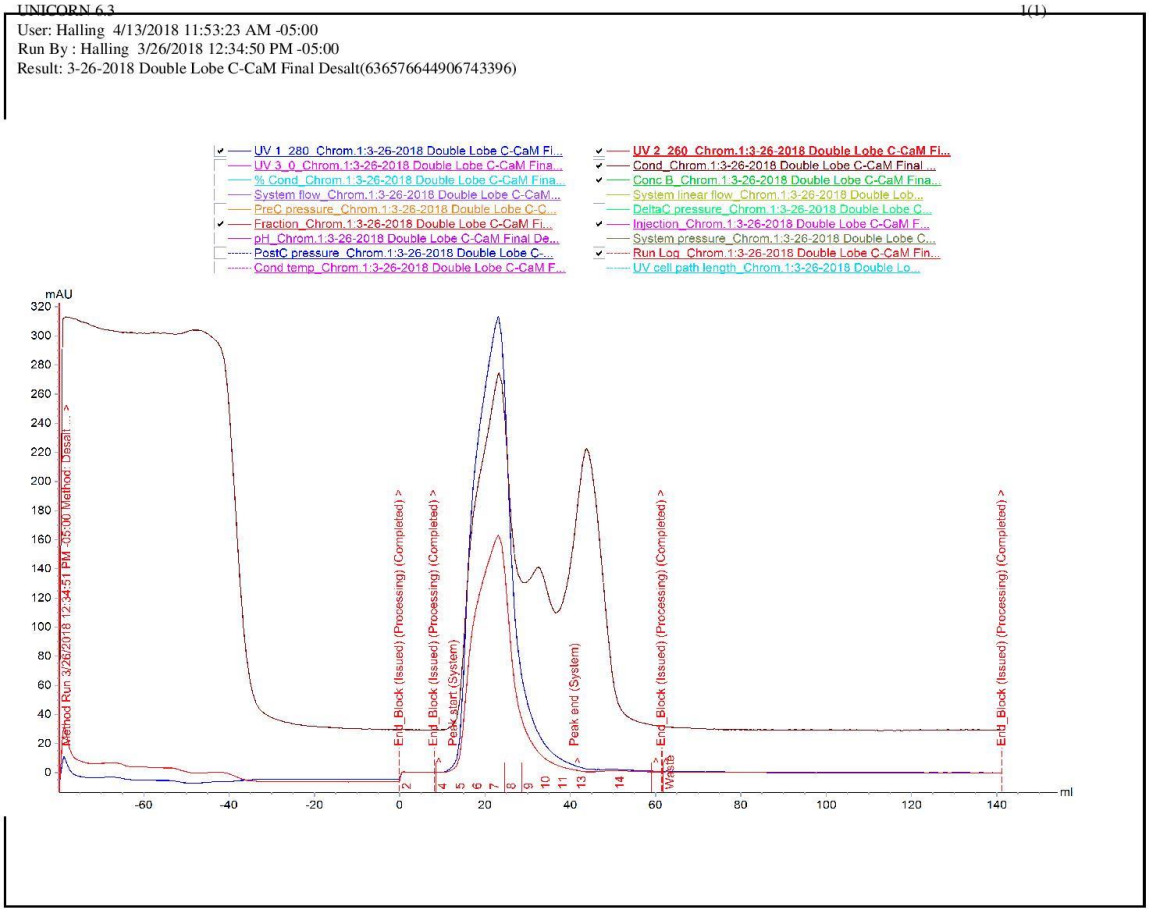

Figure 4: Double C-Lobe Calmodulin Final

Figure 4 shows HPLC data of running the double C-Lobe Calmodulin through the final desalt column. There is a clear red and blue peak at the 20 ml mark observed in the spectra that corresponds to the double C-Lobe Calmodulin. There is also a slight conductivity peak to the left of the red and blue peak which shows some leftover salt being separated from the protein. Interestingly, there is a conductivity peak within the red and blue peak. This peak has been observed in many previous Calmodulin purifications; however there is not much of an explanation for it.

