

Analysis of Calcium/Calmodulin Regulation of a Plant Kinesin Using Co-Sedimentation and ATPase Assays

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Summary

Kinesins, a superfamily of microtubule motor proteins, are implicated in regulating a number of fundamental cellular and developmental processes including intracellular transport of vesicles and organelles, mitotic and meiotic spindle formation and elongation, chromosome segregation, germplasm aggregation, microtubule (MT) organization and dynamics, and intraflagellar transport. Analysis of all the completed genomes of eukaryotes has revealed that *Arabidopsis*, a flowering plant, has more kinesins than any other organism. Although a complete inventory of kinesins in a number of organisms has been reported, the function and regulation of kinesins in general and plant kinesins in particular are poorly understood. In our screen of an expression library with a labeled calmodulin, we isolated a novel plant kinesin (kinesin-like calmodulin-binding protein, KCBP) from plants, which interacts with calmodulin in a calcium-dependent manner. This chapter describes the methods used in elucidating the regulation of this motor protein by calcium/calmodulin.

Key Words: calcium; calmodulin; KCBP, co-sedimentation assay; ATPase assay; kinesin; motor protein; microtubule (MT)

1. Introduction

Calcium, a universal messenger in eukaryotes, regulates a plethora of physiological and developmental processes in plants (**1**). It has been implicated in mediating the action of many hormonal and environmental signals (**2**). Many signals that affect plant growth and development have been shown to elevate cytosolic calcium. These changes in cytosolic calcium are transmitted to the metabolic machinery of the cell via calcium sensors. These sensors either directly or indirectly regulate cellular processes. In recent years, a large number (more than 250) of putative calcium sensors have been identified (**3**). Some of

From: *Methods in Molecular Biology*, vol. 392: *Molecular Motors*
Edited by: A. O. Sperry © Humana Press Inc., Totowa, NJ

these are enzymes (e.g., calcium-dependent protein kinases), and the activity of these enzymes is modulated directly by calcium. Other calcium sensors, such as calmodulin, have no enzymatic activity but interact and regulate the activity of a large number of diverse proteins including protein kinases, phosphatases, transcription factors, and cytoskeletal proteins in a calcium-dependent manner. To understand the calcium/calmodulin-regulated process in plants, much focus has recently been aimed at identifying the proteins that interact with activated (calcium-bound) calmodulin. These efforts have resulted in identification of more than 150 calmodulin target proteins (2,4). In our screens of expression libraries with labeled calmodulin, we isolated a novel calmodulin-binding kinesin from *Arabidopsis* and a number of other plants (5,6), raising the possibility that this kinesin is regulated by calcium via calmodulin. The methods described below have allowed us to demonstrate that activated calmodulin inhibits motor interaction with microtubules (7–9), suggesting that the elevated levels of calcium inactivate kinesin-like calmodulin-binding protein (KCBP).

2. Materials

2.1. Constructs of *Arabidopsis* KCBP and Calmodulin

1. Full-length KCBP is 1261 amino acids (aa) long. Two truncated versions of KCBP fused to different tags (7,10) are used in these experiments: (i) 1.5 C in pET-32 (aa 821–1261) and (ii) 1.0 C in pET-28 (aa 860–1210) (see Fig. 1 for the tags on each of these fusions). *Escherichia coli* BL21(DE3) was used to express these proteins.
2. pET-5 constructs of *Arabidopsis* calmodulin2 (CaM2), calmodulin4 (CaM4), and calmodulin6 (CaM6) isoform in *E. coli* BL21(DE3) (11).

2.2. Expression and Purification

2.2.1. CaM Isoforms

1. NZY medium: Dissolve 10 g NZ amine, 5 g NaCl, 2 g MgSO₄·H₂O in 1 L, adjust pH to 7.0, and sterilize by autoclaving for 20 min on liquid cycle.
2. Ampicillin: 50 mg/mL in water.
3. Isopropyl-1-thio-β-D-galactopyranoside (IPTG): 100 mM in water.
4. Buffer A: 50 mM Tris-HCl, pH 7.5.
5. Lysis buffer: 50 mM Tris-HCl, pH 7.5, 2 mM Na₂ ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 200 μg/mL lysozyme.
6. DNase.
7. Saturated (NH₄)₂SO₄. Dissolve 69.7 g ammonium sulfate in 10 mL water.
8. DTT: 1 M stock in water.

