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To cite this version:

Steven Damo, Michael D Feldkamp, Benjamin Chagot, Walter J Chazin. NMR studies of the interaction of calmodulin with IQ motif peptides.. Methods in Molecular Biology, 2013, Calcium-Binding Proteins and RAGE 963, pp.173-86. 10.1007/978-1-62703-230-8_11 . hal-01637792

HAL Id: hal-01637792

https://hal.science/hal-01637792v1

Submitted on 30 Nov 2017

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Methods Mol Biol. Author manuscript; available in PMC 2014 May 06.

Published in final edited form as:
Methods Mol Biol. 2013 ; 963: 173–186. doi:10.1007/978-1-62703-230-8_11.

NMR studies of the interaction of calmodulin with IQ motif peptides

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Summary

Calmodulin (CaM) is a ubiquitous EF-hand calcium sensor protein that transduces calcium signals in a wide range of signaling pathways. Structural analysis of complexes with peptides has provided valuable insights into the remarkable variety in the way in which CaM interacts with and activates its targets. Among these various targets, CaM has been shown to be an essential component of a calcium-sensing regulatory apparatus for a number of voltage gated ion channels. NMR spectroscopy has proven to be a powerful tool for the structural characterization of CaM-peptide complexes, in particular for the study of IQ motifs, which bind CaM at that basal level of calcium in cells and thereby serve to localize CaM to its sites of action. We describe here methods for the robust expression and purification of CaM isotopically enriched for NMR analysis, as well as for the complex of CaM with a peptide derived from the IQ motif sequence of the human cardiac sodium channel $Na_V1.5$. We also describe methods for NMR analysis of titrations of CaM with IQ motif peptides to determine the stoichiometry of the complex and to identify the residues at the binding interface.

Keywords

Calmodulin; Na 1.5; IQ motif; ^{15}N-^{1}H HSQC; NMR spectroscopy; protein complex; co-expression; calcium binding protein; calcium signaling; EF-hand

1. Introduction

EF-hand calcium binding proteins are used to regulate, shape and transduce intracellular calcium signals (1, 2). The ubiquitous calcium sensor calmodulin (CaM) integrates calcium signal transduction, modulating the activity of more than 50 different targets (3). It performs this function through coupled binding of calcium and distinct CaM-binding domains in these target proteins. CaM is composed of two classical EF-hand domains connected by a flexible linker.

Binding of calcium causes a shift within each domain from a closed to an open conformation, which leads to exposure of a substantial hydrophobic surface that is integral

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to its interaction with targets. These conformation changes combined with a high degree of flexibility in the linker between the domains provide CaM with a remarkable ability to interact with targets in a wide range of different modes. Given the significant molecular size and complexity of CaM targets, peptides derived from the CaM binding domain/s of targets have been used extensively to mimic the interaction of CaM with full length targets (4-8). NMR spectroscopy has been used to directly validate this reductionist approach for studying CaM (9).

Calcium signals are tightly regulated in time and duration because otherwise they would activate so many signaling pathways and overwhelm the cell. This tight regulation means that the calcium sensor proteins need to be able to respond rapidly to signals. One way that CaM ensures its rapid response is by pre-localizing to its sites of action. The IQ motif is a conserved sequence (IQxxxBGxxxB, B=Lys or Arg) in CaM targets that is widely used for this purpose (10). The key characteristic of IQ motifs providing this function is they bind relatively tightly to CaM at basal levels of intracellular calcium and the interaction changes when calcium levels are raised (11). Voltage-gated ion channels are one important class of targets whose activity is modulated by intracellular calcium signals through the action of CaM. The channels have IQ motifs in their C-terminal intracellular domains, which are known to recruit CaM.

One of the most well-studied voltage-gated ion channels modulated by calcium is the human cardiac voltage-gated sodium channel ($Na_V1.5$). $Na_V1.5$ is essential in the initial phase of action potential and a core regulatory element of the beating of the heart (12). CaM plays a critical role in the calcium regulation of $Na_V1.5$ and is pre-localized to the channel via a C-terminal IQ motif. Physical interaction of CaM with the IQ motif has been directly demonstrated both in the presence and absence of calcium (13). In the absence of calcium, the C-terminal domain of CaM binds the IQ motif with the N-lobe remaining free in solution (4). Although no structure has been determined at saturating calcium, NMR studies have shown that both the N- and C-terminal domains are engaged (13).

NMR spectroscopy has played a critical role in characterizing the interaction of CaM with its many cellular targets. NMR is well suited for the study of protein interactions because the resonance frequency observed is exquisitely sensitive to changes in chemical environment, for example when a ligand is titrated into a solution. In the past 10-15 years, ^{15}N-^{1}H HSQC NMR spectroscopy has become a widely used technique to observe residue specific changes in the chemical environment of backbone amide groups within proteins, and this approach has been widely used in studies of target peptide fragments binding to CaM.

