Microtubule-Associated Proteins and Tubulin Interaction by Isothermal Titration Calorimetry

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CHAPTER

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CHAPTER OUTLINE

Abstract

Microtubules play an important role in a number of vital cell processes such as cell division, intracellular transport, and cell architecture. The highly dynamic structure of microtubules is tightly regulated by a number of stabilizing and destabilizing microtubule-associated proteins (MAPs), such as tau and stathmin. Because of their importance, tubulin–MAPs interactions have been extensively studied using various methods that provide researchers with complementary but sometimes contradictory thermodynamic data. Isothermal titration calorimetry (ITC) is the only direct thermodynamic method that enables a full thermodynamic characterization (stoichiometry, enthalpy, entropy of binding, and association constant) of the interaction after a single titration experiment. This method has been recently applied to study tubulin–MAPs interactions in order to bring new insights into molecular mechanisms of tubulin regulation. In this chapter, we review the technical specificity of this method and then focus on the use of ITC in the investigation of tubulin–MAPs binding. We describe technical issues which could arise during planning and carrying out the ITC experiments, in particular with fragile proteins such as tubulin. Using examples of stathmin and tau, we demonstrate how ITC can be used to gain major insights into tubulin–MAP interaction.

INTRODUCTION

Microtubules, which consist of polymerized tubulin heterodimers, play key roles in numerous cell processes, including mitosis, active intracellular transport, and neuronal plasticity. Microtubules are highly dynamic structures that switch from elongation to shrinking phase and *vice versa,* depending on different regulatory factors (Mitchison & Kirschner, 1984). Under physiological conditions, microtubule dynamics is tightly controlled by stabilizing microtubule-associated proteins (MAPs), such as tau, and destabilizing factors or proteins, such as stathmin (Fig. 18.1). These proteins are in turn regulated by posttransitional modifications, the most studied one being phosphorylation. Targeting microtubules and their dynamics is also a well-established area of anticancer research. Indeed, altering microtubule dynamics by drugs named microtubule targeting agents (MTAs) leads to mitotic block and cell death. MTAs that can also be divided into two classes, molecules that stabilize microtubules, like taxanes, and molecules that induce microtubule depolymerization, like vinca-alkaloids, have been studied using a broad spectrum of techniques (for review, see Calligaris et al., 2010). Deciphering the molecular mechanisms of microtubule regulation by MAPs is fundamental to understanding the biology of the cell. This is of particular interest in oncology as it was proposed that resistance to anticancer drugs could be linked to the level of expression of MAPs (Alli, Yang, Ford, & Hait, 2007; Cucchiarelli et al., 2008; see Chapter 5). A better knowledge of MAP–MTA interplay would help orient treatments and possibly lead to the discovery of new therapeutic strategies. Since the discovery of the tau family
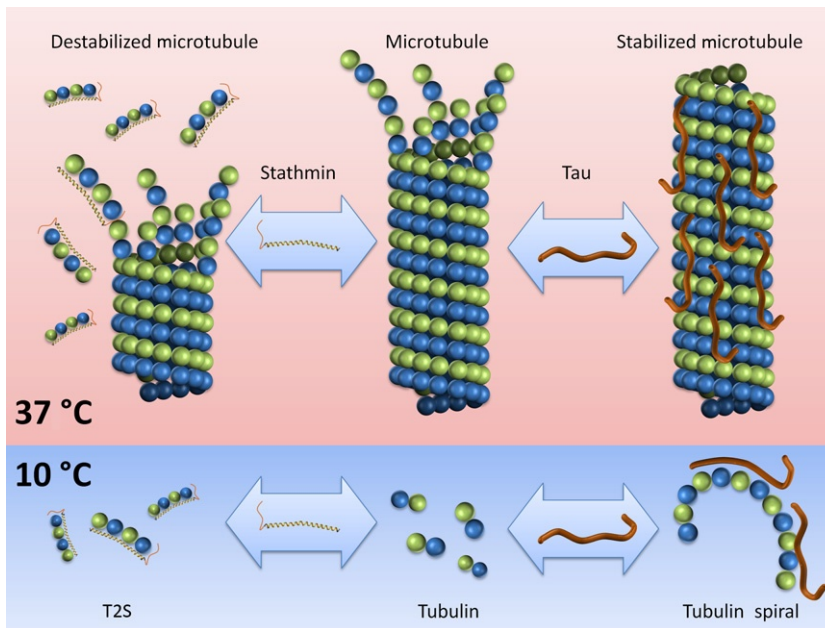
FIGURE 18.1

MAPs–tubulin interactions and their consequences on tubulin self-assembly at 10 and 37 °C. This scheme represents tubulin self-assembly in a classical polymerization buffer (in which tubulin spontaneously polymerizes into MT at 37 °C). At 10 °C, free tubulin forms T2S complex in the presence of stathmin and circular protofilaments in the presence of tau. 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.

(Weingarten, Lockwood, Hwo, & Kirschner, 1975) and until the identification of stathmin (Belmont & Mitchison, 1996), many MAPs had been discovered and investigated. Their interaction with tubulin has been and is still being studied, using numerous biochemical and biophysical methods both *in vitro* (Devred et al., 2010; Kiris, Ventimiglia, & Feinstein, 2010; Ross & Dixit, 2010; Wilson & Correia, 2010) and *in vivo* (Drubin & Kirschner, 1986; Konzack, Thies, Marx, Mandelkow, & Mandelkow, 2007; Samsonov, Yu, Rasenick, & Popov, 2004; Weissmann et al., 2009). In this chapter, we focus on the use of isothermal titration calorimetry (ITC). ITC is a very useful technique that has been extensively used in other fields, but that is still rarely utilized to study the tubulin cytoskeleton. After describing briefly the principle of ITC, we focus on the important steps (from sample preparation to data analysis) when applying ITC to the tubulin cytoskeleton and MAPs. Taking as examples the case of a destabilizing MAP (stathmin) and stabilizing MAP (tau), we illustrate the pitfalls that must be avoided and the precautions that must be taken in order to use this technique efficiently to gain new insights into the molecular mechanism of action of MAPs on MTs.

