Chapter 13

Expression and Purification of Calmodulin for NMR and Other Biophysical Applications

Benjamin M. M. Grant, Christopher B. Marshall, and Mitsuhiko Ikura

Abstract

Calmodulin (CaM) is a ubiquitous calcium-sensing protein that has one of the most highly conserved sequences among eukaryotes. CaM has been a useful tool for biologists studying calcium signaling for decades. In recent years, CaM has also been implicated in numerous cancer-associated pathways, and rare CaM mutations have been identified as a cause of human cardiac arrhythmias. Here, we present a collection of our most recent and effective protocols for the expression and purification of recombinant CaM from *Escherichia coli*, including various isotopic labeling schemes, primarily for nuclear magnetic resonance (NMR) spectroscopy and other biophysical applications.

Key words Calmodulin, CaM, Calcium, Expression, Purification, NMR, Isotopic labeling

1 Introduction

Calmodulin (CaM) was first discovered as an activator of cyclic AMP phosphodiesterase in the brain by Cheng [1] and Kakiuchi [2] independently. The Ca^{2+} dependency of CaM activity demonstrated by Kakiuchi opened a totally new field of calcium-dependent signaling regulators and their cellular pathways. CaM is a small, 16.7kD protein that acts as an important master regulator of Ca^{2+} signaling in cells [3, 4]. The CaM sequence is conserved among all vertebrates and is highly conserved throughout kingdoms Animalia and Plantae. CaM is highly acidic, soluble, and stable, both in Ca^{2+}-free and Ca^{2+}-bound states, and interacts with binding targets under both conditions [5]. CaM binds Ca^{2+} through motifs called "EF-hands," first discovered in carp parvalbumin by Kresinger et al. [6]. There are four EF-hand motifs in CaM, each of which forms a 29-amino acid helix-loop-helix structure that coordinates Ca^{2+} through side chains and backbones of five residues in a twelve-residue loop; one pair of EF-hands forms an N-terminal lobe, and the other forms a C-terminal lobe. Binding of Ca^{2+} induces a

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structural change that opens a hydrophobic pocket in each lobe, which mediates the interaction with Ca^{2+}-dependent binding partners [5]. EF-hands have been identified in 1613 protein sequences [7], including 221 proteins encoded in the human genome.

Due in part to its size and stability, CaM was an early target for solution protein NMR and was used to test and develop many of the core NMR experiments employed today [8–10]. There are currently ~200 structures of CaM in the Protein Data Bank (www.rcsb.org). In early years, CaM was purified from various tissues (e.g., the heart, brain, and red blood cells) using combinations of anion exchange, gel filtration chromatography, and ammonium sulfate precipitation, with many schemes taking advantage of the high stability of CaM through precipitation of undesired proteins by heating [11] and trichloroacetic acid (TCA), followed by hydrophobic column chromatography [12].

With the development of recombinant DNA technology, researchers moved to overexpression of CaM in bacterial cells in order to obtain large quantities of protein, as well as to produce mutants to probe structure/function relationships. In the 1990s, *Drosophila* CaM was cloned into a heat-inducible pAS system for expression in *E. coli* to allow incorporation of isotopic labels [13]. This construct did not contain any affinity tags and was purified through a multi-step process involving TCA precipitation of unwanted proteins (3% TCA), followed by TCA precipitation of CaM (6% TCA), Ca^{2+}-dependent binding of resuspended CaM to Phenyl Sepharose, and elution with ethylenediaminetetraacetic acid (EDTA) after extensive washing. The *Drosophila melanogaster* CaM sequence has three amino acid substitutions relative to vertebrate CaM; thus, many studies adopted recombinant expression of *Xenopus laevis* CaM, which has an amino acid sequence that is identical to that of all known vertebrates. However, it should be noted that recombinant CaM differs from endogenous CaM in that the initiating Met residue is not removed, and it lacks acetylation of the N-terminus and trimethylation of Lys115.

Recent implications of CaM in cancer-associated pathways have renewed and expanded interest in this protein for in vitro biophysical and biochemical characterization. Here we present our laboratory's most recent and effective protocols for the expression and purification of recombinant CaM from *E. coli* and include techniques for various isotopic amino acid labeling schemes for NMR. We use a T7-inducible promoter system to express a hexa-histidine-tagged CaM protein, which we purify through a two-step process: Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography followed by size-exclusion liquid chromatography (Fig. 1). The entire protocol can be performed within a week and yields approximately 30 mg/L of highly pure, functional protein.

