Fluorescence correlation spectroscopy analysis of the dynamics of tubulin interaction with RB3, a stathmin family protein

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Abstract **We have used fluorescence correlation spectroscopy to analyze the interaction of GTP-tubulin with rhodamine-labeled RB3, a neural protein of the stathmin family, and to determine the kinetic pathway of the association process. RB3 displayed slow association–dissociation kinetics with tubulin depending on the square of the tubulin concentration. The values of the apparent association and dissociation rate constants of the complex of two tubulin dimers and RB3 are determined to be $(3.52 \pm 0.14) \times 10^{-3}$ $\mu M^{-2}/s$ and $(1.9 \pm 0.6) \times 10^{-3}$ s^{-1} respectively. The value of the equilibrium dissociation constant for the first tubulin–RB3 interaction is estimated to be $\geqslant 7$ μM at 20°C.**

Key words: Fluorescence correlation spectroscopy; Tubulin; RB3 protein; Association–dissociation kinetics

1. Introduction

Tubulin is a 50 kDa αβ heterodimer protein that self-assembles into microtubules. The dynamic state of microtubules is finely modulated during the cell cycle by a variety of proteins that control tubulin association–dissociation at the plus and minus ends of microtubules, and by anchoring and severing proteins that control the availability of plus and minus ends. As a result, the steady-state concentration of αβ-tubulin, which reflects microtubule dynamics, varies during the cell cycle. Changes in free tubulin concentration are amplified by tubulin-sequestering proteins, which establish a pool of non-polymerized tubulin. Proteins of the Op18/stathmin family are the major tubulin-sequestering agents known so far. These small (18 kDa) proteins interact with two tubulin molecules in a stathmin–tubulin complex (T_2S) [1–5], the stability of which is negatively regulated by phosphorylation, in response to a variety of signaling pathways [6] and during mitosis [7,8]. While stathmin is ubiquitous, its relatives SCG10, SCLIP, RB3, and RB3′ are more prominently expressed in nervous tissue.

So far the interaction of stathmin with tubulin has been studied in solution by analytical ultracentrifugation [1] and by gel filtration assay [9] in the range 10^{-6}–10^{-5} M, and by measuring its effect on nucleotide exchange on tubulin in the range 10^{-7}–10^{-6} M [10]. These studies led to the conclusion that the T_2S complex was very tight and had slow association–dissociation kinetics, but the value of the equilibrium dissociation constant was too low to be determined accurately by these methods. In a more recent study, by buffering the concentration of free tubulin to below 10^{-7} M, values of the equilibrium dissociation constant of 0.7 ± 0.5 μM^2 and 0.1 ± 0.05 μM^2 were proposed for the T_2S complex in HEPES (pH 7.4) buffer and GTP-containing PIPES (pH 6.8) buffer at 37°C respectively [11]. In addition to these quantitative studies in solution, the relative stabilities of the complexes formed between tubulin and stathmin derivatives were estimated by gel filtration and surface plasmon resonance using immobilized stathmin derivatives [2,9]. However, the kinetic mechanism of association of stathmin and stathmin-related proteins with tubulin and the kinetic parameters for association–dissociation of the complex are not known. Because fluorescence correlation spectroscopy (FCS) makes it possible to examine the interaction between proteins in the 10^{-9} M range, it is the method of choice to analyze the dynamics of the T_2S complex. The stathmin core of RB3 protein, which has been crystallized in complex with tubulin [5] and interacts slightly more strongly with tubulin than stathmin [9], was first taken here as an example.

2. Materials and methods

2.1. Preparation of tubulin

Tubulin was purified by phosphocellulose chromatography in MES buffer from microtubule proteins that were isolated by two cycles of assembly–disassembly from pig brains according to Shelanski et al. [12] as modified by Engelborghs et al. [13].

Purified tubulin was concentrated in Centricon tubes and its concentration was determined spectrophotometrically using an extinction coefficient of 120 000 M^{-1} cm^{-1} at 278 nm [14]. Tubulin was stored in liquid nitrogen in 50 mM MES pH 6.4, 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM NaN_3, with the ionic strength adjusted to 0.1 M with NaCl. Prior to use, the tubulin solution was adjusted to 1 mM GTP and aggregated protein was removed by centrifugation (4°C, 15 000 rpm, 20 min).