18.1 ISOTHERMAL TITRATION CALORIMETRY

18.1.1 Principles

Interaction of molecules, including protein–protein binding, is usually accompanied by heat exchange. ITC is based on the measuring of this heat as a function of molar ratio of interacting molecules. Subsequent fitting of raw data allows one to get most thermodynamical parameters of interaction such as stoichiometry (N), enthalpy (ΔH), entropy (ΔS) of binding, and association equilibrium constant (K_a) from a single 1-h-experiment (Ladbury & Doyle, 2004). As heat exchange upon binding occurs naturally, ITC does not require immobilization, as surface plasmon resonance does, and/or modification of the reactants by addition of a fluorophore, for example. In addition, ITC does not depend on the size or mass difference between the studied interacting molecules, which enables ITC to be also used to study binding of low molecular weight drugs and metal ions to proteins (Tsvetkov et al., 2010). At last, contrary to spectroscopic methods, ITC can be used with colored, turbid or even not transparent solutions and suspensions. Continuous progress and improvements in ITC instrumentation now allow using ITC routinely to characterize thermodynamics of binding with association constants ranging from 10^3 to $10^8 \, \mathrm{M}^{-1}$. For all these reasons, ITC is a very powerful method for the study of a wide range of biological systems under near physiological conditions (Ladbury & Doyle, 2004).

18.1.2 Experimental procedure

An ITC apparatus consists of a calorimetric cell in an isothermal jacket and a syringe that is inserted in the cell (Ladbury & Doyle, 2004; Pierce, Raman, & Nall, 1999). To perform ITC experiments, the calorimetric cell is filled with protein solution at a concentration close to the expected dissociation constant, and the titration syringe is filled with about a 10-fold more concentrated solution of the second interactant (usually called ligand) in an identical buffer. During each successive injection of small aliquots of ligand into the cell, the microcalorimeter registers heat exchange. The released heat is proportional to the amount of complex formed after each injection and decreases as the protein gets saturated by the ligand (Freyer & Lewis, 2008). During final injections, as there is no more binding of the ligand to the protein, the measured heat corresponds to the heat of mixing of the two solutions, which is often referred to as heat of dilution. The signal due to dilution can be significant in the case of imbalance in compositions of buffers between the calorimetric cell and the syringe, and/or a high intrinsic dilution heat of the ligand. This is why the sample preparation is a crucial step in the experimental procedure (see Section 18.2.1). Areas under each peak corresponding to heat exchanges are then plotted against molar ratio of ligand over protein in order to obtain a thermogram or binding isotherm. To determine the thermodynamic parameters, this binding isotherm should be fitted with a theoretical binding isotherm curve. All ITC instruments are supplied with the software that offers several standard models of interaction.

18.1.3 Binding models

Generally, the list of models includes one-set-of-sites, two-sets-of-sites, and sequential-binding-sites models. To choose the appropriate binding model, reasonable assumptions should be made, based on preliminary knowledge about the investigated system and/or the shape of the binding isotherm. The first model, one-set-of-sites, is the most simple one and can be used for the systems where the ligand has one or more equal independent (noninteracting) sites on the target molecule. The binding isotherm for such a system represents a monotonic curve which has no or one inflection point (Fig. 18.2). When concentration of investigated molecule $[M]$ in the calorimetric cell is close to optimal value ($10/NK_a < [M] < 100/NK_a$), the binding isotherm has a sigmoid shape with an inflection point close to the molar ratio corresponding to the stoichiometry of the interaction (N) (Ladbury & Doyle, 2004). This value should be
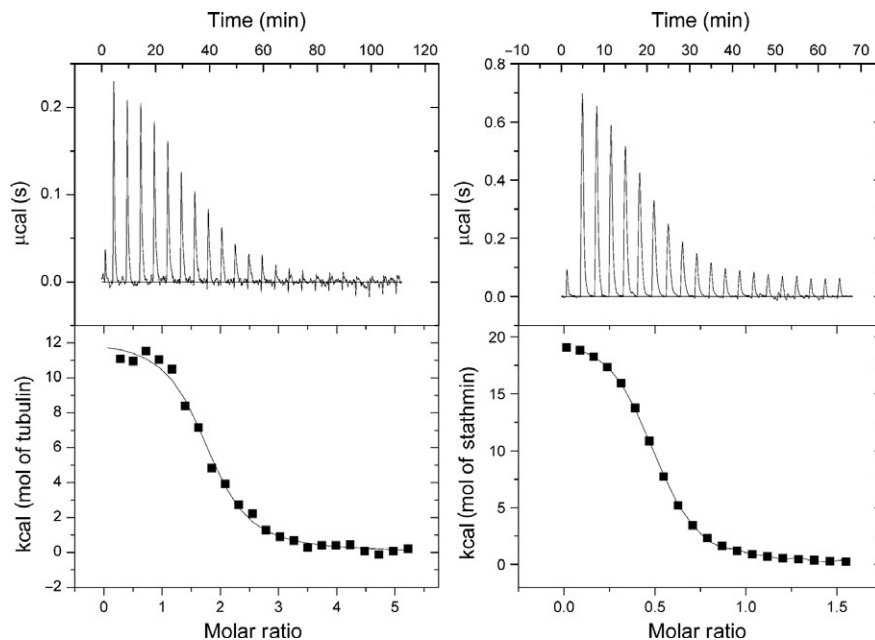
FIGURE 18.2