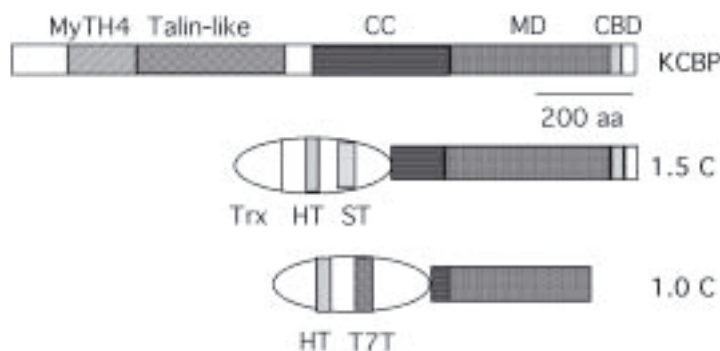


Fig. 1. Schematic representation of fusion proteins of *Arabidopsis* kinesin-like calmodulin-binding protein. KCBP, the full-length *Arabidopsis* kinesin-like calmodulin-binding protein, showing various domains that are identified based on sequence similarity or functional analysis of *Escherichia coli*-expressed protein (5,18); MyTH4, a domain present in the tail region of some members of the myosin superfamily (VIIa, IV, X, and XII); CC, α -helical coiled-coil region; MD, motor domain; CBD, calmodulin-binding domain; 1.5C, the carboxy-terminal fusion protein (amino acids 821–1261) of KCBP containing motor and calmodulin-binding domains and a limited coiled-coil stalk; 1.0C, the carboxy-terminal fusion protein (amino acids 860–1210) containing a short coiled-coil stalk and motor domain but without calmodulin-binding domain; HT, His.tag for affinity purification of fusion protein; T7T, T7.tag; Trx, thioredoxin for increased solubility of fusion protein; ST, S tag.

9. Buffer 1: 50 mM Tris-HCl, pH 7.5, 0.1 mM CaCl_2 , and 0.5 mM DTT.
10. Buffer 2: 50 mM Tris-HCl, pH 7.5, 0.1 mM CaCl_2 , and 0.5 mM DTT, 5 mM NaCl.
11. Buffer 3: 50 mM Tris-HCl, pH 7.5, 0.1 mM ethyleneglycoltetraacetic acid (EGTA), and 0.5 mM DTT.

2.2.2. KCBP

1. Luria-Bertani (LB) medium: 1% bacto-tryptone, 0.5% bacto-yeast extract, and 1% NaCl. Adjust pH to 7.0 with NaOH. Sterilize the medium by autoclaving for 20 min on liquid cycle.
2. Ampicillin: 50 mg/mL in sterile water.
3. Kanamycin: 50 mg/mL in sterile water.
4. Isopropyl-1-thio- β -D-galactopyranoside (IPTG): 100 mM in water.
5. Calmodulin-Sepharose (Pharmacia).
6. His-bind matrix (Novagen).
7. Complete protease inhibitor tablets (Boehringer Mannheim).

2.3. Co-Sedimentation of Microtubules with Motors

1. Tubulin (cytoskeleton): 10 mg/mL.
2. 5X microtubule preparation buffer: 400 mM piperazine-*N,N*-bis[2-ethanesulfonic acid] (PIPES), pH 6.8, 5 mM MgCl₂, 5 mM EGTA, 5 mM GTP, 5 μ M taxol, and 50% glycerol.
3. 10X co-sedimentation assay buffer: 200 mM PIPES, pH 6.9, 10 mM MgCl₂, 10 mM DTT, 1.5 M NaCl, 200 μ M taxol, 5 mM AMP-PNP.
4. Calcium chloride.
5. Arabidopsis CaM isoform.
6. Bovine CaM (Calbiochem).
7. Taxol (paclitaxel; Sigma T-7402): 6 mM in dimethylsulfoxide (DMSO).