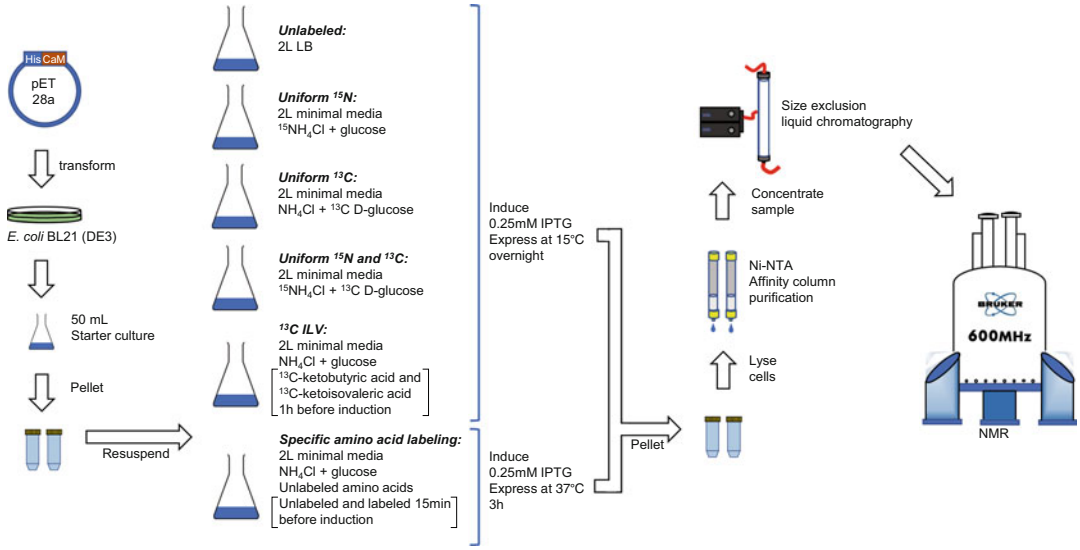

Fig. 1 Schematic flow of calmodulin expression and purification protocol

2 Materials

2.1 CaM in pET28 Vector

CaM construct: CaM has been engineered with an N-terminal hexa-histidine (His) tag and linker containing a specific cleavage motif that is recognized by thrombin. This construct was cloned into the pET28a vector with its stop codon using the NdeI and BamHI restriction endonuclease sites. This encodes a reversibly His-tagged CaM protein under the control of a T7 promoter such that expression can be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) in DE3$^+$ *E. coli* strains, with selection through kanamycin resistance. Following cleavage of the His-tag by thrombin, three extra residues (Gly-Ser-His) remain at the N-terminus.

2.2 Competent Cells

The construct is expressed in *E. coli* BL21 (DE3$^+$) which is compatible with the T7 expression system employed by pET vectors. Strains with additional copies of specific tRNA genes that are rare in the *E. coli* genome (e.g., codon + or Rosetta) can improve yields but are not necessary. These strains carry an additional antibiotic resistance marker (e.g., chloramphenicol).

2.3 Stock Solutions

1. LB broth: Dissolve in water 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, or purchase premixed broth powder and follow instructions.
2. M9 Media (10× stock): Dissolve in water Na_2HPO_4, 60 g/L, KH_2PO_4, 30 g/L, and NaCl, 5 g/L, and adjust pH to 7.4.
3. Trace elements (1000×): Add 5 mL of 0.5 M EDTA, pH 8.0, to 80 mL of water. Add trace elements one at a time in the

following order, with stirring, waiting at least 10 min between the addition of each compound: Ferric chloride ($FeCl_3 \cdot 6H_2O$) 833 mg, zinc chloride ($ZnCl_2$) 50 mg, cupric chloride ($CuCl_2 \cdot 2H_2O$) 12.6 mg, cobalt chloride ($CoCl_3 \cdot 6H_2O$) 10 mg, boric acid (H_3BO_3) 10 mg, and manganese chloride ($MnCl_2 \cdot 4H_2O$) 2500 mg. Do not readjust pH or there will be precipitation. Top up to 100 mL and filter (0.22 μm).

4. Kanamycin (1000×): Prepare 50 mg/mL stock in water; store in aliquots at −20 °C.
5. Chloramphenicol (1000×): Prepare 30 mg/mL solution in ethanol; store at −20 °C.
6. Biotin (1000×): Prepare a 1 mg/mL solution in 50% ethanol, ideally stored at 4 °C.
7. Thiamine (1000×): 1 mg/mL in water.
8. Magnesium sulfate ($MgSO_4$) (1000×): 1 M solution in water.
9. Ammonium chloride (NH_4Cl) and/or ^{15}N ammonium chloride (for uniform ^{15}N labeling).
10. 20% (w/v) glucose in water or ^{13}C D-glucose (for uniform ^{13}C labeling).
11. ^{13}C-ketobutyric acid (for methyl labeling of Ile) and ^{13}C-ketoisovaleric acid (for methyl labeling of Leu and Val).
12. ^{15}N and/or ^{13}C amino acids (for labeling of specific amino acids).
13. Minimal media: Add 200 mL of 10× M9 to 1800 mL of water in a 6 L Erlenmeyer flask and autoclave. After media has cooled, add 1 mL/L of kanamycin, [chloramphenicol for codon +], biotin, thiamine, magnesium sulfate, and trace elements (1000× stocks), as well as 0.3 mL/L of 1 M $CaCl_2$. Calcium and metals in the trace elements solution will form a visible precipitate with the phosphate in M9, which is not a cause for concern. The remaining components of the minimal media are dependent on isotopic labeling schemes which are covered in *Methods*.
14. Lysis buffer: Tris pH 8.0, 50 mM, NaCl, 150 mM, NP-40 (detergent), 0.1% v/v, glycerol, 10% v/v, imidazole, 10mM. Make 1 L and store. Before use, take 50 mL in a Falcon tube and add β-mercaptoethanol, 10 mM (pure liquid β-mercaptoethanol is 14.3 M), lysozyme (egg white), DNase I (both enzymes can be added by scooping a small amount of powder with a pipette tip), and phenylmethylsulfonyl fluoride (PMSF), 1 mM (toxic) (*see* **Note 1**).
15. His-wash buffer 1: Tris pH 8.0, 50 mM, NaCl, 500 mM, glycerol, 10% v/v, imidazole, 10 mM. Make 1 L and store.