2.2. RB3 protein and fluorescent RB3

RB3 protein is a recombinant protein containing only the core of RB3 similar to stathmin expressed in *Escherichia coli* and was purified

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Abbreviations: FCS, fluorescence correlation spectroscopy; TAMRA, carboxytetramethylrhodamine succinimidyl ester; T_2R, two tubulin dimers in complex with RB3

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Stathmin MASS----------DIQVKELFKRASGQA
RB3      MAMTLAAYKEKMKELPLVSLFCSCFLSDPLNKSSYKYEADTVDLNWCVISDMEVIELNKCTSGQS

Stathmin FELILSPRSKESVPEFPLSPP-KKKDLSLEEIQKKLEAAEERRKSHEAEVLKQLAEKREHEKEVLQKA
RB3      FEVILKPPSFDGVPEFNASLP-RRRDPSLEEIQKKLEAAEERRKYQEAELLKHLAEKREHEREVIQKA

Stathmin IEENNNFSKMAEEKLTHKMEANKENREAQMAAKLERLREKDKHVEEVRKNKESKDPADETEAD
RB3      IEENNNFIKMAKEKLAQKMESNKENREAHLAAMLERLQEKDKHAEEVRKNKELKEEASR
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Fig. 1. Amino acid sequences of stathmin and RB3.

as described [2]. Amino acid sequences of stathmin and RB3 are shown in Fig. 1.

RB3 was fluorescently labeled on the αNH_2 of the N-terminal residue using carboxytetramethylrhodamine succinimidyl ester (TAMRA). Kinetic studies of the amount of dye incorporated versus time at different concentrations of TAMRA were performed, to define the conditions under which a single residue on average was labeled in a burst phase. The reaction was carried out at 20°C in 0.1 M PIPES–KOH buffer at pH 6.5. RB3 (50 μM) was incubated for 1 h with 75 μM TAMRA. The reaction was stopped by addition of 10 mM Tris–HCl, pH 7.5. The solution was gel-filtered (Sephadex G25, PD-10, Pharmacia) in PIPES buffer to isolate the covalent rhodamine–RB3 adduct. Before each FCS experiment, the labeled protein was again gel-filtered in MES buffer at a lower concentration (10–20 μM) to remove residual non-covalently bound dye. For FCS measurements the fluorescent RB3 was diluted to 5–10 nM in MES buffer (pH 6.4) containing 50 mM MES, 1 mM $MgCl_2$, 1 mM GTP, 1 mM EGTA.

2.3. Fluorescent correlation spectroscopy

FCS was applied to the analysis of the interaction of tubulin and fluorescent RB3 protein using the instrumental setup of the LSM 510-ConfoCor 2 (Zeiss). This technique allows determining the diffusion characteristics of fluorescent molecules and their interactions with other particles at nanomolar concentrations in a femtoliter volume.

The principle of the FCS has been described by Rigler et al. [15–17] and the setup of the LSM 510-ConfoCor2 has been described in detail by Jankowski and Janka [18].

The experimentally obtained $G(t)$ function allows determining the number of particles and their diffusion time in an excitation volume and then the relation between the diffusion coefficient D and the diffusion time τ_d is used to calculate the diffusion coefficient:

$$\tau_d = \frac{\omega_1^2}{4D} \quad (1)$$

where ω_1 is the radius of the Gaussian beam profile at $1/e^2$ of its maximal intensity (in the x- and y-directions) of the laser beam that is determined from the calibration of the ConfoCor 2 with rhodamine 6G dye of known diffusion coefficient $D = 2.8 \times 10^{-10}$ m^2/s [19]. If the fluorescent molecule binds to a target of diffusion coefficient differing by at least two-fold without change in fluorescence intensity, the concentration, the diffusion times of free and bound molecules and the degree of binding can be determined directly from the correlation function [20].

All FCS measurements were done in a sample volume of 100 μl. The concentration of the fluorescent RB3 was between 5 nM and 10 nM. The concentration of tubulin was varied in the micromolar range. The measuring time per sample was 30 s and all samples were measured 20 times.

3. Results

3.1. Diffusion coefficient of RB3

FCS measurements of rhodamine-labeled RB3 yielded an autocorrelation curve that was consistent with the existence of two components. 20% of the fluorescent material had a translational diffusion time τ_1 of 33 ± 1 μs, identical to the diffusion time of the free TAMRA dye measured independently in control experiments. The value of the translational diffusion time τ_2 of the second major component was 153 ± 22 μs, which corresponds to a value of the diffusion coefficient of RB3 of $(7.3 \pm 0.2) \times 10^{-11}$ m^2/s.