The use of peptide fragments for the reductionist approach has certain advantages. Titrations of peptides into solutions of ^{15}N-enriched proteins monitored by ^{15}N-^{1}H HSQC spectra require relatively modest quantities of the peptides, which can be synthesized in a cost effective manner. However, for more in-depth NMR studies, it is advantageous to monitor changes not only in the protein but also in the peptide, which means producing the peptide with isotopic enrichment. Due to the much higher cost of producing such samples, the labeled peptide is usually produced recombinantly in a bacterial expression system. This approach faces certain technical challenges as in general it is difficult to express small

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peptides alone due to their susceptibility to proteolytic cleavage and low expression levels. These obstacles can be overcome through fusion to a carrier protein (14). Furthermore, co-expression of protein complexes offers the advantages of pre-assembly of the complex, generally accurate stoichiometry and more facile purification.

Here we describe methods for the production of ^{15}N-enriched CaM and titration of CaM with a synthetic $Na_V1.5$ IQ motif peptide ($Na_V1.5_{IQp}$) monitored by ^{15}N-^{1}H HSQC NMR spectroscopy. We show how to quantify changes in amide chemical shifts observed in the ^{15}N-^{1}H HSQC spectrum of ^{15}N-labeled apo CaM to dissect the stoichiometry of peptide binding, as well as to identify residues perturbed upon peptide binding. In addition, we describe co-expression and purification of a uniformly ^{15}N enriched CaM-IQ peptide complex. The methods described here are generally applicable to CaM complexes. Given the large and still increasing number of CaM targets, the use of these approaches to characterize of CaM-peptide complexes will continue to be valuable for understanding the molecular basis for transduction of calcium signals by CaM and the activation of intracellular signaling pathways.

2. Materials

2.1. Production of ^{15}N Apo CaM for NMR studies

Expression of ^{15}N CaM

1. CaM pET15 plasmid (see **Note 1**)
2. BL21(DE3) competent cells (Invitrogen): 100 μL aliquots stored at -80 °C.
3. Ampicillin (AMP) 1000x stock solution 100 mg/mL in Milli-Q (MQ) water (filtered to a resistance of 18.3 MΩ-cm). Sterilize by 0.2 μM filtration.
4. LB AGAR plates: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 15 g/L agar dissolved in MQ water and autoclaved at 121 °C for 20 min. Add AMP stock at a 1:1,000 dilution when the medium has cooled to 50-60 °C.
5. SOC Recovery medium: 20 g/L tryptone, 0.5 g/L NaCl, 5 g/L yeast extract, 2.5 mM KCl, 5 mM $MgCl_2$, 5 mM $MgSO_4$, 20 mM glucose dissolved in MQ H_2O and autoclaved at 121 °C for 20 min.
6. LB medium: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract autoclaved at 121 °C for 20 min.
7. ^{15}N labeled M9 medium: Per L of medium. Add 0.5 g NaCl, 3.0 g KH_2PO_4, 6.0 g Na_2HPO_4 and 1.0 g $^{15}NH_4CI$ autoclaved at 121 °C for 20 min. Add 1.0 mL of 2 M $MgSO_4$ (sterile filtered 0.2 μM), 100 μL of 1 M $CaCI_2$(sterile filtered, 0.2 μM), 20 mL of 20% glucose (sterile filtered, 0.2 μM).
8. IPTG (Gold Biotechnology): 1M solution in MQ H_2O. Sterilize by filtration at 0.2 μM.

[1]cDNA encoding for human CaM was sublconed between the NcoI and BamHI restriction sites of a pET15b vector (ampicillin resistance Novagen).

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Purification of Apo CaM

1. Refrigerated ÄKTA FPLC (GE Life Sciences) purification system and accessories (see **Note 2**).
2. Two Phenyl sepharose 6 Fast Flow columns (prepacked 25 mL, Amersham).
3. Phenyl sepharose (PS) Buffer A: 50 mM Tris pH 7.5, 1 mM $CaCl_2$.
4. Phenyl sepharose (PS) Buffer B: 50 mM Tris pH 7.5, 500 mM NaCl, and 1 mM $CaCl_2$.
5. Phenyl sepharose (PS) Buffer C: 50 mM Tris pH 7.5, 1 mM EDTA.
6. Dialysis tubing: 3 kDa molecular weight cutoff (MWCO).
7. NMR buffer N: 20 mM HEPES pH 6.8, 100 mM KCl, 1 mM EDTA, and 0.01% NaN_3. (see **Note 3**).