Before use, aliquot 100 mL and add β-mercaptoethanol, 10 mM.

16. His-wash buffer 2: Tris pH 8.0, 50 mM, NaCl, 150 mM, glycerol, 10% v/v, imidazole, 10 mM. Make 1 L and store. Before use, aliquot 100 mL and add β-mercaptoethanol, 10 mM.
17. His-elution buffer: Tris pH 8.0, 50 mM, NaCl, 150 mM, glycerol, 10% v/v, imidazole, 250 mM. Make 1 L and store. Before use, aliquot 50 mL and add β-mercaptoethanol, 10 mM.
18. General dialysis buffer: Tris pH 8.0, 50 mM, NaCl, 150 mM, glycerol, 20% v/v, DTT, 1 mM. Make 4 L in a beaker and store short term at 4 °C.
19. Gel filtration buffer: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 20 mM, NaCl, 100 mM, tris (2-carboxyethyl)phosphine (TCEP), 2 mM. TCEP is a reducing agent available as a highly acidic hydrochloride salt (TCEP-HCl); thus, the pH of TCEP-containing buffers must be adjusted before topping up to final volume. Filter (0.22 μm) and degas 20 min. Store at 4 °C.
20. Chelation buffer: HEPES pH 7.4, 20 mM, NaCl, 100 mM, EDTA, 20 mM, TCEP, 2 mM. Prepare in smaller batches as needed.

3 Methods

3.1 Transformation

1. Thaw 50 μL *E. coli* BL21 ($DE3^+$) [codon + optional] competent cells on ice.
2. Add 50–200 ng of plasmid to cells, mixing gently. Store on ice for 30 min.
3. Heat shock at 42 °C for 1 min. Return to ice for 5 min.
4. Add 450 μL of LB to tube and incubate at 37 °C for 1 h.
5. Plate 100 μL on kanamycin LB plate [add chloramphenicol for codon +], and incubate at 37 °C overnight (*see* **Note 2**).

3.2 Expression

1. Prepare a starter culture: pick one colony of transformed cells with a sterile loop and inoculate 50 mL of LB containing 1× kanamycin (50 μg/L) [add 30 μg/L chloramphenicol for codon +]. Shake at 37 °C overnight (*see* **Note 3**).
2. Prepare media based on desired isotopic labeling scheme.
   (a) *Unlabeled CaM*: LB broth is used for preparation of unlabeled protein. Autoclave 2 L of LB in a 6 L Erlenmeyer flask prior to inoculation. The volume and number of flasks can be adjusted for the desired protein yield;

