Stathmin Slows down Guanosine Diphosphate Dissociation from Tubulin in a Phosphorylation-Controlled Fashion†

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ABSTRACT: Stathmin is an important protein that interacts with tubulin and regulates microtubule dynamics in a phosphorylation-controlled fashion. Here we show that the dissociation of guanosine 5′-diphosphate (GDP) from β-tubulin is slowed 20-fold in the $(tubulin)_2$−stathmin ternary complex (T_2S). The kinetics of GDP or guanosine 5′-triphosphate (GTP) dissociation from tubulin have been monitored by the change in tryptophan fluorescence of tubulin upon exchanging 2-amino-6-mercapto-9-β-ribofuranosylpurine 5′-diphosphate (S6-GDP) for tubulin-bound guanine nucleotide. At molar ratios of stathmin to tubulin lower than 0.5, biphasic kinetics were observed, indicating that the dynamics of the complex is extremely slow, consistent with its high stability. The method was used to characterize the effects of phosphorylation of stathmin on its interaction with tubulin. The serine-to-glutamate substitution of all four phosphorylatable serines of stathmin (4E-stathmin) weakens the stability of the T_2S complex by about 2 orders of magnitude. The phosphorylation of serines 16 and 63 in stathmin has a more severe effect and weakens the stability of T_2S 10^4-fold. The rate of GDP dissociation is lowered only 7-fold and 4-fold in the complexes of tubulin with 4E-stathmin and diphosphostathmin, respectively. Sedimentation velocity studies support the conclusions of nucleotide exchange data and show that the T_2S complexes formed between tubulin and 4E-stathmin or diphosphostathmin are less compact than the highly stable T_2S complex. The correlation between the effect of phosphorylation of stathmin on the stability of T_2S complex measured in vitro and on the function of stathmin in vivo is discussed.

The regulation of the assembly/disassembly processes of microtubules is essential in a number of cellular reactions such as cell division and morphogenesis. The dynamics of microtubules is described in terms of dynamic instability (*1*). This behavior is the consequence of GTP[1] hydrolysis, which accompanies tubulin polymerization and generates nonlinearity in the assembly kinetics (*2*). It is currently accepted that microtubules at steady state have a cap of terminal slowly dissociating GTP subunits. Stochastic loss of the cap leads to the rapid depolymerization of GDP-tubulin. Exchange of GTP for GDP on tubulin regenerates polymerizable GTP-tubulin. In bulk solution, dynamic instability generates oscillatory polymerization kinetics (*3*), the period of which is controlled by the rate of GDP dissociation from tubulin (*4*). The rate of nucleotide exchange therefore appears as a key parameter in the regulation of microtubule dynamics.

Microtubule dynamics is exquisitely regulated in vivo. Three proteins so far have been demonstrated to play distinct roles in this regulation: Op18/stathmin; XKCM1, a kinesin of the KIF family (for review see ref *5*); and XMAP215 (*6*). Stathmin, a ubiquitous phosphoprotein long known to integrate a variety of intracellular signaling pathways (*7*), was lately shown to interact with tubulin and cause microtubule destabilization in a phosphorylation-controlled fashion (*8*−*10*). The phosphorylation-controlled activity of stathmin likely accounts for its regulation of microtubule dynamics in the cell cycle (*11*−*13*). Stathmin was first proposed to act as a catastrophe-promoting factor (*8*), and then it was shown to sequester tubulin in vitro, forming an unpolymerizable ternary complex of one stathmin with two $\alpha\beta$-tubulin dimers (*14*, *15*). Sedimentation velocity measurements (*14*) showed that the T_2S complex was very tight. The low value of 7.8 S found for the sedimentation coefficient of this 218 kDa molecule indicated that the complex had an elongated shape; in addition, the fact that only a ternary T_2S complex and no binary TS complex was formed, indicated that strong tubulin−tubulin contacts present in T_2S were essential in its stability. Very recent electron microscopy analysis of the structure of the T_2S complex brought support to these conclusions and further demonstrated that stathmin bound in a single helix along two tubulin subunits, interacting via polarized longitudinal bonds such as in a microtubule protofilament (*16*). The longitudinal bond $\alpha\beta-\alpha\beta$ is the most stable tubulin−tubulin bond in tubulin polymers (*17*, *18*), consistent with the high stability of the T_2S complex.