2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 30% acrylamide/bisacrylamide solution; 29.2% acrylamide, 0.8% bisacrylamide. Acrylamide is a neurotoxin. Wear a mask when weighing acrylamide. Wear gloves in handling this solution. This solution can be stored for a month.
2. 10% ammonium persulfate solution: Prepare fresh solution before use or freeze in aliquots at -20°C.
3. 1.5 M Tris-HCl, pH 8.8.
4. 0.5 M Tris-HCl, pH 6.8.
5. 2X sample loading buffer: 0.125 M Tris HCl, pH 6.8, 4% SDS, 10% β -mercapto-ethanol, 20% glycerol, 0.015% bromophenol blue.
6. 5X electrode buffer: 125 mM Tris base, 960 mM glycine, and 0.5% (w/v) SDS. Use mask while weighing SDS. Do not adjust pH. Store at room temperature.
7. Staining solution: 0.25% Coomassie blue R250, 40% methanol, and 7.5% acetic acid.
8. Destaining solution: 30% methanol and 7% acetic acid.

2.5. ATPase Assay

1. ATPase reaction buffer: 15 mM imidazole, pH 7.0, 2 mM MgCl₂, and 1 mM dithiothreitol (DTT).
2. MgATP: 100 mM in water. Make it fresh.
3. 20% (v/v) Triton X-100. Make it fresh.
4. Malachite green reagent (MGR): Dissolve 340 mg malachite green (Sigma) in 75 mL deionized water and dissolve 10.5 g ammonium molybdate in 250 mL 4N HCl. Mix these two solutions and bring the volume to 1 L with water. Keep this solution on ice for at least 1 h to clear the solution. Filter the solution through Whatman paper and store at 4°C. It can be stored up to 2 months.
5. MGR/Triton X-100: Mix 50 mL MGR prepared as above with 250 μ L freshly prepared 20% (v/v) Triton X-100.
6. 34% (v/v) citric acid: Make fresh.

3. Methods

3.1. Expression and Purification of Different CaM Isoforms

1. Inoculate 3 mL NZY medium containing 50 µg/mL ampicillin with a single colony or 5 µL glycerol stock of a CaM isoform construct and grow overnight at 37°C.
2. Add overnight culture to 500 mL fresh NZY medium containing 50 µg/mL ampicillin and incubate with shaking at 37°C until the OD₆₀₀ reaches 0.6.
3. Add IPTG to a final concentration of 1 mM (5 mL from 100 mM stock) and continue incubation for an additional 3 h at 37°C.

Perform all the following procedures at 4°C.

4. Harvest the cells by centrifugation at 5000g for 5 min (*see Note 1*).
5. Wash the cells by suspending the pellet in 10 mL buffer A and collect the cells as in **step 4**.
6. Resuspend the cell pellet in the lysis buffer, incubate for 30 min, and add DNase to 50 U/mL and MgCl₂ to 3 mM to remove DNA. Incubate for an additional 30 min.
7. Centrifuge the extract at 27,000g for 30 min.
8. To the supernatant, add saturated (NH₄)₂SO₄ dropwise to obtain 55% saturation and stir gently while adding the saturated solution (*see Note 2*). Continue stirring for 30 min to precipitate proteins.
9. Collect the precipitate by centrifugation at 27,000g for 30 min, suspend it in 50% (v/v) H₂SO₄ to bring the pH to 4, and stir it for another 30 min (*see Note 3*).
10. Collect the precipitated protein by centrifugation at 27,000g for 30 min.
11. Resuspend the pellet in 2.5 mL buffer A containing 1 mM DTT.
12. Dialyze the protein for 1 h against 1 L deionized water and then for another 1 h against 1 L buffer A containing 100 mM NaCl, 0.5 mM EGTA, and 1 mM DTT.
13. Following dialysis, centrifuge the protein at 27,000g for 15 min.
14. Add calcium chloride to a final concentration of 5 mM, mix, and load it onto a Phenyl-Sepharose CL-4B column (5 mL bed volume) that was equilibrated with buffer 1. Wash the column with the same buffer until A₂₈₀ is less than 0.01.
15. Wash the column with buffer 2 until the A₂₈₀ = 0.
16. Elute the bound protein with buffer 3.
17. Pool the peak fractions and dialyze first against 20 mM NH₄HCO₃ and then against deionized water (*see Note 4*).
18. Check the purity and concentration of protein on an SDS gel (**Fig. 2**).
19. Aliquot and freeze the dialyzed fractions and store at -80°C or -20°C. About 20 mg purified protein is obtained from 1 L culture.