3.2 Protein Concentration Analysis

Concentration of purified protein was determined immediately after going through the final desalt column.

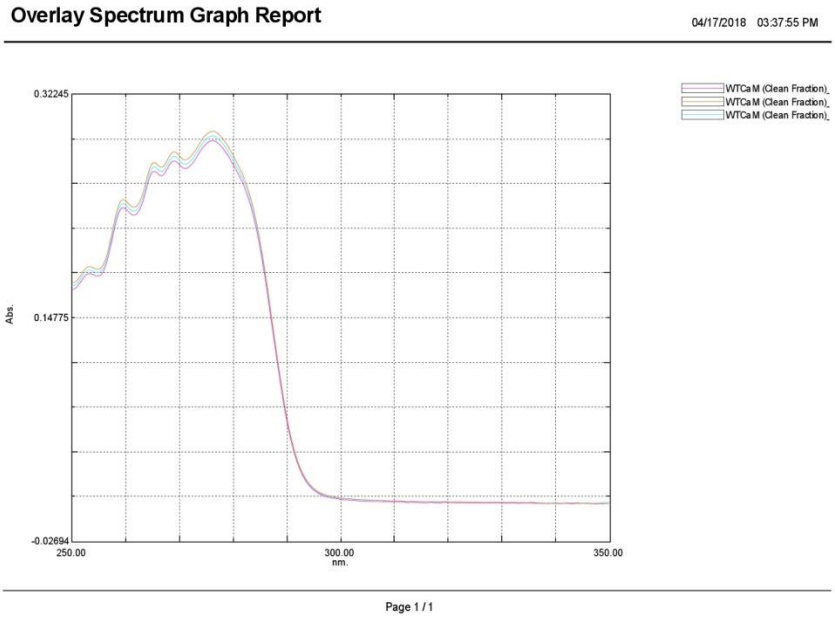

Figure 5: Absorbance Spectrum of WT CaM (250nm-350nm)

Concentration was determined by measuring absorbance of purified Calmodulin with a UV spectrometer. Absorbance was taken from 250 nm to 350 nm and it was done three times for validity. Figure 5 shows an example of the UV spectra for a purified WT CaM. Concentration was calculated by taking the average absorbance value at the highest peak, typically around 276 nm, and subtracting that value from the average absorbance value at approximately 330 nm. This was done to account for any noise in the spectrum. The resulting absorbance was then used to calculate concentration by using Beer-Lambert's Law ($A=\varepsilon lc$). The molar absorptivity coefficients for the various Calmodulin constructs purified can be found in supplemental figure 3 (S3). Once concentration was determined, Calmodulin was aliquoted for storage and labelled with the construct and concentration.

3.3 SDS PAGE

SDS PAGE was performed throughout the HPLC purification in order to keep track of where the Calmodulin was and confirm the protein was getting purified. The data presented reflects SDS GELS performed for a purification of a Calmodulin with a single N-Lobe. This is commonly referred to as an N-CaM.