Direct and reverse titration by ITC. Raw ITC curves (top panels) and binding isotherms with fitting curves (bottom panels) of stathmin titration by tubulin (left panels) and tubulin titration by stathmin (right panels). Both thermograms are monophasic curves with an inflection point at 2 for direct titration and 0.5 for reverse titration. Thermodynamic parameters of both titrations are in good agreement. Fitting binding isotherms with model of one-set-of-sites yielded the same constants for both titrations and the enthalpy of binding around 14 kcal/(mole of tubulin) for direct binding and 27 kcal/(mole of stathmin) for reverse titration. The last value corresponds to overall enthalpy of formation of the T2S complex (ΔH_{T2S}).

equal to n or $1/n$, where n is an integer (Fig. 18.2). When stoichiometry of the interaction is slightly different from these "allowed" values, it indicates that there is an error in concentration of either interacting molecules or that a portion of one of the interactants is not active (mis-folding, aggregation, etc.). In both cases, concentrations should be corrected to the closest allowed value. Substoichiometry ($1/n$) can be observed in two cases: when the titration syringe is filled with the solution of a studied molecule that has several binding sites and the ligand is placed in the calorimetric cell (usually called "reverse titration"), and/or when titrated molecules are oligomers that bind only one molecule of ligand. For stathmin–tubulin binding, direct titration leads to a stoichiometry of 2, whereas reverse titration leads to a stoichiometry of 0.5 (Fig. 18.2). It should always be kept in mind that ΔH result is given per mole of molecule in the syringe. For example, in the case of the formation of T2S complex between stathmin (S) and tubulin (T), direct titration is stathmin titrated by tubulin, and reverse titration is tubulin titrated by stathmin and $\Delta H_{T2S} = \Delta H_{revers} = 2\Delta H_{direct}$. In case of substoichiometry, it is strongly recommended to also perform the opposite titration to obtain stoichiometry more precisely. For example, the difference between a stoichiometry of three and a stoichiometry of four would correspond to a $4-3=1$ shift in direct titration, whereas it would correspond only to a $\frac{1}{4} - \frac{1}{3} = 0.08$ shift of inflection points in reverse titration. It should be noted that even if experimental data can be fitted with the model of one-set-of-sites, it does not necessarily mean that the interaction occurs through identical or noninteracting sites. For example, if there are two identical (with the same ΔH and K_a) strongly interacting sites and if the binding with the first site modifies only the constant of binding to the second site (but not the enthalpy), then the resulting binding isotherm obtained by ITC will be indistinguishable from that for "equal noninteracting sites" model with higher constants of binding. If a binding isotherm has several inflection points or extremes, it indicates that there is more than one nonequal or interacting binding site. In order to fit such binding isotherms, more complex models should be used. The standard software gives a choice between two-sets-of-sites and sequential-binding-sites models. The first one implies the existence of two sets of noninteracting sites and allows the determination of stoichiometry, binding constant, enthalpy, and entropy of binding for each set of sites ([n_1, K_{a1}, ΔH_1, ΔS_1], [n_2, K_{a2}, ΔH_2, ΔS_2]). The second one assumes the preliminary knowledge of the stoichiometry of binding, which should be set before fitting. If any reasonable assumption about stoichiometry cannot be made, then fitting with a model of interacting binding sites should be started from two sites. In the case of an unsatisfactory fitting result, the number of binding sites used in the model should be sequentially increased until a further rise in the number of sites will not give a significant drop in fitting error. Unfortunately, in some cases, the experimental binding isotherm could be well fitted using both models and additional experiments are necessary to determine the precise model of binding. If none of the models described above can be used to fit the experimental data, researchers should implement their own model of binding. The choice between models of binding could be a rather tricky problem that goes beyond the scope of this chapter. Nevertheless, authors should always take into

account that in biology, as in any other field, "all models are wrong, but some are useful" (Box & Draper, 1987).

18.1.4 Thermodynamic profile of binding (meaning of enthalpy and entropy signs)

Interactions can occur only if the variation of free Gibbs energy of the process is negative ($\Delta G < 0$). This is a fundamental thermodynamic law, which is valid for all interacting systems. Gibbs energy has two components, enthalpic and entropic: $\Delta G = \Delta H - T\Delta S$. Fitting a binding isotherm allows us to determine ΔH of binding and association constant K_a. Using the above equation and standard thermodynamic relationship ($\Delta G = -RT \ln K_a$), entropy of binding could be easily calculated (Ladbury & Doyle, 2004). Thus, contrary to other methods, ITC allows one to determine both components of Gibbs energy after one single experiment, providing us with information about the nature of the interaction. The values of enthalpy and entropy can be either positive or negative. They constitute the energetic signature of the interaction, also referred to as the thermodynamic profile of binding. Interactions are going to be favored by negative ΔH and/or positive ΔS. If ΔH is negative (exothermic reaction), the entropic component of free Gibbs energy could be either favorable ($\Delta S > 0$) or unfavorable ($\Delta S < 0$), as long as ΔG stays negative. Otherwise, if ΔH is positive (endothermic reaction), then entropy of binding should be favorable ($\Delta S > 0$). In the last case, it can be typically concluded that binding is driven by hydrophobic interactions. For example, during binding, there is a burying of hydrophobic areas in the interface of interaction or conformational changes in one interacting molecule that lead to hiding of hydrophobic surfaces. Otherwise, a highly favorable enthalpy and an unfavorable entropy of binding are usually associated with a high degree of hydrogen bonding formed upon interaction, in addition to conformational changes (Ladbury & Doyle, 2004; Ross & Subramanian, 1981). In addition to providing information about driving forces, the thermodynamic parameters of interaction of tubulin with different ligands can sometimes be correlated with the differences in biological activity between these ligands (Buey et al., 2004, 2005).