2.2. Titration of apo CaM with Peptide and Analysis of NMR Data

Sample preparation and NMR data acquisition

1. $Na_v1.5_{IQp}$ (Genscript): corresponding to residues E1901-L1927 of $Na_V1.5$.
2. NMR buffer N: 20 mM HEPES pH 6.8, 100 mM KCl, 1 mM EDTA, and 0.01% NaN_3.
3. D_2O.
4. 9 inch glass pipette (Sigma-Aldrich).
5. 5 mm OD Precision borosilicate glass tubes (for use with a 5 mm probe) (Wilmad glass company).
6. 500 MHz or higher field spectrometer equipped with 5 mm ^1H,^{13}C,^{15}N room temperature or cryo probe (e.g. Bruker Avance III).

Analysis of NMR Data

1. Computer (running Mac OS X or linux operating system).
2. NMR data processing software (e.g. NMRPipe) (15).
3. NMR spectral analysis software (eg. Sparky or NMRView) (16, 17).

2.3. Production of Apo CaM-$Na_V1.5_{IQp}$ complex for NMR studies

Co-expression of CaM-His_6-SUMO-$Na_V1.5_{IQp}$fusion complex

1. CaM pET15 plasmid.
2. IQ pBG102 plasmid (see **Notes 4** and [5]).
3. BL21(DE3) competent cells (Invitrogen): 100 μL aliquots stored at -80 °C.

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[2]To preserve columns and chromatography equipment, all buffers should be 0.2 μM filtered immediately prior to use.
[3]Due to the high Ca^{2+}-binding affinity of CaM, all buffer solutions should be prepared with MQ water to reduce the risk of Ca^{2+} contamination of apo CaM.

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4. Ampicillin (AMP) 1000x stock solution 100 mg/mL in MQ H_2O. Sterilize by 0.2 μM filtration.
5. Kanamyacin (KAN) 1000x stock solution 35 mg/mL in MQ H_2O. Sterilize by filtration at 0.2 μM.
6. LB AGAR plates: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 15 g/L agar dissolved in MQ water and autoclaved at 121 °C for 20 min. Add AMP and KAN stocks at a 1:1,000 dilution when the medium has cooled to 50-60 °C.
7. SOC Recovery medium: 20 g/L tryptone, 0.5 g/L NaCl, 5 g/L yeast extract, 2.5 mM KCl, 5 mM $MgCl_2$, 5 mM $MgSO_4$, 20 mM glucose dissolved in MQ H_2O and autoclaved at 121 °C for 20 min.
8. LB medium: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract autoclaved at 121 °C for 20 min.
9. ^{15}N labeled M9 medium: Per L of medium. Add 0.5 g NaCl, 3.0 g KH_2PO_4, 6.0 g Na_2HPO_4 , and 1.0 g $^{15}NH_4Cl$ autoclaved at 121 °C for 20 min. Add 1.0 mL of 2 M $MgSO_4$ (sterile filtered 0.2 uM), 100 μL of 1 M $CaCl_2$ (sterile filtered, 0.2 uM), 20 mL of 20% glucose (sterile filtered, 0.2 uM).
10. IPTG (Gold Biotechnology): 1M solution in MQ H_2O. Sterilize by filtration at 0.2 μM.

Co-purification of Apo CaM-$Na_V1.5_{IQp}$ complex

1. Refrigerated ÄKTA FPLC purification system and accessories (GE Life Sciences).
2. Ni-NTA (prepacked 25 mL column Amersham).
3. Ni-NTA Buffer A: 50 mM Tris pH 7.5, 300 mM NaCl, and 10 mM Imidazole (see **Note 6**).
4. Ni-NTA Buffer B: 50 mM Tris pH 7.5, 300 mM NaCl, and 500 mM Imidazole.
5. His_6-Human rhinovirus 3C (H3C) protease. 10 Units per mg of substrate (see **Note 7**).
6. Dialysis membrane (3 kDa MWCO).
7. Centrifugal concentrator (3 kDa MWCO).
8. HiLoad Superdex 75 16/600 column (GE Life Sciences).

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[4]cDNA encoding for Human $Na_V1.5$ IQ motif E1901-L1927 subcloned between BamHI and XhoI restriction sites of a pET27 derivative in house vector (L. Mizoue CSB Vanderbilt University). This derivative codes for an N-terminal His_6 tagged SUMO followed by a flexible linker containing a H3C protease cleavage site. The IQ motif is expressed as a fusion C-terminal fusion to SUMO (His_6-SUMO-$NaV1.5_{IQp}$). The final product is isolated as a 31-residue polypeptide that contains Gly-Pro-Gly-Ser fused to its N-terminus after cleavage of the His_6-SUMO.