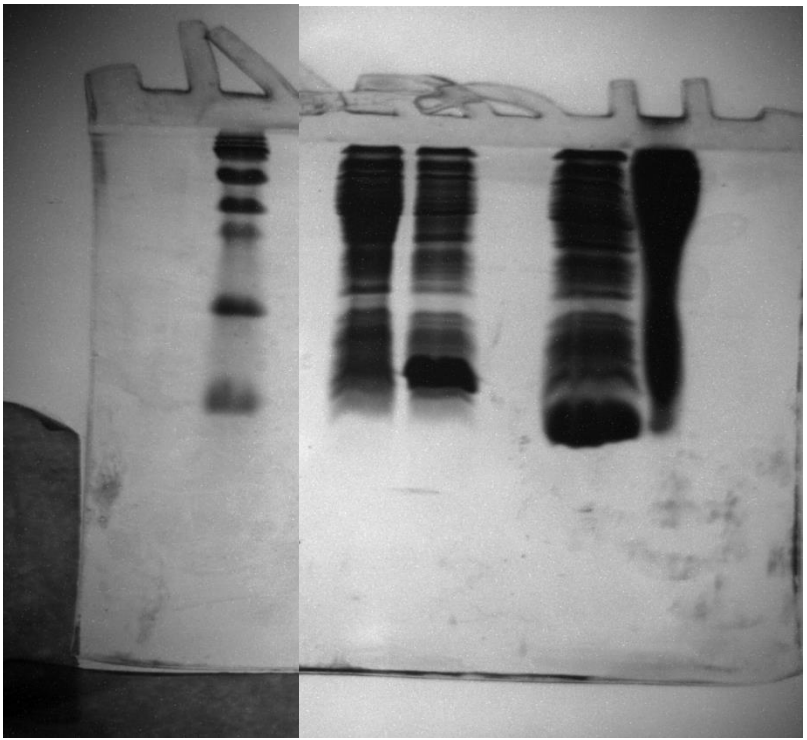

Figure 6: N-CaM Expression

An initial SDS gel was taken of the N-CaM right after the expression stage in order to confirm that N-CaM was present in the sample. A PageRuler Plus Prestained Protein Ladder was placed in Lane 1 of the gel in order to approximate the molecular weight of the bands. The bands and their corresponding molecular weight can be seen in S2. Calmodulin is approximately 16.7 kDa and presence of a band near that molecular weight mark will indicate that Calmodulin is present. In the case of single lobe Calmodulin, such as the N-CaM, their molecular weight is approximately 8 kDa and will run further down the gel. The figure was modified as the original gel included additional data on another protein expression that was being run concurrently with the N-CaM. Figure 6 also shows large bands present at almost every row indicating the sample contains a lot of other molecules besides the Calmodulin. It is important to note that the left most column shows the weight ladder which gives insight to the weight of the molecules in the sample.