3.2. Expression and Purification of 1.5C and 1.0C Constructs of KCBP

1. Inoculate 3 mL LB medium containing either 50 µg/mL ampicillin (for pET 32 constructs) or 50 µg/mL kanamycin (for pET28 constructs) with a single bacterial

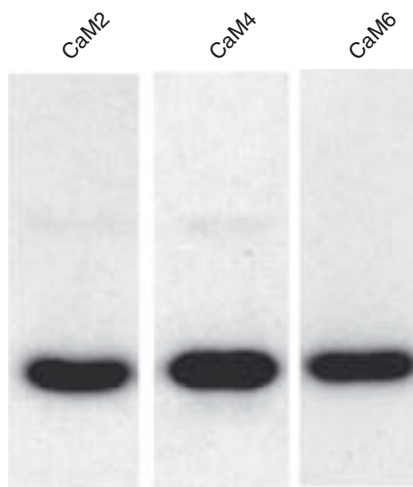


Fig. 2. Stained gel showing the purity of *Arabidopsis* calmodulin (CaM) isoforms. Purified CaM isoforms were analyzed by electrophoresis and stained with Coomassie blue (19).

- colony from a freshly streaked plate or 5 μ L glycerol stock and grow the cells overnight at 37°C.
2. Add 2 mL overnight culture to fresh 50 mL LB containing the appropriate antibiotic in a 250-mL Erlenmeyer flask. Incubate the flask at 37°C with shaking (250 rpm) until the A_{600} is about 0.6.
3. Induce the protein by adding IPTG to a final concentration of 0.5 mM (add 0.25 mL IPTG from 100 mM stock) (see **Note 5**).
4. Grow the culture for another 5 h at 30°C (see **Note 6**).
5. Harvest the cells by centrifugation at 5000g for 5 min at 4°C (see **Note 7**).
6. Resuspend the pellet in 5 mL 50 mM Tris-HCl, pH 8.0, and collect the cells by centrifugation as above.
7. Add 5 mL lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM Mg ATP, 0.5 mM β -mercaptoethanol, 0.5 mM DTT, 100 mM lysozyme, and complete protease inhibitor) and place the cells on ice for 30 min (see **Notes 8 and 9**).
8. Sonicate three to four times, 10 s each, and centrifuge at 12,000g for 30 min at 4°C.
9. Purify the protein containing the calmodulin-binding domain using a calmodulin-Sepharose 4B affinity column and the protein without the calmodulin-binding domain using a His-bind affinity column (see **Note 10**).

3.2.1. Purification of Calmodulin-Binding Domain (CBD)-Containing KCBP (1.5 C) Using Calmodulin-Sepharose 4B Affinity Column

The binding of calmodulin to its targets occurs mostly through hydrophobic interactions. The hydrophobic regions of calmodulin are exposed in the

presence of calcium. Hence, calmodulin binds to its target proteins with high specificity only in the presence of calcium. Protein extracts containing calmodulin-binding proteins are prepared in a calcium-containing buffer and passed through calmodulin-Sepharose, which allows calmodulin target proteins to bind to the affinity matrix. Following washing, the bound calmodulin-binding protein can be eluted with a buffer containing a calcium-chelating agent such as EGTA. Because of the high specificity and affinity of calmodulin to its target proteins, highly pure protein can be obtained by one-step purification. Perform all operations at 4°C.