18.1.5 Temperature dependence of ΔH

It should be noted that ΔH of binding depends on temperature. In the temperature range where interacting molecules are not denatured, this dependence is linear and usually has a negative slope. This slope corresponds to heat capacity change of binding (ΔC_p) which is generally correlated with the surface of the area buried upon complex formation (Ladbury & Doyle, 2004). A consequence of this temperature dependence is that at certain temperatures, the enthalpy of binding could be equal to zero, making such binding undetectable by ITC. In other words, the absence of signal during an ITC experiment does not necessarily mean that there is no interaction between molecules, but could signify that ΔH of binding is equal to zero at the chosen experimental temperature. In this case, entropy is the driving force of the

interaction. Fortunately, modern microcalorimeters allow carrying out titration experiments at a wide range of temperatures.

18.2 TUBULIN AND MAPs SAMPLE PREPARATION

Because of certain peculiarities of tubulin, its interaction with regulatory proteins has been studied by ITC only occasionally, despite the growing popularity of this method and its obvious advantages. The difficulties that could arise during such a study necessitate a deep knowledge of both method details and tubulin cytoskeleton regulatory mechanisms. In this section, we want to draw attention to some important points about tubulin and certain MAPs sample preparations for ITC experiments.

18.2.1 Equilibration

To minimize the heat signal due to the dilution of the samples during injections, a balance between the composition of buffers in the calorimetric cell and the syringe needs to be established. Due to the high sensitivity of microcalorimeters, the two solutions must be matched with regard to composition, pH, buffer, and salt concentrations. A slight mismatch between the two solutions may lead to heat of dilution that could overwhelm the heat of the binding reaction. Usually, to achieve the perfect match between buffers in the cell and syringe, dialysis of both interactant solutions against the same buffer is used. Unfortunately, due to tubulin instability, buffer specificity, and the necessity of keeping a high concentration of ligand, this option is not appropriate. After purification, when tubulin is stored, 1 M sucrose buffer to stabilize its conformation upon freezing (Frigon & Lee, 1972), the sucrose should be completely removed from buffer before ITC experiment, since it significantly contributes to the dilution effect. However, extensive dialysis cannot be used because of low tubulin stability over an extended period of time in the absence of a stabilizer. Previously, we described a tubulin equilibration procedure using two custom-made columns filled with Sephadex G25 (Andreu & Timasheff, 1982; Barbier, Peyrot, Leynadier, & Andreu, 1998; Devred et al., 2010; Na & Timasheff, 1982; Peyrot et al., 1992). Later, we optimized the protocol by replacing these two columns by a single desalting Hitrap column (GE Healthcare) on an AKta Purifier FPLC system. This allowed us to reduce the time and to increase the yield of tubulin preparation. Tubulin can also be commercially bought as a powder, which contains stabilizers of tubulin that should be removed by running the tubulin preparation on a desalting column. MAPs, such as stathmin and tau, can be dry-lyophilized and then stored as powders (Devred et al., 2004, 2008). Direct dilution of lyophilized proteins in experimental buffer often results in an increase in the dilution signal, even if MAPs were dialyzed against water to eliminate salt before dry-lyophilization. Thus, when used for ITC experiments, dry-lyophylized MAPs should be resuspended in the buffer of interest, centrifuged to remove aggregated proteins, and ran on the desalting column identical to the one used for tubulin.

18.2.2 Determination of protein concentrations

ITC is based on measuring the heat exchange during the interaction as a function of the ratio of interacting molecules. This is why, just like any other quantitative analysis of interaction, knowing the concentration of tubulin and tau or stathmin at the beginning of the experiment is also a critical point that should not be overlooked. Tubulin concentration is usually determined spectrophotometrically at 275 nm using an extinction coefficient of $109,000 \text{ M}^{-1} \text{ cm}^{-1}$ in 6 M guanidine hydrochloride (Andreu & Timasheff, 1982; Na & Timasheff, 1982). This determination should be done after tubulin full equilibration and as late as possible (just before the ITC experiment). Indeed, in a buffer without glycerol or other tubulin stabilizer, tubulin rapidly degrades, leading to significant errors in determination of thermodynamic parameters of interaction (Fig. 18.3). Thus, prior to each subsequent ITC titration, aggregated tubulin should be eliminated by centrifugation and concentration should be measured again. As tau protein is unstructured and elongated, it induces some scattering of light. Thus, to measure tau concentration, it is necessary to do a full UV-spectrum of the sample and then correct it for light scattering to avoid overestimation of tau concentration (Winder & Gent, 1971). It should also be noted that GTP, which has to be present in the final buffer in all binding experiments with tubulin, strongly absorbs in the range used for measurement of tau concentration. Thus, it is recommended to equilibrate tau in the absence of GTP, which should be added just prior to the ITC titration.
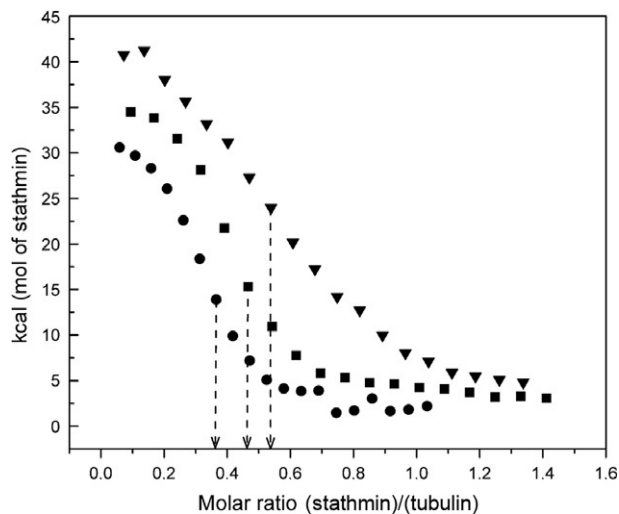

FIGURE 18.3

Effect of tubulin degradation with time on binding isotherms. Three sequential ITC experiments made with the same protein samples with a period of 1 h. The arrows indicate the stoichiometry which decreases as time goes by, which indicates that less active tubulin is available due to degradation over time.