3.2. Association and dissociation processes of fluorescently labeled RB3 and tubulin

When fluorescent RB3 (8 nM) was mixed with tubulin at a concentration higher than 3 μM, the formation of the complex was complete within the mixing time (10 s). The autocorrelation curves of the mixture were again consistent with two components, the minor component having the diffusion characteristics of the free dye, while the major component had a translational diffusion time τ_3 of 252 ± 20 μs, corresponding to a value of $(3.8 \pm 0.2) \times 10^{-11}$ m^2/s, attributed to the diffusion coefficient of the tubulin–RB3 complex (T_2R). This value was identical at different tubulin concentrations. In a range of tubulin concentrations from 0.2 μM to 3 μM, changes in the fluorescence fluctuations reflecting the binding process of tubulin to RB3 could be monitored (Fig. 2). The autocorrelation function was analyzed using a two- or three-component model. The fractions of free and bound RB3 were derived using the diffusion times determined for the isolated RB3, T_2R and free dye. The observed binding process was mono-exponential and analyzed using the following equation:

$$Y = Y_0[1 - \exp(-k_{obs}t)] \quad (2)$$

where Y is the fraction of RB3 in complex, k_{obs} is the observed first-order rate constant for the association process. The value of k_{obs} varied as a quadratic function of tubulin concentration [T], reflecting the fact that RB3 binds two tubulin molecules (Fig. 3).

To determine the rate constant for dissociation of T_2R complex, an excess of unlabeled RB3 (3 μM) was added at time zero to the preformed complex with 5 nM fluorescent

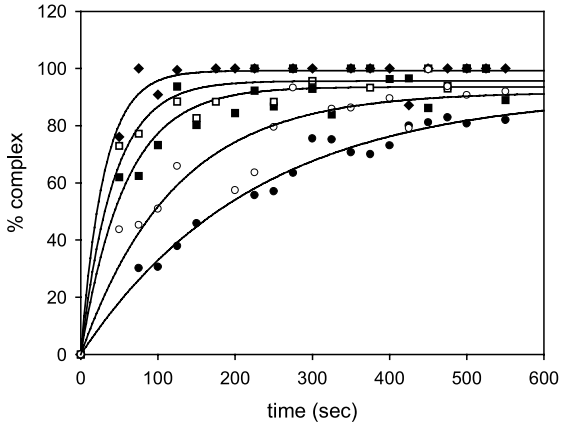

Fig. 2. Time course of an association of RB3 to tubulin at different concentrations of tubulin. Measurements were performed in MES buffer (pH 6.4) in the presence of 1 mM GTP, 1 mM $MgCl_2$, 1 mM EGTA. The concentration of fluorescent RB3 was 8 nM. The data represent the measurements by FCS in the presence of (●) 0.9 μM tubulin, (○) 1.4 μM tubulin, (■) 2 μM tubulin, (□) 2.5 μM tubulin, (♦) 3 μM tubulin. The experimental data are fitted to Eq. 2.

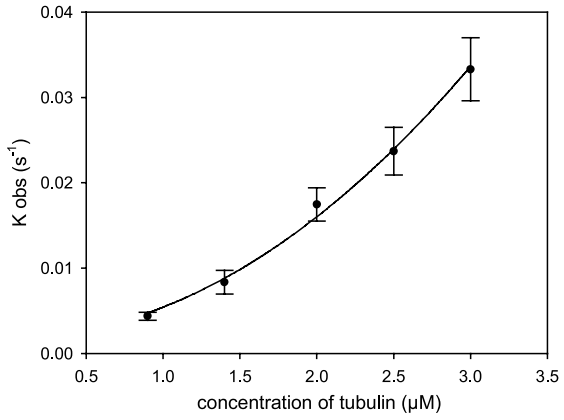

Fig. 3. Dependence of the observed rate constant k_{obs} on the concentration of tubulin. The value of k_{obs} varies as a quadratic function of tubulin concentration, reflecting the fact that RB3 binds to two tubulin molecules. The apparent association and dissociation constants are calculated to be $(3.52\pm0.14)\times10^{-3}$ $\mu M^{-2}/s$ and $(1.9\pm0.6)\times10^{-3}$ s^{-1} respectively. The equilibrium dissociation constant for T_2R is found to be 0.5 ± 0.2 μM^2.