1. Prepare a calmodulin-Sepharose 4B column (10 mL bed volume). Equilibrate the column with binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM Mg-ATP, and 1 mM CaCl₂) by passing at least 50 mL binding buffer (*see Note 11*).
2. Load the protein extract from **step 8** above onto the column and allow it to pass through the column.
3. Wash the column thoroughly with the binding buffer until there is no protein in the flow-through ($A_{280} = 0$).
4. Elute the bound protein in elution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM Mg-ATP, and 1 mM EGTA) in 2-mL fractions. EGTA strips the calcium from calmodulin and reverses the conformational change, thereby releasing the bound protein.
5. Pool the peak fractions and dialyze against motor buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM Mg-ATP) to remove EGTA.
6. Check the purity and concentration of the protein by running about 30 μ L from each fraction on denaturing gels.

3.2.2. Purification of KCBP Without a CBD (1.0C) Using the His-Bind Column

The KCBP (1.0C) that lacked the CBD contains a His-tag at the N-terminus (*see Fig. 1*). The presence of a stretch of histidines permits one-step purification of protein to near homogeneity using an affinity matrix (e.g., His-bind column) that binds the His-tag.

1. Prepare His-bind affinity (10 mL bed volume) column. Equilibrate the column with TN buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 0.5 mM Mg-ATP) containing 5 mM imidazole. Pass at least 50 mL binding buffer (*see Note 9*).
2. Load the protein extract from **step 8** above onto the column and allow it to pass through the column.
3. Wash the column thoroughly with the TN buffer containing 20 mM imidazole until there is no protein in the flow-through.
4. Elute the bound protein in TN buffer containing 100 mM imidazole. Collect 2-mL fractions. A high concentration (100 mM) of imidazole strips the bound protein from the His-bind matrix.

5. Pool the peak fractions and dialyze against motor buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM Mg-ATP) to remove EGTA.
6. Check the purity and concentration of the protein by running about 30 μ L from each fraction on denaturing gels. Use freshly purified protein for microtubule-binding and ATPase assays (*see Note 12*).

3.3. Analysis of KCBP Interaction with Microtubules

3.3.1. Calcium/Calmodulin Regulation of KCBP Interaction with Microtubules

Co-sedimentation of kinesin with microtubules (MTs) in the presence of ATP or nonhydrolyzable ATP analogs has been widely used to study the interaction of motors with MTs (*12,13*). In this simple yet powerful assay, the purified motor protein is mixed with taxol-stabilized MTs, incubated for some time, and centrifuged at high speed to separate MTs (pellet) from unbound kinesin (supernatant). If the motor is bound to MTs, it co-sediments with MTs. The presence of motor in the pellet and supernatant is then analyzed by SDS-PAGE. This method was used to identify MT-binding domains in motors and other MT-interacting proteins as well as to study the regulation of interaction between kinesins and MTs. We have used the co-sedimentation assay to study calcium/calmodulin regulation of KCBP and to identify a second MT-binding domain in the nonmotor region of KCBP (*7–9*).

3.3.2. Preparation of Microtubules

1. To 100 μ L (10 mg/mL) tubulin (*see Note 13*), add 10 μ L 10X MT preparation buffer plus 90 μ L deionized water and incubate at 35°C for 30 min.
2. Then add 2 μ L 6 mM taxol (*see Note 14*), 46 μ L 50% glycerol, 6 μ L 10X MT preparation buffer, and 6 μ L water.
3. Incubate at 35°C for 30 min.
4. Add 5 μ L 6 mM taxol (*see Note 14*) and 0.6 μ L MT preparation buffer.
5. Incubate at 35°C for 5 min.
6. Add another 5 μ L 6 mM taxol (*see Note 14*) and 0.6 μ L MT preparation buffer.
7. Incubate at 35°C for 4 h.
8. Sediment microtubules at 10,000g at 35°C for 30 min.
9. Dissolve MT pellet in 200 μ L 1X co-sedimentation assay buffer to obtain about 5 μ g/ μ L concentration (*see Note 15*).