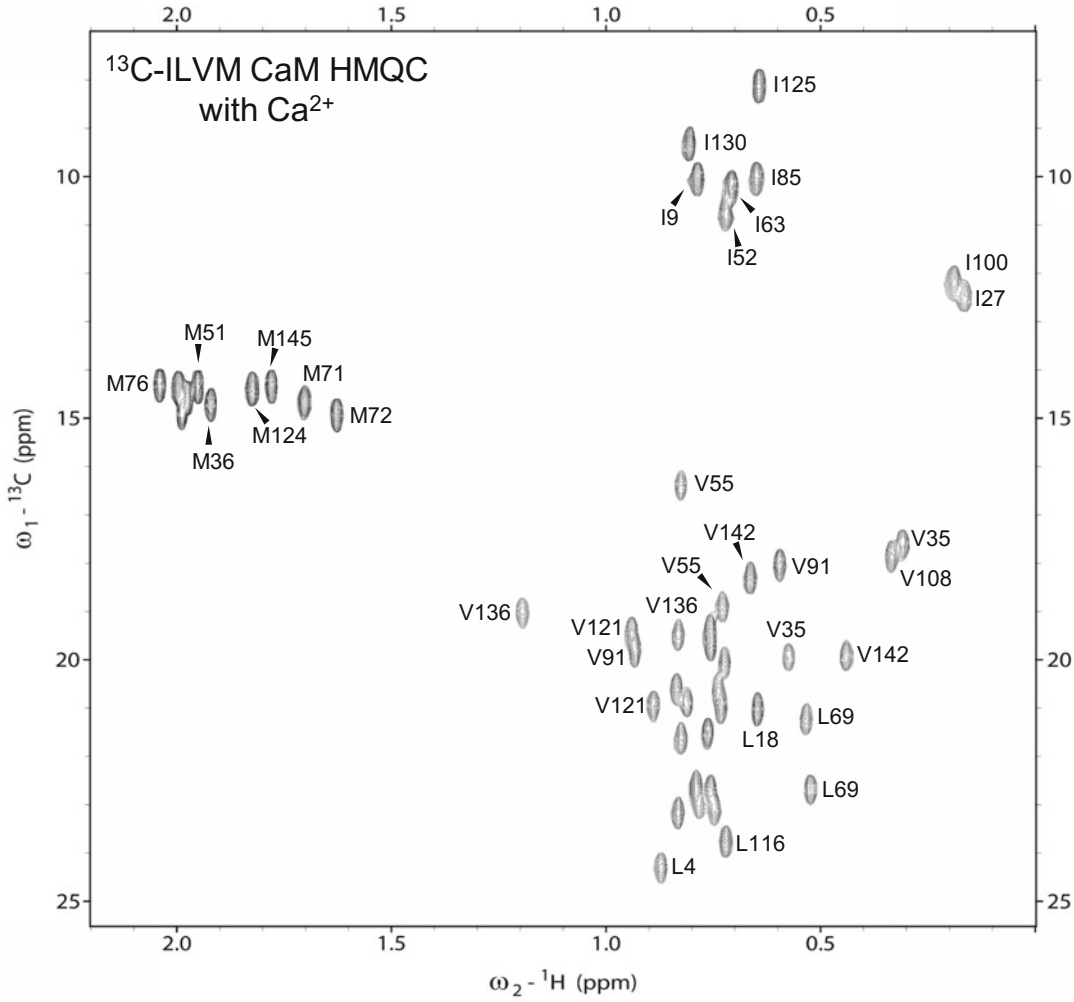

Fig. 2 ^{1}H-^{13}C HMQC of specifically labeled ^{13}C-methyls of Ile, Leu, Val, and Met in calmodulin bound to Ca^{2+}. Sample contains 0.75 mM calmodulin in gel filtration buffer (20 mM HEPES 7.4, 100 mM NaCl, 1 mM TCEP) with 10 mM $CaCl_2$. HMQC collected at 25 °C

however, for efficient aeration, the flasks should not be filled beyond ~1/3.

(b) *Uniform ^{15}N-labeling*: For preparation of isotopically labeled CaM, minimal M9 media is used to control available isotopes. In this case, $^{15}NH_4Cl$ is the only source of nitrogen present in the media and will therefore be incorporated in newly synthesized amino acids and proteins. Prepare up to 2 L of minimal media, as described in *Materials*. For uniform ^{15}N labeling, add 10 mL/L glucose (0.2% w/v) and 1 g/L $^{15}NH_4Cl$.

(c) *Uniform ^{13}C-labeling*: Prepare minimal media as described, and add 1 g/L unlabeled NH_4Cl and 2 g/L

^{13}C D-glucose. As glucose is the principle carbon source in the media, ^{13}C will be incorporated in all newly synthesized amino acids and proteins.

(d) *Uniform ^{15}N- and ^{13}C-labeling*: Prepare minimal media as outlined above using both isotopically labeled $^{15}NH_4Cl$, 1 g/L, and ^{13}C D-glucose, 2 g/L.

(e) *Specific methyl labeling—ILV*: For specific methyl labeling, prepare minimal media with unlabeled NH_4Cl (1 g/L) and glucose (0.2% w/v). Isotopic labeling of Ile, Leu, and Val (ILV) methyl groups is achieved by adding isotopically labeled metabolic precursors of these amino acids. ^{13}C-ketobutyric acid (for Ile—50 mg/L) and ^{13}C-ketoisovaleric acid (for Leu and Val—100 mg/L) are added 1 h prior to the anticipated induction of CaM expression, i.e., when the OD_{600} is approximately 0.5.

(f) *Specific amino acid labeling*: For specific labeling of amino acids, prepare minimal media with unlabeled NH_4Cl (1 g/L) and glucose (0.2% w/v) and 100 mg/L of each amino acid not to be isotopically labeled. Isotopic labeling of specific amino acids is achieved by supplying a source of the labeled amino acid(s) at a crucial time to ensure incorporation in proteins and minimize isotopic scramble to other amino acids due to metabolic processes. Immediately prior to the anticipated induction of CaM expression, i.e., when the OD_{600} is approximately 0.6–0.8, add 500 mg/L addition unlabeled amino acids and 100 mg/L of desired specific labeled amino acid(s). Wait 15 min and induce. Gly, Ala, and Ser labeling is inefficient due to rapid interconversion with metabolites, and Gln, Glu, Asn, and Asp will readily scramble.

An example of a specific isotopic labeling scheme for CaM (methyl labeling of ILVM residues) using these techniques is shown below (Fig. 2).

3. Set aside 1 mL of media for use as a spectrophotometer blank later on. Transfer the starter culture to two 50 mL Falcon tubes, and centrifuge at 2300 × *g* for 10 min. Resuspend pellets in 5–10 mL of new media and inoculate the large flask.
4. Shake flask at 37 °C and 180 rpm (*see* **Note 4**). Measure OD_{600} of cultures roughly every hour, using the blank set aside in **step 3**. Cell cultures grow much more slowly in M9 media than in LB. When OD_{600} reaches roughly 0.4, lower the temperature to 15 °C. It takes some time to cool the shaker and significantly longer for the culture to equilibrate to this temperature, during which the cells will continue to grow. The goal is to cool the culture to 15 °C when the OD_{600} reaches between 0.6 and 0.8, to optimize the yield of soluble protein. At this time, induce

protein expression by adding 0.25 mL/L 1 M IPTG, and continue shaking at 15 °C overnight (*see* **Notes 5** and **6**). For specific amino acid labeling, overnight expression promotes isotopic scrambling. In this case, induce expression at 37 °C and express protein for 3 h.