[5]The choice of His_6-SUMO as a fusion partner for $Na_V1.5_{IQp}$ simplifies the purification by taking advantage of robust Ni-NTA chromatography methods and allows for the separation of excess CaM.

[6]The use of 10 mM imidazole in the loading buffer greatly reduces nonspecific binding of endogenous *E. coli* proteins to the Ni-NTA resin.

[7]We produce N-terminal His_6-tagged H3C protease in house (L. Mizoue CSB Vanderbilt University). As a commercially available alternative (GST-tagged) PreScission Protease (GE Lifesciences) can be used.

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9. S75 buffer: 50 mM Tris pH 7.5 and 300 mM NaCl.

3. Methods

3.1 Production of ^{15}N Apo CaM for NMR studies

Here we describe our protocol for the robust expression and purification of ^{15}N labeled CaM for NMR studies. Purification of CaM involves two hydrophobic interaction chromatography steps, exploiting the significant changes in hydrophobic surface accessibility of apo CaM and calcium-loaded CaM. Typical yields are 30-40 mg/L for M9 media.

Expression of ^{15}N CaM

1. Thaw 100 uL of BL21(DE3) competent cells on ice, add 50 ng of CaM pET15b. Incubate on ice for 20 minutes.
2. Heat shock the cells at 42 °C for 30 s and incubate on ice for 2 min.
3. Add 900 uL of SOC recovery medium. Incubate for 1 hr at 37 °C with shaking (220 rpm).
4. Plate 150 uL of cells onto LB-agar ampicillin plates and incubate at 37 °C overnight.
5. Inoculate a single colony into 100 mL of LB media supplemented with AMP. Incubate overnight (12-16 hrs) at 37 °C.
6. Add 20 mL of LB starter culture to 1 L of sterile ^{15}N M9 media. Incubate at 37 °C with shaking (220 rpm) until OD_{600} reaches 0.6.
7. Induce cells by adding IPTG to a final concentration of 1 mM (1 mL of 1M IPTG solution added to 1 L of cell culture) and incubate at 37 °C for 4 hrs with shaking (220 rpm).

Purification of Apo CaM

1. Harvest cells by centrifugation at 5,000 g for 10 minutes.
2. Resuspend cell paste in PS buffer C (10-15 mL of buffer per gram of cell paste) and transfer to a stainless steel beaker. Chill this beaker in a secondary container with melting ice.
3. Sonicate the resuspended cell paste with a macrotip set to 50 W output power for total processing time of 5 min (5 sec pulse bursts with 5 sec recovery/off time).
4. Clear cell lysate by centrifugation at 48,000 g for 20 minutes.
5. Decant the lysate and filter through a 0.45-μM membrane.
6. Equilibrate one prepacked 25 mL phenyl-sepharose 6 Fast Flow column with 1 column volume (CV) of MQ H_2O and 2 CVs of PS buffer C.
7. Load the filtered lysate onto the phenyl sepharose column at a flow rate of 1.0 mL/min. Collect the flow through in one fraction.

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8. Wash the column with 1 CV of PS buffer C.
9. Combine the flow through and wash fractions, and adjust $CaCl_2$ concentration to 5 mM.
10. Equilibrate the second prepacked 25 mL phenyl-sepharose 6 Fast Flow column with 1 CV of MQ H_2O and 2 CVs of PS buffer A.
11. Load the combined flow through and wash fractions onto the second phenyl sepharose column.
12. Wash the column with 4 CVs of PS buffer A, followed by 4 CVs of PS Buffer B, and 4 CVs of PS Buffer A.
13. Elute the protein with 4 CVs of PS Buffer C with 5 mL fractionation (see **Note 8**).
14. Determine fractions containing CaM by SDS-PAGE.
15. Pool the fractions and dialyze against buffer N. Concentrate sample to 0.1-1 mM for NMR studies (see **Note 9**).

3.2.Titration of apo CaM with Peptide and Analysis of NMR Data Sample preparation and NMR data acquisition

1. Solubilize the lyophilized $Na_V1.5_{IQp}$ with buffer N containing 10% D_2O to a concentration of 1-5 mM or as high a concentration permitted by the peptide of study to minimize the dilution of apo CaM during the course of the titration series(see **Note 10**). It may be necessary to adjust the pH of the peptide solution to ensure that a pH effect is not encountered upon peptide titration. It is likely that the volume of peptide containing solution will be 1 ml or less, we recommend a micro-pH probe or spotting of the peptide solution onto pH paper to verify the solution pH.
2. Add 50 μl of D_2O to 450 μl of 100 μM ^{15}N-labeled apo CaM (>95% purity) in buffer N.
3. Using a 9 inch glass pipette, carefully transfer the CaM solution to a clean NMR tube (see **Note 11**).
4. Insert the apo CaM containing NMR tube into the sample spinner, verify tube depth, insert and lower tube into NMR spectrometer.
5. Set and equilibrate the temperature of the sample, then lock, tune and shim the spectrometer and use simple 1-dimensional ^1H and ^{15}N experiments to determine 90° pulse lengths for these nuclei.