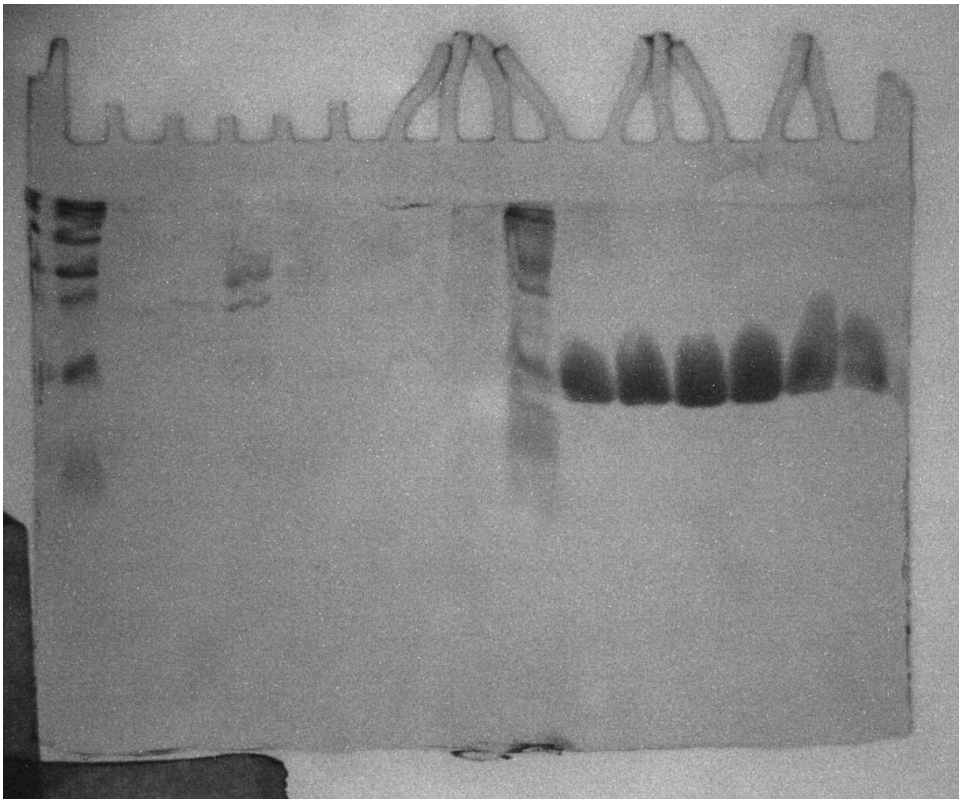

Figure 7: N-CaM Anion Exchange

Another SDS Gel was run on selected fractions from the anion exchange run. These fractions were selected based on the absorbance measured in them and Figure 7 displays the results of the gel. As observed, many of the bands present in Figure 6 did not appear in the Figure 7 gel. This indicates that the desalt and anion exchange columns were effective in removing many of the contaminants in the N-CaM sample. This is explained by the clearer band present at near the 10 kDa marker. The bands were still little stretched meaning there is still some junk in the protein sample.

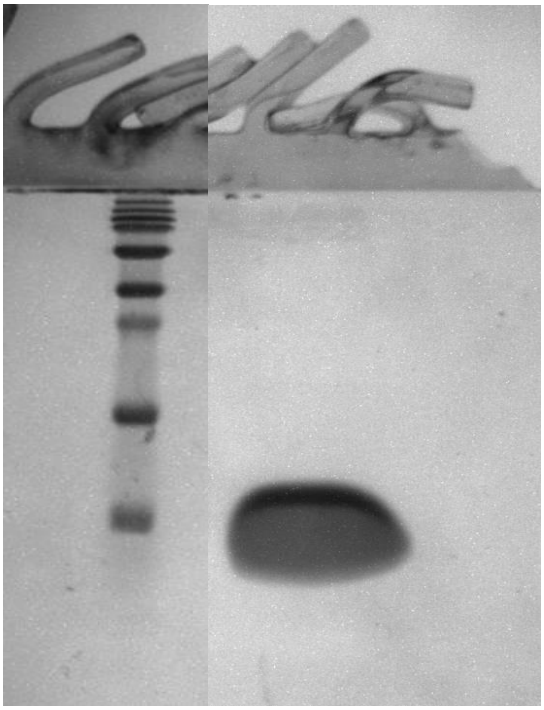

Figure 8: N-CaM Final Desalt (Modified)

A final gel was taken at the end of the HPLC purification protocol and the results are seen in Figure 8. As shown, a clear band was present at near the 10 kDa marker. The band itself was round and dark implying a high concentration of N-CaM in the samples run through the gel. The gels in Figures 6-8 serve as another way of visualizing the purification process for Calmodulin and confirm that protein was indeed purified throughout the HPLC procedure.

3.5 CG-MALS

Data was collected using light scattering in order to study the interaction between Wild-Type Calmodulin (WT CaM) and the SK channel peptide (SKp) in zero Ca^{2+} (EDTA) conditions and saturating Ca^{2+} (2 mM Ca^{2+}) conditions. As mentioned before, light scattering intensity can be used to determine the molar mass of the complexes formed, and if the molar mass of the proteins injected into the machine are known, stoichiometries of the complexes can be determined.