5. The following morning, harvest cells by centrifugation in 1 L centrifuge bottles at 8500 × *g* for 20 min. Discard supernatant and transfer cell pellets into 50 mL Falcon tubes. These cell pellets can be used for protein purification immediately or frozen at −80 °C for later use (*see* **Notes 7** and **8**).

3.3 Purification

1. Add DNase I, lysozyme, PMSF (*see* **Note 1**), and β-mercaptoethanol to 50 mL lysis buffer. Add fresh lysis buffer to cell pellets, frozen or fresh, leaving 5–10 mL in the Falcon tube for air. Rock or rotate the tube at 4 °C to resuspend the pellet for at least 30 min but no more than 2 h. Lysis efficiency is improved when the DNase I and lysozyme have time to work. Before continuing, ensure the remaining cell pellet is fully resuspended by pipetting until homogenous.
2. Lyse cells by sonication on ice using intermittent pulses (e.g., 0.5 s on and 2.0 s off) to prevent heating of the lysate (*see* **Notes 9** and **10**). Sonication can be performed twice to ensure complete lysis.
3. Transfer lysate to 35 mL ultracentrifuge bottles, and spin at 45,000 × *g* for 40 min (*see* **Note 11**). Samples can be collected from the pellet and supernatant to check for effective lysis (*see* **Note 12**).
4. Wash 5–10 mL of Ni-NTA resin by pouring the resin slurry (in 20% ethanol storage solution) into a 50 mL Falcon tube and pelleting gently at 200 × *g* for 3 min. Pour off the ethanol, rinse with water, and repeat centrifugation. Resuspend Ni-NTA resin with the lysate supernatant, and stir at 4 °C for 1 h.
5. Pour lysate-resin slurry into a gravity flow column, and collect flow-through as Ni-NTA resin settles. Resin should not be allowed to run dry. Samples can be collected throughout purification for subsequent analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).
6. Wash Ni-NTA resin with His Wash 1 (high salt). Slowly pipette wash buffer down the side of the column to prevent disruption of the resin bed (*see* **Note 13**). Wash with approximately 10–20 column volumes (volume of resin bed) of His Wash 1 until clean (*see* **Note 14**).
7. Wash Ni-NTA resin with His Wash 2 (low salt) for 10 column volumes.

8. Elute CaM from Ni-NTA resin by slowly adding His-elution buffer, 2 column volumes at a time. Monitor protein levels (*see* **Note 14**) until no more protein is liberated. Elution volume is generally around 40 mL (*see* **Notes 15** and **16**).
9. Add 10 U thrombin (bovine thrombin is commercially available at low cost) to elution fraction for cleavage of His-tag *during* dialysis. Collect samples for SDS-PAGE before addition of thrombin, and after dialysis, to confirm complete cleavage of the His-tag. If downstream applications require immobilization through the His-tag, perform dialysis without thrombin to remove imidazole.
10. Prepare 4 L general dialysis buffer (stored at 4 °C). Measure a sufficient length of 10,000 molecular weight cutoff (MWCO) dialysis membrane to accommodate the sample with space for clamps. Wet the dialysis membrane in dialysis buffer, and fill with elution fraction after clamping one end (*see* **Note 17**). Clamp other side, and affix a buoyant object to keep the dialysis membrane clear of the magnetic stir rod. Dialyze overnight.
11. Carefully empty dialysis bag into a beaker on ice. At this point, check pre/post cleavage samples by SDS-PAGE before continuing. Concentrate sample to less than 10 mL using 50 mL 10,000 MWCO centrifugal concentrators (*see* **Note 18**). Centrifuge at 2300 × *g* for 10 min at a time, pausing between spins to thoroughly mix the sample to prevent aggregation and to add more protein. Before discarding the flow-through, check for protein by testing with Bradford dye, to ensure the membrane has not ruptured. As the protein becomes more concentrated, the flow rate will decrease. The protein solution may also become visibly viscous or appear faint yellow, which are not causes of concern; however, it should not become cloudy.
12. Prepare, filter, and degas gel filtration buffer (this can be performed in advance), and equilibrate size-exclusion (also known as gel filtration) chromatography column such as Superdex 75 26/60 run on an AKTA (GE Healthcare Life Sciences), or similar fast protein liquid chromatography (FPLC) (*see* **Note 19**). Filter the sample (0.22 μm), load into a 10 mL loop, and run 1.2 column volumes (~350 mL) of buffer at <2 mL/min. CaM should elute at a volume of ~200 mL.
13. Collect elution fractions, and concentrate once more to a working concentration of CaM for the given purpose. CaM is stable for NMR at concentrations above 1 mM, though 100–200 μM is sufficient for most experiments (*see* **Note 20**). Samples can be aliquoted, flash frozen in liquid nitrogen or ethanol/dry ice bath, and stored at −80 °C indefinitely.