3.3.3. Co-Sedimentation Assays

Perform the MT-motor (KCBP) binding assays in a reaction volume of 100 μ L.

1. Add 10 μ L 10X pelleting buffer, 2.5 μ M purified KCBP (*see Note 16*), and 10 μ M taxol-stabilized MTs (co-sedimentation buffer in controls), and bring the final

volume to 100 μ L. Run assays with MT alone and protein alone in parallel as controls. To test the effect of calcium (100 μ M), calmodulin (15 μ M), and calcium/calmodulin on motor binding to MTs, add these test compounds to appropriate assay tubes.

2. Incubate the reaction mixture at 22°C for 20 min.
3. Centrifuge the tubes for 20 min at 100,000g at 35°C.
4. Collect the supernatant (*see Note 17*) and add an equal volume of 2X SDS sample loading buffer. Dissolve the pellet in 1X SDS sample loading buffer.
5. Boil the protein samples for 5 min and analyze them by SDS-PAGE.
6. Visualize the amount of motor in the pellet and supernatant by Coomassie blue staining.

3.4. Denaturing SDS-PAGE

1. Assemble the glass plate sandwiches with two clean glass plates and two 1-mm-thick spacers for each gel and lock them into a casting stand.
2. Prepare 10% separating solution by mixing 15 mL 1.5 M Tris-HCl, pH 8.8, 20.1 mL 30% acrylamide/bisacrylamide solution, 600 μ L 10% SDS, 24.3 mL deionized water, and degas this solution for 10 min. Then add 300 μ L 10-ammonium persulfate and 30 μ L TEMED (tetramethylethylenediamine), and mix the solution by swirling gently (*see Note 18*).
3. With a pipet pour 4.5 mL separating gel solution along the edge of one of the spacers in each minigel, and with a Pasteur pipet gently overlay the separating gel solution with water-saturated isobutyl alcohol; this will leave enough space for the stacking gel. Allow the gel to polymerize for 30–60 min.
4. While the separating gel solution polymerizes, prepare 4% stacking solution by mixing 5 mL 0.5 M Tris-HCl, pH 6.8, 2.6 mL 30% acrylamide/bisacrylamide solution, 200 μ L 10% SDS, 12.2 mL deionized water, and degas this solution for 10 min. Then add 100 μ L 10-ammonium persulfate and 20 μ L TEMED, and mix the solution by swirling gently (*see Note 18*).
5. Pour off the isobutyl alcohol and rinse the top of the separating gel with water to completely remove any residual isobutyl alcohol.
6. Using a Pasteur pipet, pour the stacking solution and insert 1-mm-thick combination into the stacking solution. Allow stacking solution to polymerize for about 30 min.
7. Prepare 1X electrode buffer by diluting 100 mL 5X buffer with 400 mL deionized water.
8. Carefully remove the comb from the stacking gel, rinse the wells with 1X electrode buffer, and fill the wells with the same buffer.
9. Attach the gel sandwich to the upper buffer chamber and place it in the lower buffer chamber with 1X electrode buffer so that the electrode wire is immersed in the buffer. Then fill the upper chamber with 1X electrode buffer.
10. Carefully load the protein samples (prepared as above) into the wells. Load prestained molecular weight markers in one of the lanes (*see Note 19*).