Determination of stathmin concentration is even more challenging, as stathmin bears no tyrosine or tryptophane residues. We tested several approaches, including colorimetric methods (DC Protein Assay, Biorad) with BSA as standards. As none of these techniques proved to be satisfactory enough for ITC, we often had to adjust stathmin concentration after ITC experiments in order to reach the expected stathmin:tubulin stoichiometry of 0.5. Ideally, the most precise method would be to constitute a stock of stathmin of known concentration (e.g., previously determined by amino acid composition) and aliquot them to use as standards for colorimetric methods (instead of BSA) every time stathmin concentration is measured.

18.2.3 Temperature

As described above, the enthalpy of binding (ΔH) depends on experimental temperature. At a certain temperature, when $\Delta H = 0$, it is impossible to carry out ITC experiments (Fig. 18.4). This is why it is necessary to collect ITC titration at least at two different temperatures before considering that an interaction cannot be measured using ITC. In case of stathmin–tubulin interaction, the absolute value of ΔH is minimal at a temperature close to 25 °C, which is traditionally used as a standard temperature for ITC experiments. This means that for temperatures lower than 25 °C, the stathmin–tubulin interaction can be monitored by the endothermic signal ($\Delta H > 0$), whereas above 25 °C the interaction will be monitored by the exothermic signal ($\Delta H < 0$).
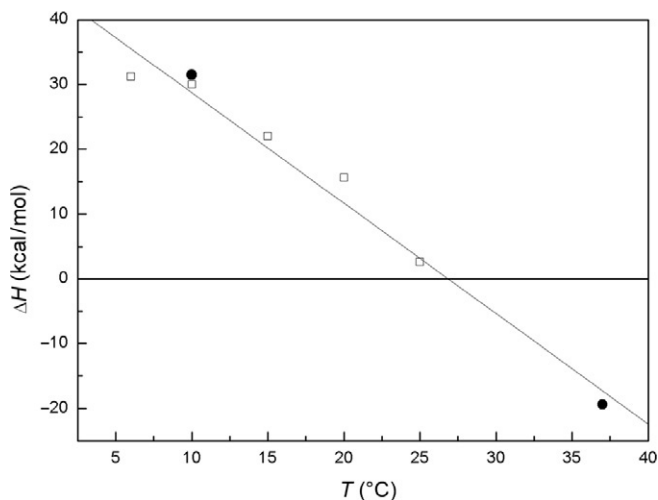

FIGURE 18.4

Temperature dependence of enthalpy of stathmin binding to tubulin. Plot is based on data obtained by Honnappa with coauthors (Honnappa, Cutting, Jahnke, Seelig, & Steinmetz, 2003) (open squares) and our data (circles). The slope of temperature dependence of enthalpy corresponds to molar heat capacity change of interaction (ΔC_p).

For the study of the interaction of tubulin with MAPs, the choice of temperature is also critical, due to temperature dependence of the self-assembly properties of tubulin (see Fig. 18.1). Indeed, in a minimum buffer (Devred et al., 2004) in the presence of tau, tubulin forms rings at 10 °C and microtubules at 37 °C. Stability of the proteins should also be taken into account when one chooses the experimental temperature. For tubulin, whenever it is possible, lower temperatures should be chosen in order to guarantee as little degradation as possible. In summary, to study the binding of depolymerizing MAPs, such as stathmin, or the binding of ligands to free tubulin, a temperature of 10 °C presents the advantage of guarantying better/longer stability for tubulin. When studying a stabilizing MAP, such as tau, or the binding of ligands to tubulin in microtubules, the temperature should be as close as possible to 37 °C.

18.2.4 Buffer conditions

As equilibrium between free tubulin dimers and microtubules can be easily perturbed by buffer components, the investigation of tubulin binding with proteins that regulate its assembly/disassembly should be performed in the minimum buffer to avoid contribution of cofactors to the thermodynamics of interaction. In our studies of MAPs binding to tubulin, we use a minimum buffer containing only phosphate (NaPi), which does not favor tubulin self-assembly, and GTP, which is necessary for tubulin structural integrity and stability, especially in the absence of any stabilizer. To study the binding of monomeric tau with tubulin, reducing agents such as DTT or TCEP should be present in the buffer to prevent formation of tau intra- or inter-molecular S–S bridges. Using buffers that stabilize microtubules, such as PIPES or MES, or molecules known to favor tubulin self-assembly, such as Mg^{2+} or glycerol, will increase the stability of tubulin compared to the so-called nonpolymerizing buffer (Devred et al., 2004). However, these molecules might also completely change the mode of binding.

