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Chapter 12

Characterization of Microtubule-Associated Proteins (MAPs) and Tubulin Interactions by Isothermal Titration Calorimetry (ITC)

Philipp O. Tsvetkov, Romain La Rocca, Soazig Malesinski, and François Devred

Abstract

Microtubules are highly dynamic structures which play a central role in many cellular processes such as cell division, intracellular transport, and migration. Their dynamics is tightly regulated by stabilizing and destabilizing microtubule-associated proteins (MAPs), such as tau and stathmin. Many approaches have been developed to study interactions between tubulin and MAPs. However, isothermal titration calorimetry (ITC) is the only direct thermodynamic method that enables a full thermodynamic characterization of the interaction after a single titration experiment. We provide here the protocols to apply ITC to tubulin interaction with either stathmin or tau, which will help to avoid the common pitfalls in this very powerful and sensitive method.

Key words Tubulin, Microtubule, Isothermal titration calorimetry, Stathmin, Tau

1 Introduction

Microtubules are highly dynamic structures [1] that play an important role in many vital cell processes such as division, migration, intracellular transport, and architecture. Their dynamicity is tightly regulated by microtubule-associated proteins (MAPs), which can be separated into stabilizing and destabilizing MAPs, such as tau protein and stathmin, respectively (Fig. 1). When dephosphorylated, both tau and stathmin bind tubulin and/or microtubules and detach upon phosphorylation [2, 3]. Interactions between these MAPs and tubulin have been intensively studied using many biochemical and biophysical methods [4–6], yet for tau, which is probably the most studied MAP, the community has not reached a unifying model [7–9].

Microtubules and their dynamics are also the target of exogenous microtubule-targeting agents (MTAs) that are part of the

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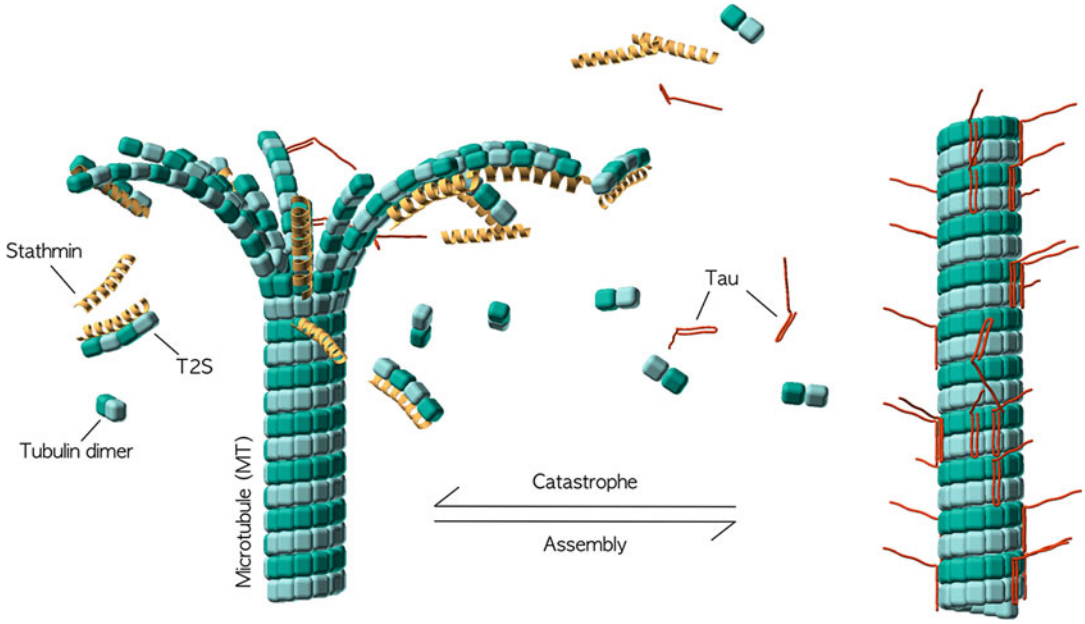

Fig. 1 MAP-tubulin interactions and their consequences on tubulin self-assembly and catastrophe. This scheme represents tubulin self-assembly in a classical polymerization buffer (in which tubulin spontaneously polymerizes into MT at 37 °C). MTs depolymerize in the presence of stathmin, and tubulin and stathmin form T2S complex, whereas MTs are stabilized in the presence of tau

main anticancer arsenal. Indeed, using MTAs that stabilize microtubules (taxanes) or MTAs that induce microtubule depolymerization (vinca alkaloids) usually alters microtubule dynamics and leads to mitotic block and cell death [10, 11]. In this context, MTA binding to tubulin has also been extensively studied using a wide variety of methods [12]. Characterizing this interaction is an essential step to test whether a suspected novel anticancer agent is an MTA [13–15].