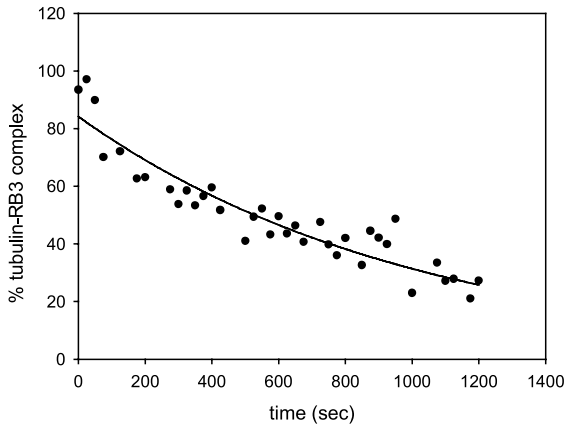

Fig. 4. Dissociation of fluorescent RB3 from tubulin in the presence of non-fluorescent RB3. Following formation of the fluorescent RB3–tubulin complex by mixing 8 nM RB3 and 2 μM tubulin, a chase of 3 μM non-fluorescent RB3 was applied to the sample. The experimental data are fitted to an exponential decay and the dissociation rate constant is determined to be $(1.1\pm0.2)\times10^{-3}$ s^{-1}.

RB3 and 2 μM tubulin, to displace bound fluorescent RB3 from tubulin. The decrease in the fraction of fluorescent tubulin–RB3 complex reflected the rate-limiting step in the replacement of fluorescent by non-fluorescent RB3 in the T_2R complex, i.e. dissociation of T_2R (Fig. 4). The data were consistent with a monoexponential process of rate constant $=(1.1\pm0.2)\times10^{-3}$ s^{-1}.

3.3. Data analysis and modelling

The general scheme for binding of RB3 to tubulin is the same as for stathmin, as presented by Amayed et al. [11], and features the formation of intermediate 1:1 complexes RT and TR, followed by binding a second tubulin molecule leading to the final T_2R state. In this isoenergetic square, the two pathways leading from T+R to T_2R are energetically equivalent, but kinetically distinct. The 3D structure of crystallized T_2R complex [5] shows that RB3 consists of two consecutive helices that bind to two αβ-tubulin protomers arranged in a polarized fashion. Whether the N-terminal or the C-terminal helix of RB3 binds one molecule of αβ-tubulin first, in an RT or TR complex, is not known. For simplicity we assume that one of the two pathways, e.g. $R\rightarrow RT\rightarrow T_2R$ is kinetically privileged. A second likely hypothesis is that the RT complex has a low stability and that a steady state develops so that $d[RT]/dt=0$, therefore:

$$k_{+1}[R][T]=k_{-1}[RT]+k_{+2}[T][RT]= [RT]\{k_{-1}+k_{+2}[T]\} \quad (3)$$

Then,

$$[RT]=k_{+1}[R][T]/(k_{-1}+k_{+2}[T])=a[R] \quad (4)$$

where $a=k_{+1}[T]/(k_{-1}+k_{+2}[T])$.

Mass conservation is expressed as follows:

$$[R_0]=[R]+[RT]+[T_2R] \quad (5)$$

$$d[T_2R]/dt=k_{+2}a[T]\{[S_0]/(1+a)-[T_2R]/(1+a)\}-k_{-2}[T_2R] \quad (6)$$

The observed rate constant is:

$$k_{obs}=k_{-2}+k_{+1}k_{+2}[T]^2/\{k^{-1}+(k_{+1}+k_{+2})[T]\} \quad (7)$$

If $k_{+2}\gg k_{+1}$, the steady assumption reduces to the simpler case of a rapid-preequilibrium of R and T in the RT complex, and Eq. 7 reduces to $k_{obs}=k_{-2}+k_{+2}[T]^2/(K_1+[T])$.

At zero tubulin concentration, the extrapolated value of k_{obs} is k_{-2}.

In a range of low tubulin concentrations, Eq. 7 is a quadratic function of [T]:

$$k_{obs}=k_{-2}+k_{+2}[T]^2/K_1 \quad (8)$$

where $K_1=k_{-1}/k_{+1}$.

At high concentration of tubulin, k_{obs} varies linearly with [T] as follows:

$$k_{obs}=k_{+1}k_{+2}[T]/(k_{+1}+k_{+2}) \quad (9)$$

Note that the slope of k_{obs} vs. [T] is k_{+2} if $k_{+1}\gg k_{+2}$, which corresponds to a rapid pre-equilibrium for the RT complex.