18.2.5 Reaction volume and duration

One of the downsides of this technique has always been the amount of protein necessary for the measure of the interaction. For the longest time (for older generation MCS ITC and VP ITC machines), the volume of reaction chamber had to be 1.4 mL with tubulin in the 10 μM scale and syringe volume of 500 μL in the 100 μM range for ligand. Therefore, experiments could not be performed for a number of proteins only available in small quantities. For our studies, we used tubulin concentration in the 5–20 μM range in the cell, whereas stathmin or tau was in the 15–100 μM range (Devred et al., 2008; Tsvetkov, Makarov, Malesinski, Peyrot, & Devred, 2012). Practically, 2.5 mL of tubulin sample needed to be prepared to fill the 1.4 mL cell, as well as a minimum of 750 μL of ligand (tau or stathmin) to fill the injection syringe. Another limiting factor was the duration of the titration (around 60 min), which rendered the work with tubulin difficult to reproduce during the same day. The new generation model iTC200 has drastically improved both time and quantity requirements. With this new apparatus, experiments require 300 μL to fill the sample

cell and 70 μL to fill the injection syringe. With fast equilibration times, up to two runs per hour can be accomplished. Nevertheless, in some cases, a large reaction volume, as in VP ITC, is still necessary. For example, it might be difficult to obtain or to work with high concentrations of the ligand due to the possibility of aggregation. Also, larger volumes of reaction might be needed for a low binding constant, or when the ΔH is small and requires the sum of many interactions to be detected. In these latter cases, the ITC200 can still be used, but it will imply conducting several consecutive experiments with the resultant curves concatenated (for a comparison, see Fig. 18.5).

18.3 RESULTS: TUBULIN/MAPs BY ITC

All the requirements described above may explain why ITC has not been used more often to study such complex systems as the cytoskeleton network. Nevertheless, ITC has been used to study the mechanism of bacterial tubulin homologue FtsZ assembly (Caplan & Erickson, 2003; Huecas et al., 2007) or to characterize the binding of several modulators of FtsZ assembly in order to use them in new anti-bacterial treatments (Chen, Milam, & Erickson, 2012; Domadia, Bhunia, Sivaraman, Swarup,
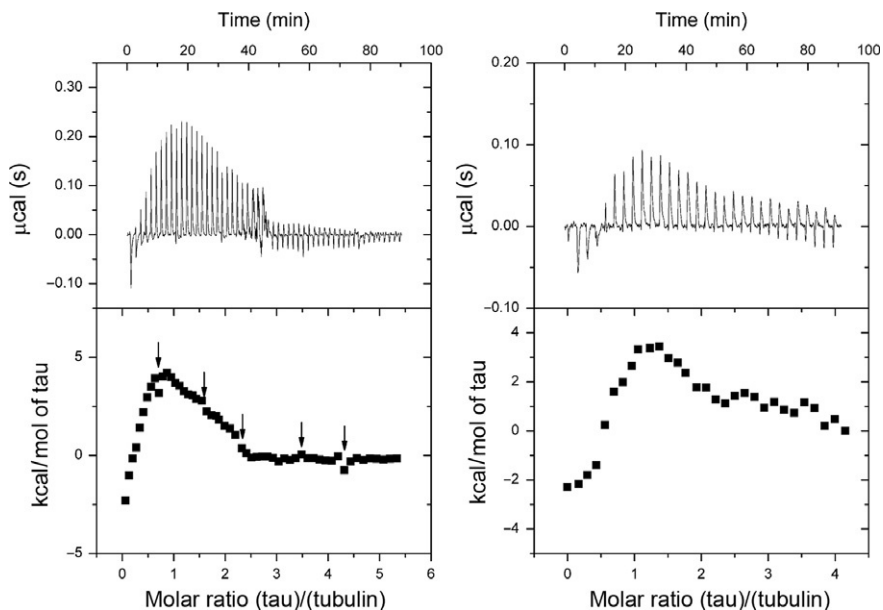

FIGURE 18.5

ITC titration curves (upper panels) and binding isotherms (low panels) of tau–tubulin interactions registered on ITC200 (left panels) and on VP ITC (right panels) at 10 °C in 20 mM NaPi, 0.1 mM GTP, 1 mM TCEP, buffer at pH 6.5. The arrows show syringe refilling with the same tau solution.
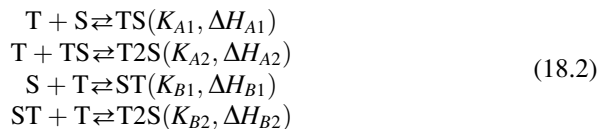
& Dasgupta, 2008; Domadia, Swarup, Bhunia, Sivaraman, & Dasgupta, 2007). It has also been used to characterize the binding of modulators of microtubule assembly on tubulin (Banerjee et al., 2005; Das et al., 2009; Gupta et al., 2003; Menendez, Laynez, Medrano, & Andreu, 1989; Rappl et al., 2006; Tsvetkov et al., 2011), to study stathmin–tubulin binding (Honnappa et al., 2003) and more recently tau–tubulin binding (Tsvetkov et al., 2012).
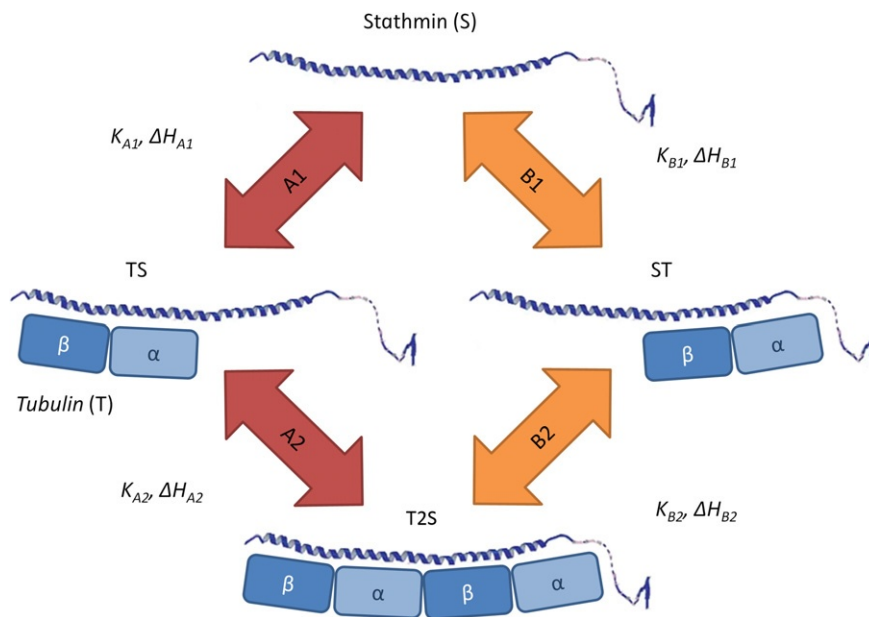
18.3.1 Stathmin–tubulin interaction