Isothermal titration calorimetry (ITC) is a powerful method to study directly the thermodynamics of MTA or MAP interaction with tubulin. It was first used to give a full thermodynamics characterization of stathmin-tubulin interaction [16] and later to describe the impact of the phosphorylation of the four individual serine residues of stathmin on its structure and on its interaction with tubulin [17]. It was also used to show by which molecular mechanism overexpression of stathmin could potentiate the anticancer activity of vinca alkaloids and decrease the activity of taxanes [13–15] opening new perspectives to cancer therapies.

ITC was also used to study tau binding to tubulin [9]. While it turned out to be more complex in terms of finding the appropriate model to fit the obtained calorimetric data, it did nevertheless showed that tau was binding in a similar fashion to tubulin rings which are formed at 20 °C [18] and to microtubules.

We have previously described the important steps and precautions take into consideration as well as pitfalls to avoid when studying tubulin binding by ITC [19, 20]. Here we describe the detailed protocols to characterize MAP-tubulin interaction by ITC, for tau and stathmin.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

2.1 Tubulin Purification

The detailed protocol of tubulin purification from bovine brain was described earlier [21].

2.2 Tau Purification

The detailed protocol of recombinant human tau purification was described earlier [21].

2.3 Stathmin Purification

2.3.1 Reagents and Buffers

1. Transformed bacteria *Escherichia coli* BL21DE3 with pET11c plasmid containing stathmin cDNA.
2. Luria Broth (LB) Miller medium (200 mL). Weigh 5 g medium in a 200 mL glass beaker and add ultrapure water. Adjust volume to 200 mL in a graduated flask and transfer in a glass bottle for sterilization. Store at 4 °C.
3. Luria Broth (LB) buffered medium (1 L). Weigh 25 g medium in a 1 L glass beaker and add ultrapure water. Adjust volume to 1 L in a graduated flask and transfer in a bottle for sterilization. Store at 4 °C.
4. Ampicillin 100 mg mL^{-1}. Weigh 1 g ampicillin in 15 mL tube, and add ultrapure water. Adjust volume to 10 mL in a graduated flask. Make 1 mL aliquots, and store them at −20 °C.
5. 1 M Isopropyl-beta-d-1-thiogalactopyranoside (IPTG). Weigh 2.4 g in a 15 mL tube and add ultrapure water. Adjust volume to 10 mL in a graduated flask. Make 1 mL aliquots and store them at −20 °C.
6. Lysis buffer (100 mL): 20 mM Tris–HCl pH 7.8, 1 mM EDTA, 8 mM Triton X-100. Weigh 0.5 g Triton and 0.32 g Tris–HCl. Mix in a 100 mL glass beaker with 95 mL of ultrapure water. Adjust volume to 100 mL in a graduated flask. Make 10 mL aliquots, and store them at −20 °C.
7. Ion-exchange activation buffer (500 mL): 20 mM Tris–HCl, 1 M NaCl, pH 7.8. Dissolve 1.6 g Tris–HCl and 29 g NaCl in 450 mL of ultrapure water in a 500 mL glass beaker. Adjust pH to 7.8 with NaOH, and complete volume to 500 mL in a graduated flask. Store at 4 °C.