According to Eq. 7, the data (Fig. 3) indicate that $k_{-2}=(1.9\pm0.6)\times10^{-3}$ s^{-1}, in good agreement with the value of $(1.1\pm0.2)\times10^{-3}$ s^{-1} derived from the displacement experiment. The value of k_{+2}/K_1 was 3.5×10^9 M^{-2}/s. The value of k_{+2} derived from the extrapolation of the curve in the high

Table 1
Comparison of the rate constants of binding of fluorescent RB3 to tubulin and stathmin to tubulin obtained by FCS and by Amayed et al. [10]

	k_{+2}/K_1 ($\mu M^{-2}/s$)	k_{-2} (s^{-1})	K_D (μM^2)
FCS, RB3 protein	$(3.52\pm0.14)\times10^{-3}$	$(1.9\pm0.6)\times10^{-3}$ $(1.1\pm0.2)\times10^{-3}$	0.5 ± 0.2
Amayed et al., stathmin	3×10^{-3}	0.8×10^{-3}	0.7 ± 0.5

tubulin concentration range was $\sim 2\times10^4$ M^{-1}/s. The value of K_1 therefore is ~ 7 μM, consistent with previous rough estimates [10]. The overall equilibrium dissociation constant for the T_2R complex is $K_D = K_1K_2$. A value of 0.5 ± 0.2 μM^2 was derived for K_D from the data. This value compares well with the value of 0.7 ± 0.5 μM^2 obtained for stathmin [11]. All values of kinetic and equilibrium parameters are summarized in Table 1.

4. Discussion

The main conclusion of the present work is that the T_2R complex of GTP-tubulin with the stathmin variant RB3, a member of the tubulin-sequestering protein family, is in slow association–dissociation equilibrium. The rate parameters that have been evaluated for the first time are consistent with a binding scheme in which RB3 binds one molecule of GTP-tubulin in a rapid equilibrium low affinity TR complex, followed by binding of a second GTP-tubulin molecule with an association rate constant of $\sim 2\times10^4$ M^{-1}/s. This value is so low for a bimolecular reaction that it probably reflects a more complex binding scheme in which the second tubulin molecule binds rapidly to TR leading to T_2R, and a subsequent slower intramolecular structural rearrangement leads to the final very tight T_2R^* complex. Within this hypothesis the apparent dissociation rate constant of $(1.9 \pm 0.6)\times10^{-3}$ s^{-1} would represent the rate-limiting reverse isomerization of the T_2R^* complex. The overall scheme then becomes as follows:

$$T + R \leftrightarrow TR$$

$$T + TR \leftrightarrow T_2R \leftrightarrow T_2R^*$$

Several lines of evidence indicate that stathmin proteins have a higher affinity for GDP-tubulin than for GTP-tubulin. Therefore it is expected that the dynamics of the T_2R complex must be slower in the presence of GDP.

The RB3 protein was chosen for these experiments because it displays the tightest binding to tubulin among all stathmin family members and comes just ahead of stathmin itself [9]. The value of the rate constant for the rate-limiting step in the dissociation of GTP-bound T_2R complex at 20°C is about 10^{-3} s^{-1}. A similar maximum limit value of 10^{-3} s^{-1} had been postulated for the dissociation of the GDP-bound T_2S complex at 20°C, because evidence indicated that the dissociation of GDP from tubulin in the complex, which took place with a rate constant of 0.01 s^{-1}, was not limited by complex dissociation [10], implying that complex dissociation had to be an order of magnitude slower than nucleotide dissociation. In conclusion, the quantitative evaluation of the dynamic parameters of the T_2R complex is fully consistent with the interpretations of previous works. We anticipate that the dynamics of the T_2S complex must be about an order of magnitude faster in GTP, but as slow in GDP as the one measured here for GTP-bound T_2R. Available records indicate that faster dynamics are expected for the complexes formed with tubulin by other members of the stathmin family such as SCLIP and SCG10, which bind tubulin less tightly than stathmin and RB3 [9].

The fact that with different stathmin derivatives tubulin forms complexes that have different dynamics may have physiological relevance regarding the regulation of the cell cycle by stathmin, for instance, or the regulation of the pool of unassembled neuronal tubulin by SCG10.

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