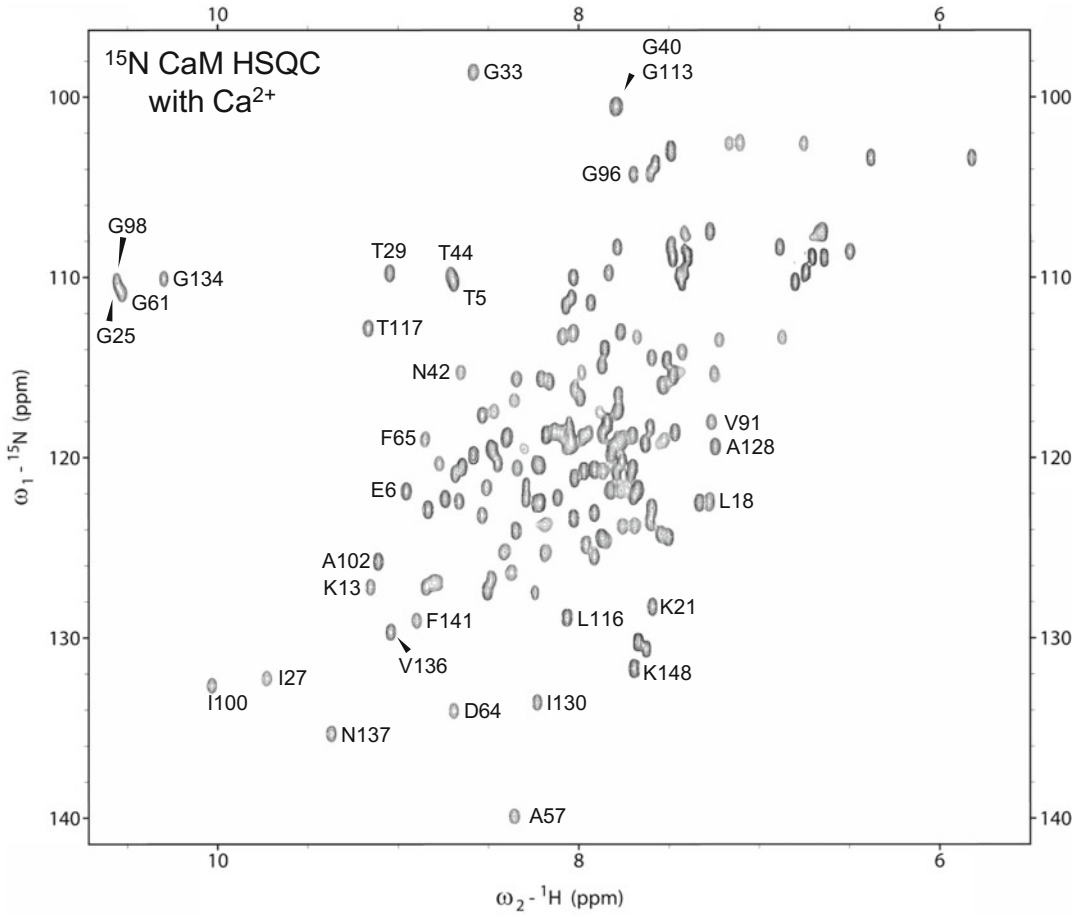

Fig. 3 ^{1}H-^{15}N HSQC of uniformly ^{15}N-labeled calmodulin bound to Ca^{2+}. Sample contains 0.75 mM calmodulin in gel filtration buffer (20 mM HEPES 7.4, 100 mM NaCl, 1 mM TCEP) with 10 mM $CaCl_2$. HSQC collected at 25 °C in 10 min

3.4 Preparation Methods

1. CaM from *E. coli* will be purified bound partially to Mg^{2+}. If Ca^{2+}-bound CaM is required, add 10 mM $CaCl_2$ to sample before use. Binding is fast and will displace other cations that may be present in the sample. An example of a ^{1}H-^{15}N heteronuclear single-quantum correlation (HSQC) spectrum of Ca^{2+} loaded, uniformly ^{15}N-labeled CaM is included (Fig. 3). If apo-CaM is required, a buffer exchange must first be performed, either on a single sample or with a batch, into chelation buffer. Use 10,000 MWCO centrifugal concentrators for buffer exchange (available in 15 mL, 4 mL, or 500 μL sizes). Add chelation buffer until it makes up at least 50% of the volume before switching back to gel filtration buffer. Thoroughly exchange buffer back into gel filtration buffer to remove the EDTA. An example of a ^{1}H-^{15}N HSQC of uniformly ^{15}N-labeled apo-CaM is shown below (Fig. 4) (*see* **Note 21**).