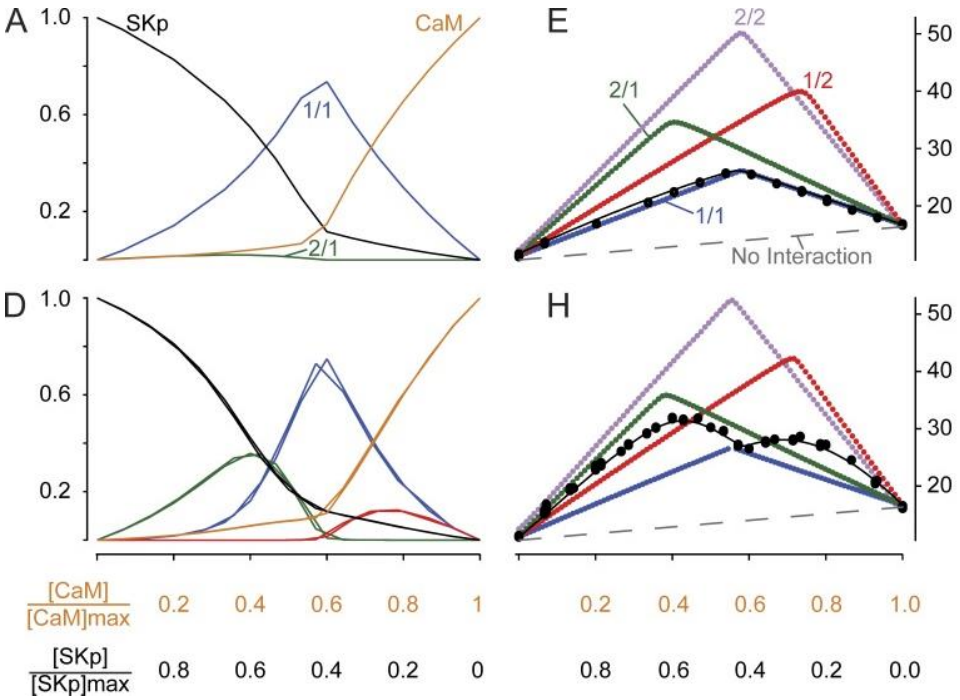

Figure 9: CG-MALS Data (Adapted from Halling et al. 2014)

Figure 9 displays data collected with WT CaM and SKp in low and saturated concentrations of Ca^{2+}. Light scattering signal was measured over time as the proteins were pumped in and out of the machine resulting in different molar ratios of SKp to Calmodulin as shown at the bottom of the figure. Figures 9A and 9D show the molar fraction of the proteins and the stoichiometries of the complexes that formed. The black and gold lines represent the mole fraction of SKp and

CaM respectively. The blue line represents a 1:1 ratio (1 SKp to 1 CaM), the green line represents a 2:1 ratio (2 SKp to 1 CaM), the red line represents a 1:2 ratio (1 SKp to 2 CaM), and the purple line represents a 2:2 ratio (2 SKp to 2 CaM). Figures 9E and 9H display the actual data points, represented by the black dots, and the colored lines are simulations that assume all of the SKp/CaM complexes forming are in their respective corresponding stoichiometry. The right side of the graph also shows the apparent Molar Mass of the complexes formed in kilodaltons. Between Figures 9E and 9H, there are significant noticeable differences. Low Ca^{2+} conditions was conducted with 5 mM EGTA, and the majority of the complexes that formed were in a 1:1 stoichiometry with a small amount of 2:1 forming when [SKp] > [CaM]. There is no presence of 2:2 or 1:2 complexes forming in these conditions. Figure 8H shows the interaction between SKp and Calmodulin under saturated Ca^{2+} conditions (2 mM Ca^{2+}). In the figure, a great amount of 2:1 complexes formed when [SKp] > [CaM], which is explained by the large initial bump. When concentrations of SKp and CaM are relatively equal, the 1:1 formation is dominant. Interestingly, when [SKp] < [CaM], there is a considerable amount of 1:2 complexes forming in solution, which is also shown by the presence of another bump. This stoichiometry was not present under low Ca^{2+} conditions suggesting the 1:2 stoichiometry is calcium dependent.

4. Discussion

4.1 Conclusions

From the CG-MALS data, there were only 1:2, 1:1, and 2:1 stoichiometries observed to form in both low and high Ca^{2+} concentrations. Multiple trials confirmed that these were the stoichiometries occurring between the proteins. This is unexpected since multiple crystal structures show a 2:2 stoichiometry that occurs between the SK peptide and Calmodulin. Variances between crystallography and light scattering could be responsible for the differences

in results. However, our results do propose that the 1:2 and 2:1 stoichiometries could be significant in gating the SK channel. Additionally, the Ca^{2+} dependent 1:2 stoichiometry suggests there could be another binding site on the SK peptide. This would reasonably explain how two Calmodulins could bind to a single peptide.