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[8]Typically, CaM will elute completely within the first 40 mL.
[9]CaM can be quantified by measuring the absorbance at 276 nm and using the extinction coefficient 3006 M^{-1} cm^{-1}.
[10]In cases of extremely hydrophobic peptides, an organic solvent such as dimethyl sulfoxide (DMSO) can be used as an additive to increase solubility.
[11]Clean and rinse the NMR tube with a 1% solution of nitric acid to remove any Ca^{2+} contamination present within the NMR tube followed by extensive rinsing with buffer N to remove any residual 1% nitric acid.

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6. Load a suitable ^{15}N-^{1}H HSQC pulse program, input 90° ^{1}H and ^{15}N pulse lengths and set the receiver gain. Collect an initial ^{15}N-^{1}H HSQC spectrum to access the quality and signal strength of the apo CaM sample. Typically increment values of 1,024 and 128 points are used in the direct and indirect dimensions respectively with a scan setting of 16 to obtain a reasonable spectrum. Due to the expected chemical shifts to be observed upon peptide addition, it is recommended to set the spectral width in ^{1}H and ^{15}N dimensions be extended beyond the apo CaM peaks observed in this initial spectrum. Although the spectral widths required are peptide dependent we suggest extending the spectral width 1 and 5 ppm beyond that of the observed apo CaM crosspeaks in the ^{1}H and ^{15}N dimensions as a starting point.

7. Collect an ^{15}N-^{1}H HSQC spectrum of apo CaM using the optimized settings determined in step 5 to serve as a reference point.

8. Eject the sample from the NMR spectrometer and make the first peptide addition. We have found removal of the sample with a 9 inch glass pipette into a 1.5 ml Eppendorf tube allows for confident addition and mixing of the peptide. Anticipating a 1:1 peptide:apo CaM binding stoichiometry, we suggest making additions in an incremental manner, where a minimum of 4 additions (0.25 molar equivalents of peptide) are used to reach a 1:1 ratio.

9. After transfer of the sample back to the NMR tube and insertion into the NMR spectrometer, allow for the temperature to equilibrate, re-shim the sample and acquire the first titration point. Repeat this process until apo CaM amide crosspeaks cease to shift (fast exchange) or that peaks corresponding to apo CaM are no longer present (slow exchange) in the^{15}N-^{1}H HSQC spectrum upon addition of peptide. We suggest at least one additional point where an excess amount of peptide is added to ensure saturation.

Analysis of NMR Data

1. Process the ^{15}N-^{1}H HSQC titration data using NMRpipe (15) or other software.

2. Assign amide crosspeaks of apo CaM in titration spectra using spectral viewing and analysis software such as Sparky or NMRView (16, 17).

3. An incremental change in apo CaM amide cross peak position in response to peptide addition is indicative of fast chemical exchange (typically associated with Kd ~10 μM or weaker binding affinity), assignment of the peptide bound state can be made by simply tracking the incremental movement of perturbed peaks (**Figure 1**). If slow chemical exchange is observed (typically associated with Kd ~1 μM or stronger binding affinity), the titration spectra will display population-weighted sets of amide cross peaks whose intensity correspond to the relative amount of free and peptide bound apo CaM present in the sample. Intermediate exchange is observed in between the fast and slow exchange regimes and is characterized by broadening and disappearance of signals over the course of the titration, followed by reappearance of signals at a new location, as in the case of slow exchange. In the case of intermediate and slow exchange, three-dimensional NMR experiments to assign the new cross peaks from the complex are required to determine the identity

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of the apo CaM signals in the complex. It is common to increase temperature in an attempt to shift peaks in intermediate chemical exchange to fast chemical exchange in an effort to characterize binding in detail and to simplify the resonance assignment in the peptide bound state.

4. Quantify the peptide binding induced perturbation of apo CaM amide cross peaks with **Equation 1** and plot the Δ*ppm* value determined (y-axis) versus residue number (x-axis) as shown in **Figure 2**.

$$\Delta ppm = \sqrt{(\Delta\delta_H)^2 + (\Delta\delta_N \bullet 0.2)^2} \quad (1)$$

In this equation, Δ*ppm* refers to the linear change of an amide cross peak from its initial starting position in the reference spectrum, while $\Delta\delta_H$ and $\Delta\delta_N$ refer to the individual changes in the ^{1}H and ^{15}N dimensions. A scaling factor of 0.2 is used to account for differences in the span of ^{1}H and ^{15}N chemical shifts.