In 2003, Steinmetz and coauthors from Paul Scherrer Institute published an extensive characterization of the thermodynamics of the stathmin–tubulin interaction (Honnappa et al., 2003). They determined the stoichiometry, binding constant, variation of enthalpy and of entropy under different conditions of pH, temperature, and nucleotide presence (GTP/GDP). Under all investigated conditions, they obtained simple sigmoid binding isotherms, which can be well fitted with a simple one-set-of-sites binding model, described by following equations:

$$\begin{aligned} T + S &\rightleftarrows TS(K_0, \Delta H_0) \\ T + TS &\rightleftarrows T2S(K_0, \Delta H_0) \end{aligned}$$
(18.1)

They reported two binding sites of equal affinity with an equilibrium binding constant of $K_0 = 6.0 \times 10^6 \, \text{M}^{-1}$ and large negative molar heat capacity change ($\Delta C_p = -860 \, \text{cal mol}^{-1} \, \text{K}^{-1}$), which suggest that the major driving force of the binding reaction was hydrophobic interactions (Fig. 18.4). Nevertheless, earlier studies using several techniques, including pull-down assays (Holmfeldt et al., 2001; Larsson et al., 1999) and analytical ultracentrifugation (Amayed, Carlier, & Pantaloni, 2000; Jourdain, Curmi, Sobel, Pantaloni, & Carlier, 1997), suggested the existence of two highly cooperative binding sites. These findings led Honnappa and coauthors to conclude that ITC data contrasted with earlier studies proposing that the second tubulin subunit is bound distinctly tighter than the first one. Nevertheless, several models can fit the same curve. Indeed, the fact that ITC titration results in a simple thermogram does not guarantee that the simplest model is the real one. In other words, in this case, the principle of Occam's razor could be summarized as "other things being equal, a simpler explanation is better than a more complex one." As mentioned above, the sigmoid form of binding isotherm could also be observed for more complex models in the case of degenerate parameters. For example, for a model of nonequal interacting sites (Fig. 18.6) described by the following equations

$$\begin{aligned} T + S &\rightleftarrows TS(K_{A1}, \Delta H_{A1}) \\ T + TS &\rightleftarrows T2S(K_{A2}, \Delta H_{A2}) \\ S + T &\rightleftarrows ST(K_{B1}, \Delta H_{B1}) \\ ST + T &\rightleftarrows T2S(K_{B2}, \Delta H_{B2}) \end{aligned}$$
(18.2)

if there is strong cooperativity ($K_{A2} \gg K_{A1}$ and $K_{B2} \gg K_{B1}$) between two equal sites ($\Delta H_{A1} = \Delta H_{B1}, K_{A1} = K_{B1}$) and if binding of first ligand molecule does not change the

FIGURE 18.6

Schema of tubulin binding to stathmin. The general model of tubulin interaction with stathmin supposes or assumes the existence of two nonequal interacting sites described by six independent parameters K_{A1}, K_{B1}, K_{A2}, ΔH_{A1}, ΔH_{B1}, and ΔH_{A2} (although in the full thermodynamic cycle K_{B2}, ΔH_{B2} are not independent).

enthalpy of binding for the second site ($\Delta H_{A1}=\Delta H_{A2}=\Delta H_{B1}=\Delta H_{B2}$). In this case, treating a binding isotherm with a model of one-set-of-sites results in the determination of wrong binding constants (K_0) for each site. However, in both cases, the overall reaction can be written as follows:

$$2T + S \rightleftarrows T2S (K_{T2S}, \Delta H_{T2S}) \qquad (18.3)$$
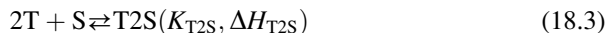
with an overall constant of formation of the T2S complex $K_{T2S}=K_0*K_0=K_{A1}*K_{A2}=K_{B1}*K_{B2}$.

Considering all the earlier evidence about the cooperativity, the current structural view of the asymmetric T2S complex (Gigant et al., 2000), and the fact that ITC cannot distinguish between two binding sites that would have the same ΔH, it is likely that ΔH of binding of individual tubulin to stathmin changes neither with the position on stathmin, nor with the presence of another tubulin on stathmin. A possible way to explain how ΔH can be similar despite the asymmetry of stathmin would be to consider that the heat exchanged during tubulin–stathmin interaction is mostly due to the lateral interaction of tubulin with the long alpha helix of stathmin, with very little (negligible) heat exchanged at the interface between β-tubulin and the consecutive α-tubulin or