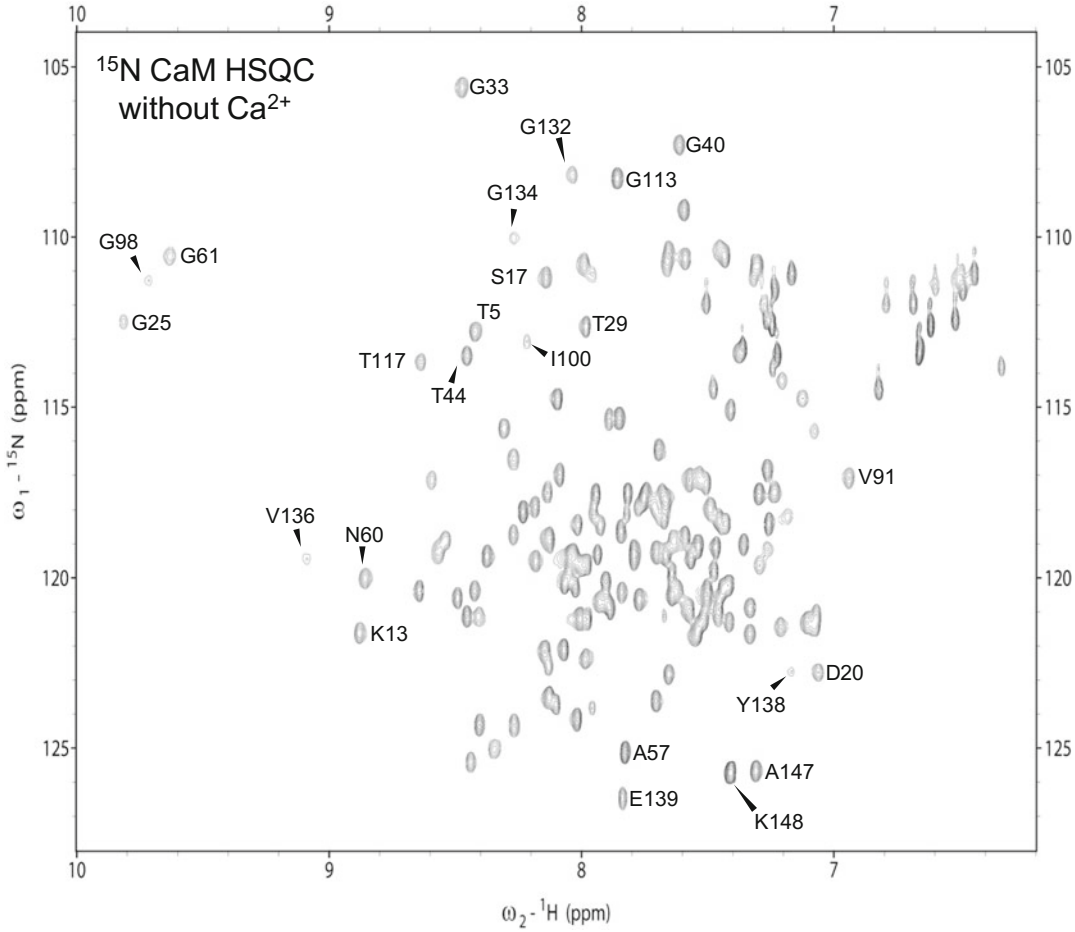

Fig. 4 ^{1}H-^{15}N HSQC of uniformly ^{15}N-labeled Ca^{2+}-free calmodulin. Sample contains 0.5 mM calmodulin in gel filtration buffer (20 mM HEPES 7.4, 100 mM NaCl, 1 mM TCEP). HSQC collected at 25 °C in 20 min

2. For formation of a CaM-peptide complex, mix Ca^{2+}-bound or apo-CaM and peptide at low concentrations, and then co-concentrate the mixture in a 3000 MWCO centrifugal concentrator to the working concentration. Mixing at low concentrations reduces the risk of precipitation.
3. Lyophilization allows long-term storage of CaM preparations. First, exchange apo-CaM (Ca^{2+}-containing buffers are not amenable to this technique) into a buffer of 25 mM ammonium bicarbonate. Ammonium bicarbonate provides sufficient buffering capacity but is volatile and thus dissipates during lyophilization. Following freeze-drying, pure apo-CaM powder can be stored at −20 °C for very long periods. To resuspend, place a drop of buffer beside the CaM powder, and allow

the powder to be slowly "drawn in." Adding buffer too quickly produces clumps that are difficult to dissolve.