5. Determine the Δ*ppm* value to use as a cut off for what is classified as significant chemical shift perturbation to identify apo CaM residues that are either directly or allosterically involved in peptide binding. We suggest using a value of one standard deviation over the average of all chemical shifts as a cutoff (**Figure 1**).

6. Different treatments are required to examine the stoichiometry of peptide binding to apo CaM when rate of chemical exchange is in the fast or slow exchange binding regime. In the case of fast exchange, the position of the amide cross peak is the weighted average of the chemical shifts of the peptide free and peptide bound apo CaM states. During slow exchange, separate apo CaM amide cross peaks whose intensity is directly proportional to the ratio of peptide free to peptide bound apo CaM are observed. To determine the binding stoichiometry for peptide binding in fast chemical exchange, plot the chemical shift for each titration point (y-axis) against the ratio of [peptide]/[apo CaM] (x-axis). When slow chemical exchange is observed, plot the normalized ratio of the intensities of the peptide free to peptide bound apo CaM amide crosspeaks for a given resonance (y-axis) versus the ratio of [peptide]/[apo CaM] (x-axis).

7. The stoichiometry of the peptide:apo CaM complex can then be determined by as illustrated in **Figure 2**.

3.3. Production of Apo CaM-$Na_V1.5_{IQp}$ complex for NMR studies

This section describes the co-expression and co-purification of uniformly ^{15}N labeled CaM$Na_V1.5_{IQp}$ complex for NMR studies. The $Na_V1.5_{IQp}$ is expressed as an N-terminally His_6-tagged SUMO fusion protein (His_6-SUMO-$Na_V1.5_{IQp}$). The complex is formed *in vivo* and isolated via a 3 step purification procedure. The CaM-His_6-SUMO-$Na_V1.5_{IQp}$ fusion complex is purified by Ni-NTA chromatography. This is followed by cleavage and removal of the His_6-SUMO fusion tag by another Ni-NTA column followed by size exclusion chromatography. Typical yields are 5-10 mg of complex per L of M9 medium.

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Co-expression of CaM-His_6-SUMO-$Na_V1.5_{IQp}$ fusion complex

1. Thaw 100 uL of BL21(DE3) competent cells on ice, add 100 ng each of CaM pET15b and IQ pBG102 vectors. Incubate on ice for 20 minutes (see **Note 12**).
2. Heat shock the cells at 42 °C for 30 s and incubate on ice for 2 min.
3. Add 900 μL of SOC recovery medium. Incubate for 1 hr at 37 °C with shaking (220 rpm).
4. Plate 150 μL of diluted cells onto LB-agar AMP/KAN plates and incubate at 37 °C overnight.
5. Inoculate a single colony into 100 mL of LB media supplemented with AMP/KAN. Incubate overnight (12-16 hrs) at 37 °C.
6. Add 20 mL of LB starter culture to 1 L of sterile ^{15}N M9 media. Incubate at 37 °C with shaking (220 rpm) until OD_{600} reaches 0.6.
7. Incubate cells at 20 °C for 1 hour with shaking at 220 rpm (see **Note 13**).
8. Induce cells by adding IPTG to a final concentration of 1 mM (1 mL of 1M IPTG solution added to 1 L of cell culture) and incubate at 20 °C overnight with shaking (220 rpm).

Co-purification of Apo CaM-$Na_V1.5_{IQp}$ complex

1. Harvest cells by centrifugation at 5,000 g for 10 minutes.
2. Resuspend cell paste in Ni-NTA buffer A (10-15 mL of buffer per gram of cell paste) and transfer to a stainless steel beaker. Chill this beaker in a secondary container with melting ice (see **Note 14**).
3. Sonicate the resuspended cell paste with a macrotip set to 50 W output power for total processing time of 5 min (5 sec pulse bursts with 5 sec recovery/off time).
4. Clear cell lysate by centrifugation at 48,000 g for 20 minutes.
5. Decant the lysate and filter through a 0.45-μM membrane.
6. Equilibrate the prepacked 25 mL Ni-NTA column with 1 CV of MQ H_2O and 2 CVs of Ni-NTA buffer A.
7. Load the filtered lysate onto the Ni-NTA column at a flow rate of 1.0 mL/min.
8. Wash unbound lysate from the column with 4 CVs of Ni-NTA buffer at a flow rate of 2.5 mL/min.
9. Elute protein complex with a 4 CV gradient (0-100% Ni-NTA Buffer B, 10-500 mM imidazole), with 5 mL fractionation.