4.2 Future Studies

If anything, our results show that the interaction between Calmodulin and the SK channel may be more complex than anticipated. The stoichiometries determined from CG-MALS and the evidence supporting the 2:2 stoichiometry gating model require us to conduct more testing in order to fully understand the interaction and the mechanism in which gating occurs. We plan on and are currently running trials with single lobe Calmodulins and double lobe Calmodulins with SKp under low and saturating Ca^{2+} conditions. This is done in an effort to understand lobe dependent interactions. Hopefully these trials will show us exactly how these lobes influence the Calmodulin-SKp complex.

One limitation of CG-MALS is that we aren't able to see where binding occurs. In order to address, there are plans to conduct FRET (Fluorescence Resonance Energy Transfer) studies. FRET is a useful technique that can measure change in distances between two reporting fluorophores. The distance change is sensitive to the Angstrom level and we believe that it will allow us to identify where binding might be occurring. Overall, we hope these techniques can be used to help gain more information on the SKp-CaM complex.

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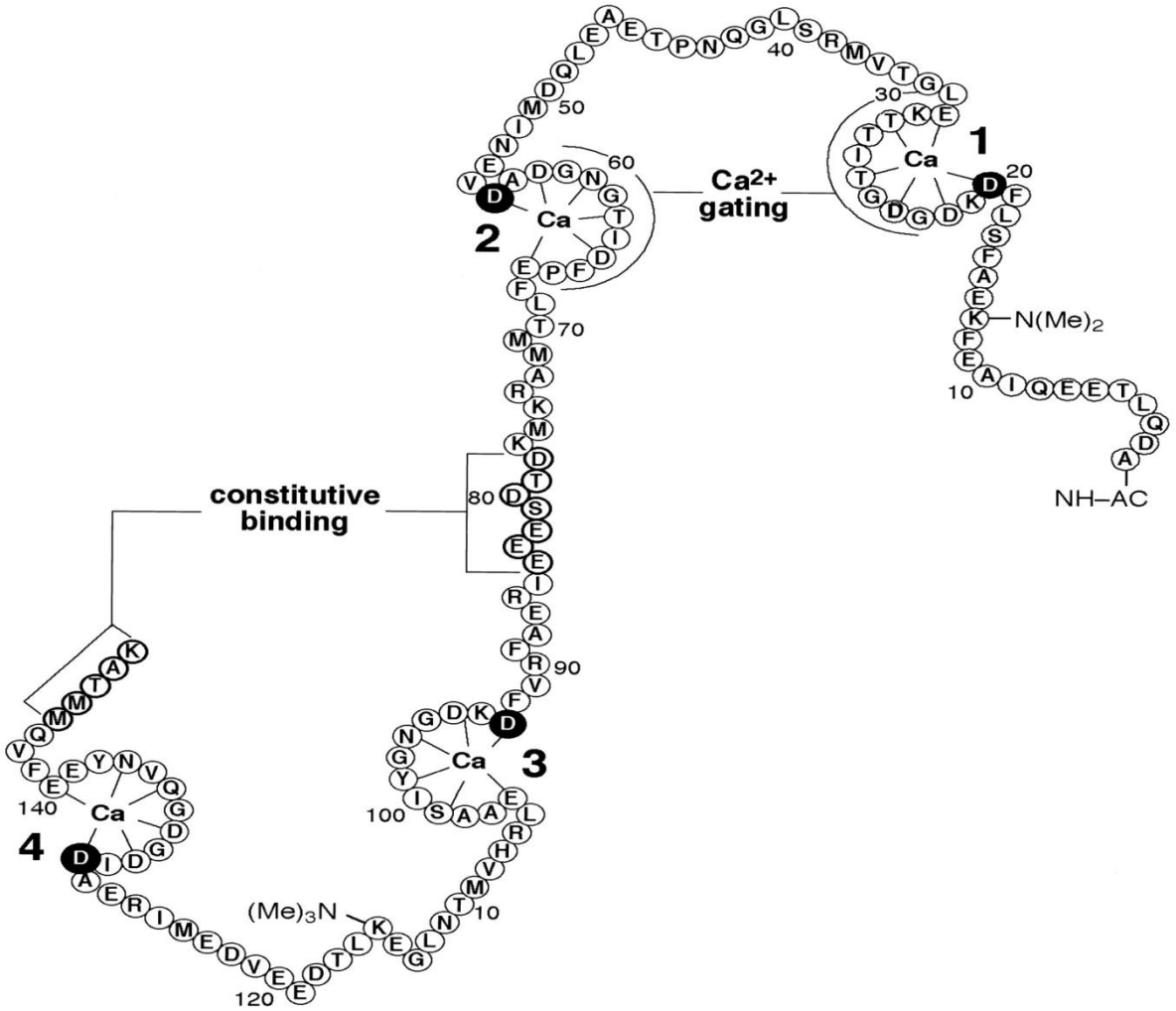
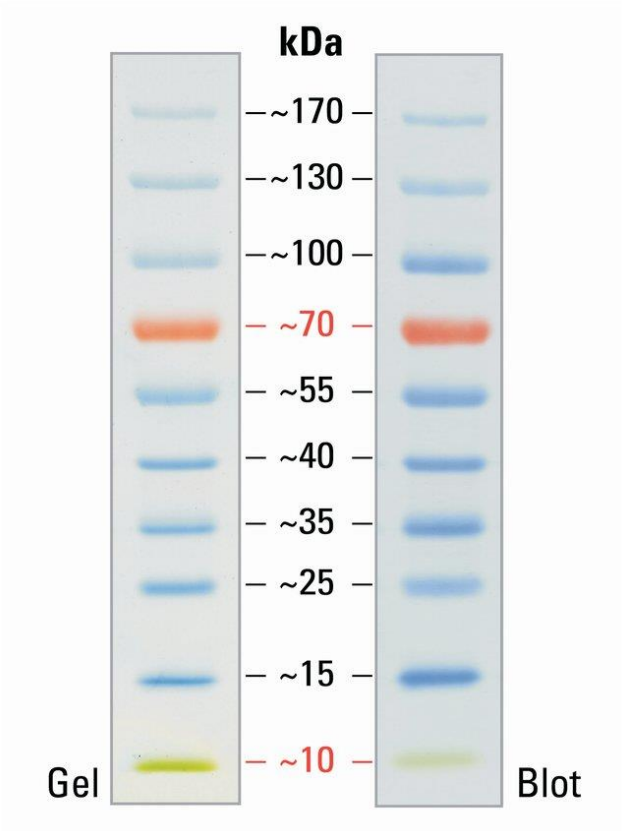
6. Supplemental Figures



S1: Calmodulin Schematic (Keen J et al. J. Neurosci. 1999)



S2: PageRuler Plus Prestained Protein Ladder

CaM	ε	Molecular Weight (MW)
WT	3020	16709
W1	8480	16791.5
W2	8480	16791.5
W3	6990	16729.4
W4	8480	16764.4
N-CaM	975	9039
C-CaM	3020	8407
CY3 (Y138F)	1510	8391
Double N-Lobe CaM	1950	16420
Double C-Lobe CaM	5960	16017

S3: Molar Absorptivity Values (ε) and Molecular Weight (MW) of various Calmodulins purified. W1-4 denotes an added tryptophan to EF hands 1-4 in Calmodulin. N-CaM and C-CaM are the single lobe Calmodulins. CY3 represents an added Tyrosine to the third EF hand of Calmodulin.