between α-tubulin and the N-terminal cap of stathmin. In this context, whichever model is hypothesized, only the overall constant of formation of T2S complex (K_{T2S}) can be determined via K_0, as K_{A1}, K_{A2}, K_{B1}, or K_{B2} cannot be determined from the model of nonequal interacting sites if the binding isotherm is a degenerate sigmoid curve. Measurement of K_0 or further extrapolation to K_{T2S} enables only the characterization of T2S complex formation (described by Eq. 18.3) with values of stoichiometry, entropy, enthalpy, and free energy. The knowledge of these thermodynamic parameters allows one to characterize the nature of the forces involved in the interaction. In the example presented in Fig. 18.4, below 28 °C, since $\Delta H > 0$ (Fig. 18.4), the only driving force of T2S complex formation is hydrophobic interactions ($\Delta S > 0$), whereas above 28 °C, the reaction is enthalpy ($\Delta H < 0$) and entropy driven ($\Delta S > 0$). Rather than the intrinsic values of these parameters, which can vary greatly depending on the buffer conditions and temperature, it is the comparison of the parameters obtained in different conditions that will bring new information about the interaction. And despite the open question about the true nature (cooperative vs. noncooperative) of stathmin–tubulin binding, ITC enabled investigators to quantify the impact of each one of the four stathmin phosphorylations and different combinations of them, on its affinity for tubulin (Honnappa, Jahnke, Seelig, & Steinmetz, 2006). It enabled the authors to provide *in vitro* the biophysical basis for understanding the mechanism by which stathmin activity gradients will regulate local microtubule growth. This approach has also been used to determine the consequences of the presence of anticancer agents, such as vinblastine, on the activity of stathmin (Devred et al., 2008). Comparison of stathmin–tubulin binding in the presence or absence of vinblastine revealed an increase in the stathmin affinity for tubulin in the presence of vinblastine, setting the molecular basis of a new or revised mechanism of action of this MTA.

18.3.2 Tau–tubulin

ITC has also been used to study the interaction of tau with tubulin. We can expect that the binding of a stabilizing MAP such as tau, whose individual repeat domains can bind and stabilize microtubules (Aizawa et al., 1989; Butner & Kirschner, 1991; Devred, Douillard, Briand, & Peyrot, 2002; Ennulat, Liem, Hashim, & Shelanski, 1989; Goedert, Wischik, Crowther, Walker, & Klug, 1988; Gustke, Trinczek, Biernat, Mandelkow, & Mandelkow, 1994), is more complex to study than the binding of a destablizer, such as stathmin. The presence of any factor that favors or inhibits tubulin polymerization may have an impact on the extent of the tau-induced self-assembly and potentially on the thermodynamic parameters determined. This is why the recent ITC study of tau–tubulin interaction was conducted in a minimum phosphate-GTP buffer in the absence of Mg^{2+} (Tsvetkov et al., 2012). Even though tau has been studied for more than 40 years, very little is known about its structure (Harbison, Bhattacharya, & Eliezer, 2012). In addition, there are several discrepancies regarding its mode and parameters of binding to tubulin, probably in part due to the fact that microtubules can induce the formation of tau filaments (Duan & Goodson, 2012). Nevertheless, several studies have

suggested the existence of two binding sites, one that may overlap the paclitaxel binding site and that would be located in the lumen, and another one on the outside wall of MT (Ackmann, Wiech, & Mandelkow, 2000; Kar, Fan, Smith, Goedert, & Amos, 2003; Makrides, Massie, Feinstein, & Lew, 2004). These two sites would not be equally accessible depending on the nature of experimental study, such as tau-induced MT self-assembly versus tau binding to stabilized MT. ITC titration of tubulin by tau results in a complex two-phase binding isotherm that could be well fitted using two-sets-of-sites model, compatible with the two types of tau–tubulin binding modes described in the literature: one corresponding to a high affinity binding site with a tau:tubulin stoichiometry of 0.2 and the other one to a low affinity binding site with a stoichiometry of 0.8. Nevertheless, it cannot be excluded that tau–tubulin binding follows a more complex model. To assign the real model, many complementary experiments will need to be performed. Like in the case of stathmin–tubulin interaction, even the simplest binding model which resulted in the determination of only apparent thermodynamic parameters helped us to gain new insights into the mechanism of tau binding to tubulin. Indeed, even though tau induces the formation of curved tubulin protofilament at 10 °C, and the formation of microtubules at 37 °C (Devred et al., 2004; Fig. 18.1), tau–tubulin binding isotherm obtained at 10 and 37 °C were both biphasic with a maximum at a tau:tubulin molar ratio of one, indicating a similar binding model (Fig. 18.5). The fact that tau would bind similarly on an MT and on a circular protofilament indicates that on MT the interaction is longitudinal (along the same protofilament) and not transversal (bridging several parallel protofilaments). This allowed us to rule out the models which hypothesized that tau stabilizes MT by binding across several protofilaments on the MT lattice.

CONCLUSION

ITC is one of the latest and most powerful techniques to be used in characterizing the binding affinity of ligands for proteins or proteins for proteins. But like most techniques, it would be useless without other methods. ITC measures the heat exchange and thus often relies on complementary studies to hint at or confirm what reaction is really happening in the calorimetric cell. For example, analytical ultracentrifugation is a technique of choice to determine stoichiometry, changes in conformation or assembly state of the molecules studied (Correia & Stafford, 2009; Demeler, Brookes, & Nagel-Steger, 2009; Lebowitz, Lewis, & Schuck, 2002; Schuck, 2003). Through the examples detailed in this chapter, we have shown that if a certain number of precautions, due mostly to the nature of tubulin, are taken, ITC can be used to thermodynamically characterize molecular interactions between tubulin and MAPs. If stathmin binding to tubulin is now well characterized by ITC, there is still a lot to understand about tau binding to tubulin. In summary, even though the tubulin cytoskeleton is a challenging system to work on, ITC is a powerful technique able to provide significant advances in our understanding of tubulin interaction with its partners.

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