8. Ion-exchange equilibration buffer (500 mL): 20 mM Tris–HCl, pH 7.8. Dissolve 1.6 g Tris–HCl in 450 mL of ultrapure water in a 500 mL glass beaker. Adjust pH to 7.8 with NaOH, and complete volume to 500 mL in a graduated flask. Store at 4 °C.
9. Ion-exchange elution buffer (500 mL): 20 mM Tris–HCl, 0.2 M NaCl, pH 7.8. Dissolve 1.6 g Tris–HCl and 5.8 g NaCl in 450 mL of ultrapure water in a 500 mL glass beaker. Adjust pH to 7.8 with NaOH, and complete volume to 500 mL in a graduated flask. Store at 4 °C.
10. Reverse-phase equilibration buffer (500 mL): 0.065% trifluoroacetic acid (TFA) in water. Add 325 μL of TFA in 450 mL of ultrapure water in a 500 mL glass beaker. Complete volume to 500 mL in a graduated flask. Store at 4 °C.
11. Reverse-phase elution buffer (500 mL): 0.05% trifluoroacetic acid in acetonitrile. Add 250 μL of TFA in 450 mL of acetonitrile in a 500 mL glass beaker. Complete volume to 500 mL in a graduated flask. Store at 4 °C.

2.3.2 Special Equipment

1. WiseCube incubator.
2. Seretech Gensablen French press.
3. Beckman Coulter Avanti J-26S XP centrifuge, with JA 16.250 and JLA 25.50 rotors.
4. AKTÄ purifier FPLC purification system.
5. Savant SpeedVac SVC 100H.
6. HiTrap DEAE FF 5 mL column (GE Healthcare), Source 15 RPC 7.5 × 150 mm column (Pharmacia Biotech).
7. Dialysis tubing: cellulose molecular weight cutoff 3500 Spectra/Por® molecular porous n°3 18 mm × 50 ft.

2.4 Sample Preparation

2.4.1 Reagents and Buffers

1. 0.1 M guanosine triphosphate sodium salt hydrate (GTP): weigh 0.0567 g GTP in a microtube, and add 970 μL of ultrapure water. Check the concentration by measuring the absorbance at 256 nm with a molecular extinction coefficient $\varepsilon_{256\ nm} = 13{,}700\ M^{-1}\ cm^{-1}$, and store at −20 °C (*see* **Note 1**).
2. 1 M TCEP stock: weigh 1.4 g of tris(2-carboxyethyl) phosphine in a 5 mL plastic tube, and add 4.5 mL ultrapure water. Use a vortex to strongly mix the solution for few minutes (*see* **Note 2**). Make 500 μL aliquots and store them at −20 °C.
3. 400 mM sodium phosphate buffer stock (1 L): dissolve 27.60 g NaH_2PO_4 and 28.39 g Na_2HPO_4 in ultrapure water in a 1 L glass beaker. Complete volume to 1 L in a graduated flask. Store at 4 °C.

4. P buffer: 20 mM phosphate buffer, pH 6.5. In a 100 mL glass beaker, add 5 mL of 400 mM phosphate buffer to 94 mL of ultrapure water. Verify that pH is 6.5, and transfer the buffer in a 100 mL graduated flask, and complete volume to 100 mL (*see* **Note 3**).
5. PG buffer is P buffer +0.1 mM GTP added extemporaneously.
6. PT buffer: 20 mM phosphate buffer, 1 mM TCEP, pH 6.5. In a 100 mL glass beaker, add 5 mL of 400 mM phosphate buffer and 100 µL of TCEP stock to 94 mL of ultrapure water. Adjust pH to 6.5 with 10 M NaOH. Transfer the buffer in a 100 mL graduated flask, and complete volume to 100 mL (*see* **Note 4**).
7. PGT buffer is PT buffer +0.1 mM GTP added extemporaneously.
8. Reagent A (DCTM protein assay): alkaline tartrate copper solution.
9. Reagent B (DCTM protein assay): Folin reagent.
10. Reagent S (DCTM protein assay): surfactant solution.
11. Reagent A′: add 1 mL of Reagent A to 20 µL of Reagent S′ (*see* **Note 5**).

2.4.2 Special Equipment

1. LLG uniCFUGE 3 mini centrifuge.
2. AKTÄ Purifier FPLC purification system (GE Healthcare).
3. LabSystems Multiskan RC microplate reader (Artisan TG).
4. Lambda 800 spectrophotometer (Perkin Elmer).
5. HiTrap Desalting 5 mL column (GE Healthcare).