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[12]Induction at lower temperature greatly enhances the solubility of the His_6-SUMONaV1.5$_{IQp}$ fusion.
[13]The use of higher concentrations of plasmid DNA increases the efficiency of the double transformation.
[14]It is critical to ensure the resuspended cell paste remains at cold temperatures during lysis or protein precipitation will occur leading to reduced yields.

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10. Determine fractions containing CaM-His_6-Sumo-$NaV1.5_{IQp}$ complex by SDS-PAGE.
11. Prepare 4 L of S75 buffer in a glass beaker equilibrated at 4 °C.
12. Pool relevant fractions, combine with H3C protease, and seal in dialysis tubing (3 kDa MWCO). Transfer dialysis tubing to glass beaker containing 4L of S75 buffer. Incubate at 4 °C overnight.
13. Filter the dialyzed sample through a 0.45-μM membrane.
14. Equilibrate the prepacked 25 mL Ni-NTA column with 1 CV of MQ H_2O and 2 CVs of Ni-NTA buffer A.
15. Load the cleaved dialyzed solution onto the Ni-NTA column at a flow rate of 1.0 mL/min. Collect the flow through.
16. Wash unbound protein from the column with 4 CVs of Ni-NTA buffer at a flow rate of 2.5 mL/min with 5 mL fractionation.
17. Determine fractions containing CaM-IQ complex by SDS-PAGE.
18. Equilibrate S75 with 1 CV of MQ H_2O and 1 CV of S75 Buffer.
19. Pre-rinse two centrifugal concentrators (15 mL, 3 kDa MWCO) with MQ H_2O) by centrifuging at 3,700xg for 15 min at 4 °C. Concentrate the pooled Cam-IQ complex fractions to ~3-5 mL.
20. Load the concentrate onto the Superdex 75 colum at 1 mL minute. Elute the protein isocratically for 1 CV with 5 mL fractionation.
21. Determine fractions containing CaM-IQ complex by SDS-PAGE.
22. Pool the fractions and dialyze against 20 mM HEPES pH 6.8, 100 mM KCl, 1 mM EDTA, and 0.01% NaN_3. Concentrate sample to 0.1-1 mM for NMR studies.

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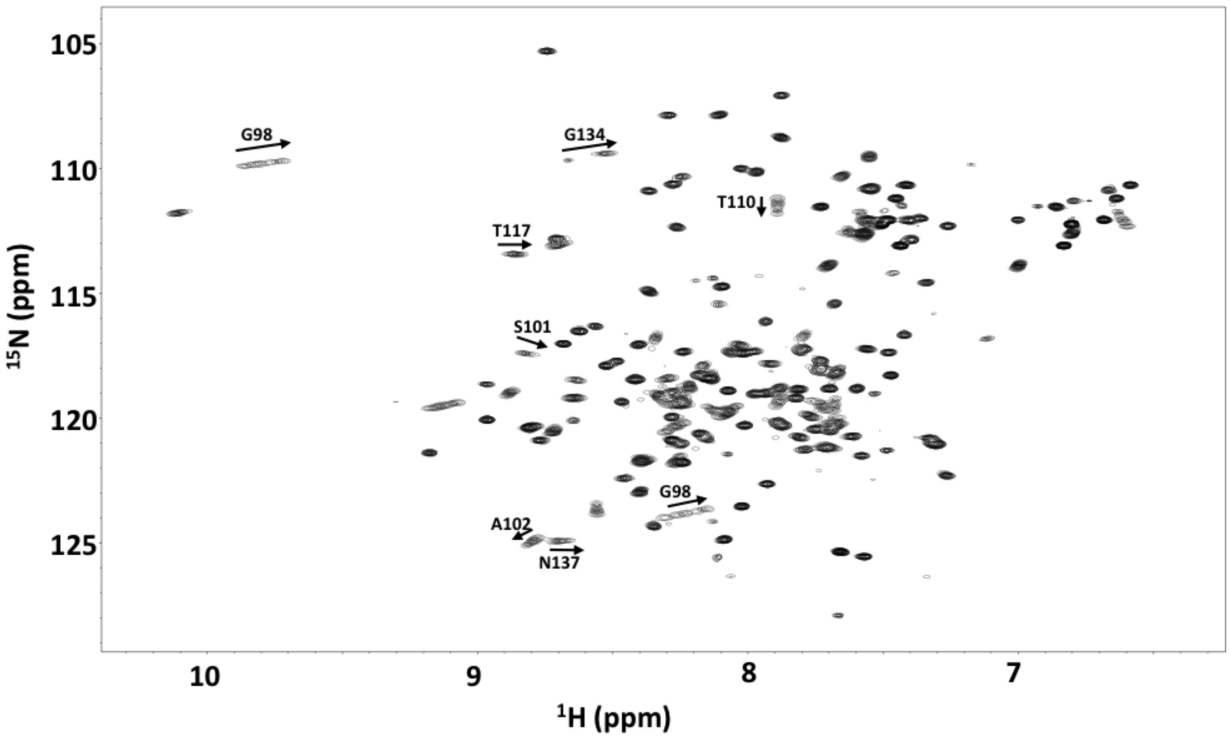