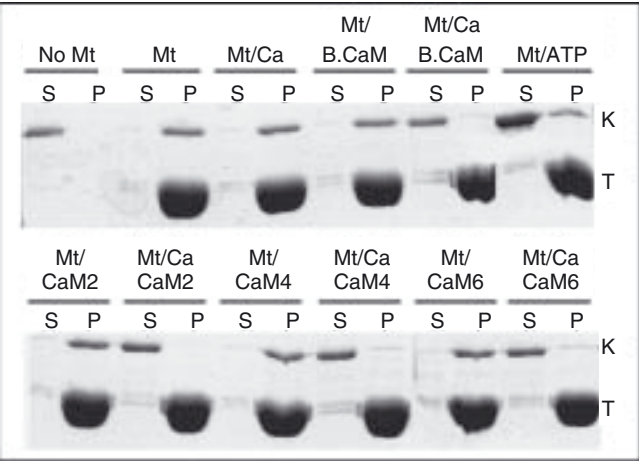


Fig. 3. *Arabidopsis* CaM isoforms regulate the interaction of KCBP containing the CBD with MTs. Purified 1.5C KCBP with the CBD (K) was incubated with MTs (Mt) for 20 min at room temperature (RT) in the presence of Ca^{2+} (Mt/Ca), various CaMs (Mt/B.CaM, Mt/CaM2, Mt/CaM4, or Mt/CaM6) alone or in the presence of both Ca^{2+} and various CaMs (Mt/Ca B.CaM, Mt/Ca CaM2, Mt/Ca CaM4, or Mt/Ca CaM6). Following centrifugation, the supernatant (S) and pellet (P) fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained (stained gel). Except in the Mt/ATP reaction, in which ATP was used, all reactions were performed in the presence of AMP-PNP. No Mt, motor protein without MTs; CaM2, CaM4, and CaM6, three isoforms of *Arabidopsis* CaM; B.CaM, bovine CaM; Mt, microtubules; K, motor; T, tubulin subunits (9).

- 11. Connect the power supply to the electrophoresis apparatus and run at 50 V per gel until the bromophenol blue reaches the bottom of the separating gel.
- 12. Disconnect the power supply, remove the upper buffer chamber with the attached gels, and discard the buffer.
- 13. Remove the gel sandwich from the casting stand and lay it on a paper towel. Carefully pry open the glass plates, and cut a small triangle at one corner of the gel to mark the orientation of the gel.
- 14. To visualize the proteins, immerse the gel in Coomassie blue staining solution for 1 h with gentle shaking, and then briefly rinse the gel with deionized water and transfer it to destaining solution until the gel is completely destained. **Figure 3** shows the binding of 1.5C KCBP to microtubules in the presence of calcium, calmodulin, and calcium/calmodulin.

3.5. Calcium/Calmodulin Regulation of ATPase Activity of KCBP

There are a number of methods (colorimetric and radioactive) to quantify ATPase activity of an enzyme (14,15). Some colorimetric assays are based on

the properties of the complex formed between the inorganic phosphate and molybdate under acid conditions. These assays are useful to study the regulation of MT-dependent and MT-independent ATPase activity of kinesins by other macromolecules that interact with motors. The ATPase assay described here is a simple colorimetric assay based on the change in malachite green with the release of Pi from ATP. The hydrophobic surface of the phosphomolybdate complex binds malachite green dye and shifts the wavelength for the maximum absorbance (**16,17**). We have used this assay to demonstrate that the MT-dependent ATPase activity of KCBP is inhibited by activated calmodulin.

1. Perform the ATPase assays in a final volume of 50 μ L (*see Note 20*). Perform all assays in triplicate in plastic tubes (*see Note 21*).
2. To each tube, add 5 μ L 10X ATPase reaction buffer, 1.5 μ L 100 mM Mg-ATP (final concentration of ATP is 3 mM), and 2 μ M Taxol-stabilized microtubules and 50–300 nM purified motor protein (KCBP). Bring the volume of all assays to a final volume of 50 μ L. Control assays have no motor protein.
3. To test the effect of EGTA (2 mM), calcium (100 μ M), calmodulin (1 μ M), and calcium/calmodulin on microtubule-dependent and -independent activity of the motor, add these test compounds in the appropriate tubes.
4. Incubate the tubes at 30°C for 20 min.
5. Then add 800 μ L of freshly prepared malachite green reagent/Triton X-100 (*see Note 22*) and 100 μ L 34% citric acid in this order and mix the solutions by vortexing.
6. Incubate the tubes at room temperature for 10 min.
7. Measure the O.D. at 660 (*see Notes 23 and 24*).
8. Subtract the O.D. value of the control (no motor protein) from all assays with motor protein.
9. Calculate the average of triplicates and estimate the released Pi using $1 \text{ OD}_{660} = 9.45 \text{ nmoles Pi}$ and use this value to calculate the specific activity of the motor (i.e., micromoles of Pi released per milligram of motor protein per minute).