2.5 MAP-Tubulin Interaction by ITC

All equipment and materials that we used for ITC experiment have been provided by MicroCal (Malvern Panalytical). It includes:

1. Isothermal titration calorimeter (VP-ITC, iTC_{200} or PEAQ-ITC).
2. Origin 7.0 data analysis software (OriginLab Northampton, Massachusetts, USA).
3. Hamilton filling syringe with a long needle with silicon tube on the tip in order to fill and empty the calorimetric cell (*see* **Note 6**).
4. Plastic 200 µL thin wall PCR tubes for filling titration syringe.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Tubulin Purification

The detailed protocol of tubulin purification from bovine brain was described earlier [21].

3.2 Tau Purification

The detailed protocol of recombinant human tau purification was described earlier [21].

3.3 Stathmin Purification

3.3.1 Day 1: Small-Scale Bacterial Culture

In a 500 mL Erlenmeyer flask, mix 100 mL sterilized LB Miller medium with 100 μL ampicillin 100 mg mL^{-1} and 100 μL glycerol stock of stathmin-BL21DE3. Incubate at 37 °C overnight in the WiseCube incubator under a 150 rpm agitation (*see* **Note 7**).

3.3.2 Day 2: Bacterial Growth

1. Start 1 L of BL21(DE3) bacterial culture in LB medium: in two 2 L Erlenmeyer flasks, mix 500 mL sterilized LB Miller medium with 500 μL ampicillin 100 mg mL^{-1} and 2 mL of overnight small-scale culture. Incubate at 37 °C under a 150 rpm agitation (*see* **Note 7**).
2. Check growth by measuring the absorbance at 600 nm of an aliquot of the culture every hour.
3. When bacterial culture A600 reaches 0.8–1.0 (usually about 3 h after), induce protein production by addition of 0.5 mM IPTG (500 μL of 1 M IPTG stock per liter of LB).
4. Continue the bacterial culture for 3 h.
5. Collect bacterial pellet by centrifugation at 5000 × *g* for 10 min at 4 °C using JA 16.250 rotor. Pellets are recovered and resuspended in 10 mL of lysis buffer (*see* **Note 8**).

3.3.3 Protein Extraction

1. Apply on the resuspended pellet a four tons pressure in French press, and slowly open the French press fosset to gradually release the samples while maintaining a four tons pressure on the left over.
2. Repeat this extraction step by loading the French press with the same sample again.
3. Boil the bacterial lysate at 90 °C for 10–15 min, to select thermal-resistant proteins like stathmin.
4. Centrifuge for 30 min at 30,000 × *g* at 4 °C using a pre-thermostated JLA 25.50 rotor, and keep the supernatant.

3.3.4 Ion-Exchange Chromatography

1. A HiTrap DEAE FF 5 mL column (GE healthcare) is activated by running 15 mL of activation buffer at a flow rate of 1 mL min^{-1}. Before sample injection, column is washed with 20 mL of equilibration buffer.
2. Load the sample in a 10 mL FPLC loop and switch it to inject. Start to collect 5 mL fractions, and inject sample with equilibration buffer at a flow rate of 0.5 mL min^{-1}. Absorbance

increases during sample deposit. Rinse column with equilibration buffer until absorbance comes back to baseline level.

3. Apply a 50% NaCl gradient: increase the elution buffer part from 0 to 50% in 10 min with a flow rate of 1 mL min^{-1}. Stathmin is released at 48% of elution buffer. When stathmin protein has been released from the column, 100% of elution buffer are applied to wash the column for few minutes at a flow rate of 1 mL min^{-1}.
4. To check in which fractions stathmin is present, 20 μL of each fraction is mixed with 5 μL of 5× SDS loading gel buffer and then loaded on a SDS-PAGE 12% acrylamide.
5. Fractions containing stathmin are pooled and loaded on the reverse-phase column.