4 Notes

1. PMSF is a protease inhibitor and is extremely toxic. Make a stock solution in ethanol to reduce routine exposure to the powder, and always wear personal protective equipment (PPE). It is rapidly degraded in water and thus must be added to lysis buffer immediately before use. There are also commercially available protease inhibitor cocktails that contain PMSF and are easier to handle.
2. This is a very flexible protocol that can be adjusted according to time constraints. Initial incubation on ice should be at least 15 min. The heat shock is the most critical step and should be performed at 42 °C for 1 min before returning the tube to ice, but the post-heat shock incubation on ice can be reduced to as little as 2 min. After addition of LB, shaking at 37 °C should be longer than 15 min but less than 1.5 h. Resuspension and plating volumes can be adjusted to optimize colony density. The plate can be incubated at room temperature over a weekend in place of overnight incubation at 37 °C.
3. Expression can be expedited by resuspending the entire plate, rather than a single colony, with 1–2 mL of media and inoculating the 50 mL LB starter culture. Shake at 37 °C for approximately 3 h (rather than overnight) to reach an appropriate cell density to pellet and proceed.
4. Alternatively, aeration and mixing of the *E. coli* culture can be achieved by bubbling compressed air in a system such as a LEX bioreactor (Epiphyte3).
5. Due to slow growth in M9 media, the one-day expression method described (*see* **Note 3**) can take as long as 12 h to achieve the OD required for induction.
6. The expression system used is highly efficient and can produce very large quantities of recombinant protein, which can form insoluble inclusion bodies. Often the overall yield of soluble protein can be improved by reducing the IPTG concentration and lowering the induction temperature. If a refrigerated incubator is not available or time is an issue, the induction of expression can also be performed at 37 °C for 3–4 h. Because CaM is highly soluble, this produces acceptable protein yields.
7. Freezing the cell pellets at −80 °C has no adverse impact on protein yield or quality and promotes cell lysis.
8. It is important, especially when unfamiliar with the protocol, to monitor every stage of the expression and purification by SDS-

PAGE. Several samples should be collected during expression and purification. The first is to test that induction of protein expression occurred. A useful trick is to save a cuvette used to measure OD_{600} preinduction until the following morning, so that samples of the culture both pre- and post-induction have similar cell densities. Take 100 μL of each sample, and centrifuge in Eppendorf tubes at max speed for 1 min. Discard supernatant and resuspend pellets in 50 μL of 1× SDS-PAGE sample buffer. When run on a gel, these samples should have similar background protein levels and a single strong band in the post-induction sample around 17 kD corresponding to CaM.

9. Heat generated by the sonication process will melt the ice around the sample beaker. Periodically check that the beaker is supported and that the tip of the sonicator remains ~1 cm from the bottom. A plastic beaker is preferable to glass, which may break.
10. Alternative lysis methods such as a French press are suitable.
11. Lipids can be loosely pelleted and contaminate the supernatant. To avoid clogging columns and filters, decant supernatant carefully and immediately following centrifugation.
12. Similar to monitoring expression as outlined above (*see* **Note 8**), it is important to check the proportion of protein found in the supernatant. For this to be reliable, the pellet and supernatant fractions need to be normalized: before centrifugation, take 50 μL of lysate, and centrifuge separately in an Eppendorf tube, max speed for 1 min. Separate supernatant from pellet, and solubilize both in SDS-PAGE sample buffer, to the same volume. As a highly soluble protein, CaM should only be present in the supernatant. If a large amount of CaM is found in the pellet, next time try slower expression (lower IPTG induction concentration) and/or more thorough lysis.
13. The resin bed is easily disrupted by the flow of buffer, which can create voids in the resin bed, allowing the buffer to channel rather than flowing evenly through the resin. If this happens, it is best to let the resin settle before continuing.
14. Bradford dye can be used to monitor the washing or elution progress. Make 10 mL of fresh Bradford dye before beginning column purification, and aliquot 400 μL into Eppendorf tubes. Collect one drop from the gravity flow column in a tube to qualitatively assess protein levels and decide when washing or elution is complete. When protein is present, Bradford dye turns blue.
15. The most efficient method to elute protein is to let one column volume elute at a time, with brief incubations in between to allow for protein dissociation from the resin. As the protein

becomes more dilute, extend the wait period. Elution is complete when these flow samples do not react with Bradford dye.

16. The Ni-NTA purification step can be automated using a prepacked Ni-NTA column on an FPLC system, such as AKTA (GE Healthcare Life Sciences).
17. Wet the dialysis membrane in dialysis buffer, and tease one side between fingers until it opens. Pipette buffer through the open membrane, making sure that it can pass freely through the entire length before clamping and adding sample. To monitor thrombin cleavage, run samples taken before and after dialysis on SDS-PAGE. Cleaved CaM runs with an approximate molecular weight just below 15 kD on SDS-PAGE due to its high negative charge.
18. Samples may be concentrated by alternative means to centrifugal concentrators.
19. Size-exclusion chromatography can be performed without an FPLC system, using a pump (e.g., peristaltic) and a column manually packed with size exclusion resin. The UV absorbance of each fraction can be measured manually to detect protein.
20. We use two methods to determine the concentrations of CaM, which are generally consistent: UV absorbance at 280 nm and Bradford. CaM lacks tryptophan; thus, its UV absorbance is relatively low, with predicted extinction coefficient at 280 nm of 2980 $M^{-1}cm^{-1}$. For Bradford reagent, the concentration can be determined using a standard curve (e.g., Abs = 0.0447 [concentration (mg/mL)] – 0.0597).
21. For sensitive analysis of apo CaM, it is important to decalcify all buffers. Ca^{2+} contamination can be present even in highly purified water and high-grade reagents. Buffers can be passed through a Chelex® 100 Resin (Bio-Rad) column to remove residual cations. This is not required for all purposes; however, the affinity of CaM for Ca^{2+} is enhanced in the presence of high-affinity Ca^{2+}-dependent target peptides; thus, traces of Ca^{2+} contamination may be sufficient to support Ca^{2+}-dependent interactions.

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