4. Notes

1. The cell pellet can be stored at –20°C for several days.
2. Use freshly prepared solution. It is important to gently stir the solution while adding the saturated ammonium sulfate solution to the protein solution; this eliminates spatial nonuniformity in the salt concentration.
3. It takes only a drop or two to lower the pH to 4.
4. Because of the low content of aromatic amino acids, the UV absorbance of CaMs is low. Do not discard the fractions that have low absorbance. Check the concentration of protein by running an aliquot on SDS gels and staining with Coomassie blue. Run a known concentration of bovine serum albumin (BSA) in parallel to estimate the concentration of protein.

5. Induction at room temperature is important to obtain good quantities of motor in the soluble fraction. A high level of protein is induced at 37°C, but most of it goes into the insoluble fraction.
6. Limit the induction time to 3–4 h. Longer induction times result in the loss of protein from the soluble fraction.
7. The cell pellet can be frozen at –80°C for several days.
8. Inclusion of complete protease inhibitor cocktail is necessary to prevent the degradation of motor protein.
9. It is important to include ATP in the buffer to keep the motor protein active.
10. Do not store the protein extract at this stage. Storing of the protein at this step leads to degradation and inactivation of the motor.
11. Inclusion of 150 mM NaCl eliminates nonspecific binding of the proteins to the column.
12. Freezing and thawing of purified motor results in precipitation of protein and loss of MT binding and ATPase activities.
13. Use highly purified tubulin that is devoid of any microtubule-associated proteins (MAPs). The presence of MAPs interferes with the motor activity.
14. Taxol is sparingly soluble in water. Hence, to avoid precipitation mix the solution after adding taxol.
15. Prepare MTs on the day of co-sedimentation or ATPase assays. Polymerized MTs are stable for several hours at 25°C.
16. Before co-sedimentation assays, centrifuge the purified motor protein at 100,000g for 1 h at 4°C to remove any precipitated protein.
17. The MT pellet is very small. Care should be taken to not disturb the pellet while collecting the supernatant. Small (1.5-mL) tubes are better than the larger tubes for these assays.
18. Separating and stacking gel solutions should be prepared fresh. These solutions should be used immediately to prevent polymerization before pouring the gels. For running CaM, prepare 12% running gel.
19. Add 1X sample buffer to any empty wells to prevent distortion of lanes.
20. If needed, the volume can be scaled up to 100 µL.
21. Use disposable plastic reaction tubes as they are devoid of any phosphate residues.
22. Prepare this solution fresh during the incubation time of ATPase assays. Avoid pipeting any precipitated material in the MGR bottle in preparing this reagent.
23. Take the O.D. within 3 min. Microtubules precipitate after 13 min.
24. Use disposable plastic cuvetts for taking the O.D. Reusable glass or quartz cuvetts tend to accumulate color stain on the walls.

Acknowledgments

The author thanks Dr. Raymond E. Zielinski (University of Illinois) for *Arabidopsis* CaM isoform constructs and methodologies pertinent to purifying these isoforms; Dr. Gero Steinberg (University of Colorado) for advice on the ATPase assays; Irene Day (Colorado State University) for carefully reading the

manuscript. This work was supported by grants from the National Science Foundation (No. MCB-9630782 and No. MCB-0079938).

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Molecular Motors

Methods and Protocols

Sperry, A.O. (Ed.)

2007, XII, 252 p. 37 illus., Hardcover

ISBN: 978-1-58829-665-8

A product of Humana Press