3.3.5 Reverse-Phase Chromatography

1. A Source 15 RPC 7.5 × 150 mm is equilibrated by running 30 mL of activation buffer at a flow rate of 1.5 mL min^{-1}.
2. 1 mL of sample from ion-exchange chromatography is loaded in a 1 mL sample loop. Start to collect 5 mL fractions, and inject sample with equilibration buffer at a flow rate of 1 mL min^{-1}. Absorbance increases during sample deposit. Rinse column with equilibration buffer until absorbance comes back to baseline level.
3. Apply a 5% acetonitrile step: increase the elution buffer part to 5% at a flow rate of 1.5 mL min^{-1} for 10 mL.
4. Apply a 40% acetonitrile gradient: increase the elution buffer part from 5 to 40% in 27 min with a flow rate of 1.5 mL min^{-1}.
5. Stathmin is the second pic released. When stathmin protein has been released from the column, 100% of elution buffer are applied to wash the column for few minutes at a flow rate of 1.5 mL min^{-1}.
6. To check in which fractions stathmin is present, 20 μL of each fraction is mixed with 5 μL of 5× SDS loading gel buffer and then loaded on a SDS-PAGE 12% acrylamide.
7. Fractions containing stathmin are collected and frozen at −80 °C to be freeze dried.
8. Once freeze dried, stathmin can be stored at −20 °C for several months.

3.4 Sample Preparation

Ideally tubulin concentration should be around 10–20 μM so that there is enough signal yet keeping the concentration of the ligand reasonable. Indeed, the ligand (here tau or stathmin) is usually tenfold more concentrated, and above 200 μM, there is a risk of oligomerization or precipitation.

3.4.1 Protein Resuspension

1. Resuspend the protein in its buffer (stathmin in P buffer and tau in PT buffer) until complete dissolution of protein (*see* **Note 9**).
2. Centrifuge at 1000 × *g* for a few seconds to collect all drops on the walls of the tube.
3. Centrifuge at 2000 × *g* for 15 min to eliminate potential aggregates.
4. Throw away the pellet and keep supernatants in the ice.

3.4.2 Protein Equilibration

The same procedure is used for the three proteins, but with different PX buffers: P buffer is used for stathmin, PT buffer is used for tau, and PG buffer is used for tubulin before a tubulin-stathmin interaction of PGT buffer in case of a tubulin-tau interaction.

1. Equilibrate the 5 mL HiTrap Desalting column by running 30 mL of PX buffer at a flow rate of 1 mL min^{-1} on the FPLC.
2. Load previous supernatants manually with a syringe in a 1 mL FPLC loop.
3. Start collecting fractions immediately as PX buffer continues to run through the column at a flow rate of 1 mL min^{-1}, with the UV detector set to follow absorbance at 215 nm, 260 nm, and 280 nm (*see* **Note 10**).
4. Collect the peak as precisely as possible (*see* **Note 11**).
5. Pool the fraction(s) containing the protein, and store them on ice before concentration determination.

3.4.3 Determination of Tau and Tubulin Concentration

As described in the previous detailed protocol [21]:

1. Tau concentration is determined in a P buffer using the extinction coefficient of 7700 M^{-1} cm^{-1} at 280 nm (calculated from protein sequence using Protein Sequence Analysis Tool [22]).
2. Tubulin concentration is determined in 6 M guanidine hydrochloride using the extinction coefficient of 109,000 M^{-1} cm^{-1} at 275 nm.

3.4.4 Determination of Stathmin Concentration

Determination of stathmin concentration is more challenging, as stathmin bears no tyrosine or tryptophan residues. We use colorimetric methods (DC Protein Assay, Bio-Rad) with BSA as standard as a first estimation. Since BSA does not behave the same way as stathmin, we usually have to adjust stathmin concentration after ITC experiments in order to reach the expected stathmin/tubulin stoichiometry of 0.5 (*see* **Note 12**).

1. The entire colorimetric test should be done at room temperature.
2. Add 5 µL of standard protein at different known concentrations in each well and 5 µL of stathmin sample in another well.

3. Repeat twice in order to have triplicates.
4. Add 25 μL of Reagent A′ in each well; never put B before A′ (*see* **Note 13**).
5. Finally, add 200 μL of Reagent B in the same wells.
6. Incubate sample for 15 min in the dark.
7. Read the plate with the microplate reader at 600 nm.
8. Trace standard curve with each known concentration point, and mark the stathmin value on it (*see* **Note 14**).
9. You can now estimate stathmin concentration using the X-axis.

3.5 MAP-Tubulin Interaction by ITC

Configure iTC200 control software using the following settings:

3.5.1 Instrument Settings

1. 20 injections
2. Initial delay 60 s.
3. Spacing 180 s.
4. Filter period 5 s.
5. Injection volume 2 μL.
6. Measurement temperature of 37 °C (*see* **Note 15**).
7. Reference power of 10 μcal s^{-1}.
8. Stirring speed of 200 rpm.
9. Feedback mode—high.