Figure 1. Overlaid ^{15}N-1H HSQC Spectra of apo CaM Titrated with $Na_v1.5_{IQp}$

$Na_v1.5_{IQp}$ is a known binding partner of apo CaM, which upon addition to apo CaM shifts a select number of apo CaM amide crosspeaks. The direction and magnitude of the chemical shift perturbation of a select number of apo CaM amide cross peaks are indicated with arrows.

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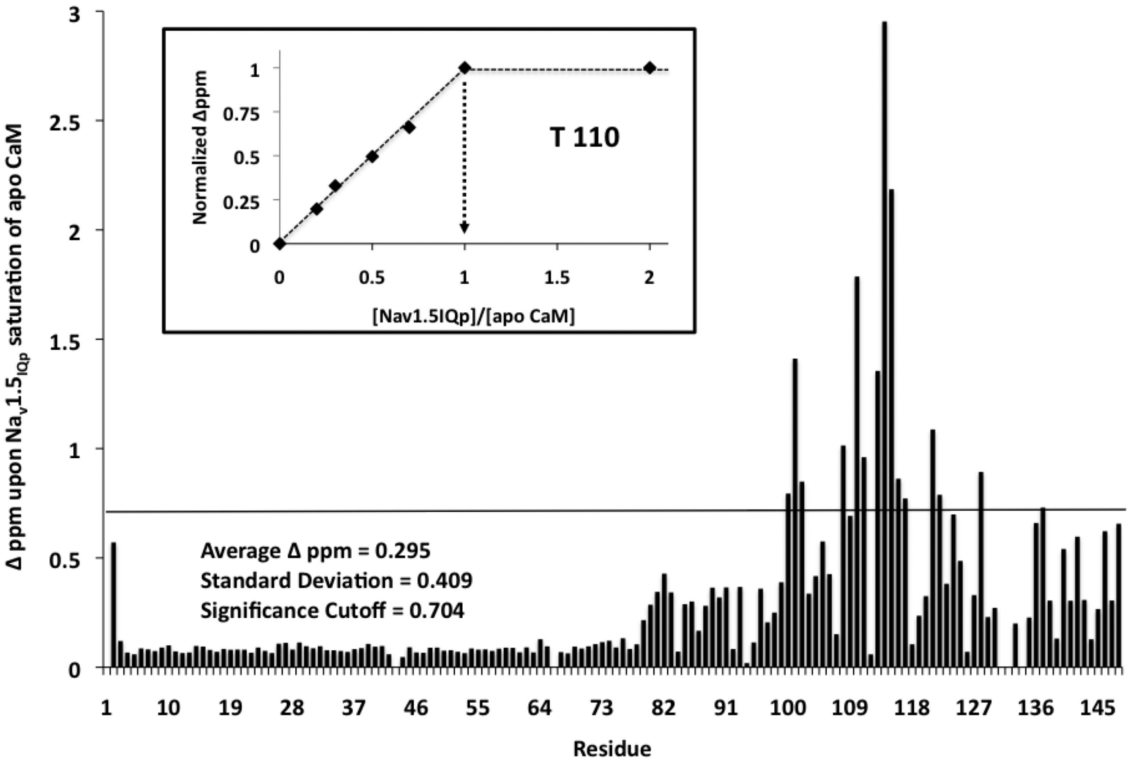

Figure 2. Quantified apo CaM Chemical Shift Perturbations Induced upon $Na_v1.5_{IQp}$ Binding

Using **Equation 1**, the change in chemical shift of apo CaM amide cross peaks were calculated and plotted. The horizontal bar at 0.704 represents the significance cutoff value used to identify apo CaM residues whose chemical shift were deemed significant. Shown in the inset in the upper left is a stoichiometry plot calculated for residue T110 over the course of the titration series. To help guide the eye, a line and arrow are placed in the data to highlight the 1:1 stoichiometry achieved once the normalized change in chemical shift has reached a plateau, signifying a $Na_v1.5_{IQp}$ saturation of apo CaM.