3.5.2 Cell Filling

All manipulations with calorimetric cell should be performed with extra care to avoid damaging cell with filling syringe.

1. Fill the Hamilton syringe ("filling syringe") with at least 300 μL of 37 °C preheated buffer (*see* **Note 16**).
2. Introduce the filling syringe all the way to the bottom of the calorimetric cell without injecting, then lift silicon tip of the syringe needle up about 1 mm above the bottom of the cell (*see* **Note 17**).
3. To inject in the calorimetric cell, push the syringe piston slowly until half of solution volume is injected, then inject the rest by doing several small but rapid injections (*see* **Note 18**).
4. Proceed with the injection up to the point when the sample overflows the calorimetric cell (*see* **Note 19**).
5. Remove this overflowing liquid.

3.5.3 Titration Syringe Filling

1. Put titration syringe piston in position "Open fill port" in control software (tab "Instrument control") when syringe is on the rest position.
2. Fill PCR plastic tube with 200 μL of buffer, and place it in the instrument holder.

3. Put syringe of "filling" position and submerge syringe needle into buffer.
4. Connect 1–5 mL plastic syringe to the titration syringe using dedicated adapter (*see* **Note 20**).
5. Then pull up plastic syringe piston to pump all the buffer from PCR tube through titration syringe, and empty it.
6. Refill PCR tube with buffer, and carefully draw buffer into titration syringe until it fills completely (*see* **Note 21**).
7. Close titration syringe choosing "Close fill port" in tab "Instrument control" of control software, disconnect filling syringe and put titration syringe in the rest position.
8. Carefully remove the drops of the liquid from titration syringe needle using Kimberly-Clark precision Wipes without bending the needle.
9. Place titration syringe into calorimetric cell and push it down to be sure that syringe properly gained the lower position.

3.5.4 Buffer-to-Buffer Titration

1. Start ITC titration with calorimetric cell and titration syringe filled with buffer solution.
2. Titration peaks should not exceed 0.1. Otherwise, calorimetric cell and titration syringe should be cleaned using detergent, and buffer-to-buffer titration should be repeated.

3.5.5 MAP-Tubulin Interaction Measurement

When performing MAP-tubulin interaction, it is better to put the tubulin in the calorimetric cell and the MAP in the titration syringe (*see* **Note 22**).

1. Empty calorimetric cell and titration syringe, and fill them with tubulin and MAP solution, respectively, as described in Subheadings 3.5.2 and 3.5.3.
2. If saturation is not reached after first titration is finished (*see* **Note 23**), refill the titration syringe by placing the needle into MAP solution and pressing button "close filing port" in control software. Otherwise go directly to Subheading 3.5.6.
3. Remove excess of the solution from the borders of calorimetric cell.
4. Place the syringe in titration position and run the experiment.
5. Repeat **steps 2–4** until titration peaks will stay the same in several consecutive injections.

3.5.6 Baseline Titration

1. Rinse the calorimetric cell with buffer.
2. If saturation was not reached at the end of the first titration, repeat the same number of consecutive titrations of MAP

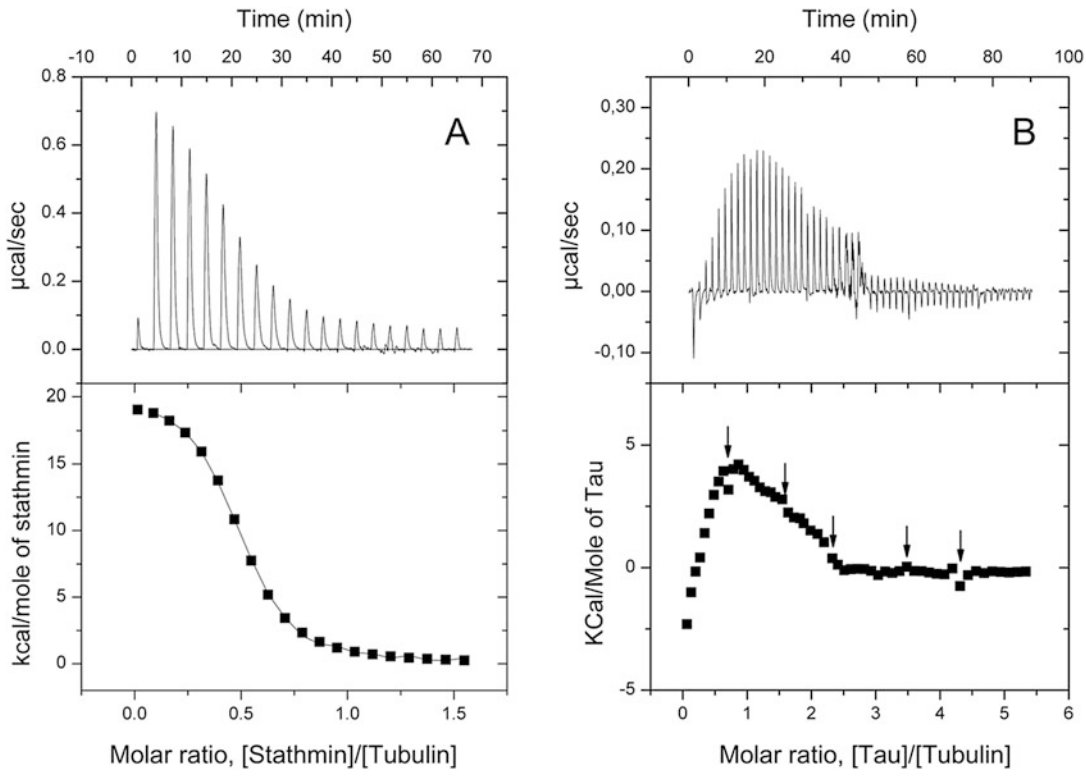

Fig. 2 Raw ITC curves (top panels) and binding isotherms with fitting curves (bottom panels) of tubulin titration by MAPs. Titration of tubulin by stathmin (**A**) and tau (**B**) proteins obtained at VP-ITC and iTC_{200}, respectively, in 20 mM NaPi, 0.1 mM GTP, and 1 mM TCEP, buffer at pH 6.5. (**A**) is an example in which saturation was reached at the end of the first titration, while (**B**) is an example in which saturation was only reached after the sixth consecutive titration. The arrows show syringe refilling with the same tau solution. Adapted from Tsvetkov et al. 2013 [20]

solution into buffer filled in the cell the same number of times as for tubulin titration by repeating **steps 15–18**.

3. Clean calorimetric cell and titration syringe using detergent as described in instrument manual.

3.5.7 Data Processing and Fitting

1. If saturation was reached at the end of the first titration (Fig. 2a), go directly to point 4.
2. If saturation was not reached at the end of the first titration (Fig. 2b), combine data from consecutive titrations of MAP in buffer solution into single titration file using ConCat32 tool provided by iTC200 manufacturer.
3. If saturation was not reached at the end of the first titration, combine data from consecutive titrations of MAP in tubulin solution into single titration file using ConCat32 tool provided by iTC200 manufacturer.

4. Subtract baseline titration from experiment titration. For that purpose:
   (a) In Origin software, open baseline titration file obtained above by choosing "Read data" from side menu "ITC Main Control."
   (b) Go back to raw data window choosing from top menu "Window/mRawITC."
   (c) Press again "Read data" from side menu "ITC Main Control," and choose the sample titration obtained above.
   (d) Repeat **step (b)**.
   (e) From top menu, choose "Math/Simple math. . ."
   (f) In pop-up window, choose "DATA2RAW_CP" in the field "Y1" and "DATA1RAW_CP" in the field "Y2" by scrolling "Available data" list and pressing corresponding button with arrow "=>." Then put "−" (minus) sign in "Operator" field and press "Ok."
5. Prepare data for fitting by integrating titration peaks, removing bad data points, and subtracting baseline. For that purpose:
   (a) In "ITC Main Control" on the left from plot, select "Auto baseline."
   (b) If necessary, press "Adjust integration," and move baseline points using mouse.
   (c) Select "Integrate All Peaks" from side menu "ITC Main Control."
   (d) Select "Remove bad data" from side menu "Data Control," and delete bad data point by clicking it (*see* **Note 24**).
   (e) Subtract baseline if the titration curve does not tend to zero at the end of the experiment by performing the following steps:
      - Press "Data selector" tool on side toolbar, and two selection markers will appear on the curve.
      - Select final linear region of the titration curve by dragging, and drop first selection marker and then press "Enter."
      - Select "Math/Linear regression" from top menu.
      - Press "Data selector" tool on side toolbar and select entire curve.
      - Select "Math/Simple math" and subtract linear regression (LinearFit1_Data2) from experimental data (DATA2_NDH).

6. Fit experimental data using "One Set of Sites" for tubulin/stathmin interactions and "Two set of Sites" for tubulin/tau interactions by choosing appropriate model in "Model Fitting" side menu. In fact the mode of tau binding to tubulin is still under debate; thus the appropriate fitting model is also still questionable (*see* **Note 25**).
7. Since the stoichiometry of tubulin/stathmin is well-known and concentration of stathmin could be determined with significant error, correct stathmin concentration multiplying it by factor corresponding to the found stoichiometry divided by 2 and then redo Subheading 3.5.7 starting from **step 4** (*see* **Note 26**).

4 Notes

1. GTP is dissolved in water. Make aliquots of 100 μL and store them at −20 °C. GTP should be added freshly to buffer to avoid hydrolysis of GTP into GDP, and make sure that tubulin is always with GTP.
2. TCEP, which is a little difficult to dissolve at this concentration, is preferred to DTT because it is more stable. It is used to reduce disulfide bonds and maintain tau monomeric.
3. This buffer will be used to resuspend and equilibrate stathmin before ITC experiment.
4. This buffer will be used to resuspend and equilibrate tau before ITC experiment. Even though TCEP is rather stable, PT buffer should be prepared extemporaneously. pH should always be adjusted after TCEP since TCEP usually changes the pH.
5. A′ should be prepared extemporaneously.
6. The silicone tubing is fitted on the needle tip in order to protect the bottom of the ITC calorimetric cell from repeated contact with the needle.
7. All bacterial culture must be prepared near a Hoffman Heater to prevent contamination.
8. Sample can be kept in lysis buffer for a few weeks at −20 °C. For prolonged storage (superior to 6 months), samples need to be stored at −80 °C.
9. Pipet up and down to make sure all the powder is dissolved.
10. The strongest signal will be observed at 215 nm. No major signal will be observed at 280 nm for stathmin since it has no aromatic residues.
11. Depending on how concentrated was your initial supernatant and how concentrated you want your final protein, you might want to collect only the central fraction of the peak.

12. Ideally, the most precise method is to constitute a stock of stathmin of known concentration (e.g., previously determined by amino acid composition) and aliquot it to use as standards instead of BSA.
13. If you put B before A′, the protein concentration of the sample cannot be measured.
14. If the absorbance of the stathmin samples is too high, restart the whole process (including standards) with a dilution of the sample.
15. If necessary, change to the temperature at which the interaction is measured.
16. Preheated buffer is needed to prevent the formation of bubbles on the wall of the cell upon heating. This can be done by holding tight the filling syringe with buffer solution in your hand.
17. If there is a tight contact between tip of the needle and the bottom of the cell during injection, it might generate solution jets that could result in bubble creation.
18. Short rapid injections will help remove bubbles attached to the cell walls if they exist.
19. This procedure also decreases the chances of having bubbles stuck in the cell.
20. Do not tighten the screw, since it can damage the titration syringe.
21. The small bubble on the top of the titration syringe close to piston is acceptable. Pay attention not to draw air in titration syringe, since even small bubble injected in the calorimetric cell can dramatically influence the experiment.
22. The opposite can be done, but since the concentration in the syringe is usually at least tenfold higher than in the cell, it is better to put the molecule that has the smallest tendency to self-assemble or aggregate in the syringe.
23. Saturation is reached when the signal reaches the baseline and is constant.
24. On one hand, usually the first point of each titration should be removed. On the other hand, be careful not remove too many points, especially on the slope of the titration curve, since this can lead to serious mistakes in data fitting.
25. For analyzing experimental data with more complex models, use dedicated software [23].
26. There are some ITC fitting software that allow fitting concentration in the cell or in the syringe by fixing stoichiometry.

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