Whether the simple tubulin-sequestering activity of stathmin is sufficient to account for its regulation of microtubule dynamics in vivo or whether another, yet nonelucidated property is responsible for its microtubule destabilizing

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; S6-GTP, 2-amino-6-mercapto-9-β-ribofuranosylpurine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 4-morpholinoethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TLC: thin-layer chromatography; PKA, protein kinase A.

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function has been a subject of debate (see ref *19* for review). The mutated stathmin in which all four phosphorylatable serines have been substituted for glutamate residues, thus mimicking phosphorylated stathmin, sequesters tubulin as efficiently as wild-type stathmin in vitro but shows a greatly reduced ability to destabilize microtubules in vivo (*20*). These puzzling results led the authors to propose that stathmin would possess tubulin-directed activities that would not require its binding to tubulin and might be responsible for destabilization of microtubules in vivo.

Here we show that the dissociation of GDP from tubulin is slowed by stathmin in a phosphorylation-controlled manner. Nucleotide exchange experiments are combined with a comparative study of the hydrodynamic properties of complexes of tubulin with stathmin and its phosphorylated or pseudophosphorylated variants. The observed differences in the dynamics of those complexes may be relevant in the in vivo regulation of stathmin function. A preliminary account of the present work was presented at the Biophysical Society Meeting last year (*21*).

MATERIALS AND METHODS

Proteins. Tubulin was purified from bovine brain by three cycles of assembly followed by phosphocellulose chromatography (*14*) and stored at −80 °C in M buffer (50 mM MES−KOH buffer, pH 6.8, with 0.5 mM $MgCl_2$, 0.5 mM EGTA, and 4.2 M glycerol) supplemented with 0.1 mM GTP. Before each experiment, tubulin was thawed and submitted to a 4th cycle of assembly in M buffer supplemented with 0.5 mM GTP and 6 mM $MgCl_2$.

Recombinant wild-type stathmin and the mutated protein in which all four phosphorylatable serines (S16, S25, S38, and S63) were substituted with glutamate (4E-stathmin) were prepared as described (*22*).

Stathmin was phosphorylated on serines 16 and 63 by protein kinase A (Sigma) as described (*23*), with the following modifications. Wild-type stathmin (4 mg/mL, 0.25 mL) was incubated with 0.5 unit of PKA/μg of stathmin (i.e., a 5-fold lower PKA:stathmin ratio than in ref *23*) for 45 min at 30 °C in 8 mM Tris-Cl^-, pH 7.5, containing 10 mM $MgCl_2$ and 0.5 mM ATP. The reaction was stopped by boiling for 10 min. Preliminary experiments showed that this PKA:stathmin ratio and this reaction time were the optimum conditions leading to full phosphorylation of serine 63 (which reacted first) and serine 16. The product of the reaction was homogeneous in nondenaturing gel electrophoresis and was identified as (S63, S16)-diphosphostathmin by immunoblotting (not shown). In our hands, using higher amounts of PKA led to phosphorylation of a third serine on about 30% of the material.

Synthesis of S6-GTP and S6-GDP. S6-GMP was synthesized and purified by DEAE-cellulose as described by Eccleston and Trentham (*24*) and reacted either with diphenyl phosphochloridate or with pyrophosphate, with fresh, nonhydrolyzed 1,1′-carbonyldiimidazole as an activator, to obtain S6-GDP and S6-GTP, respectively (*25*). The thionucleotides were then purified by DEAE-cellulose chromatography with a triethylammonium bicarbonate gradient. Purity was checked by TLC on poly(ethylenimine)−cellulose.

Kinetics of GDP and GTP Dissociation from Tubulin in the Absence and Presence of Stathmin. The fluorescence decrease associated with the fluorescence resonance energy transfer from tryptophans to S6-GDP (*26, 27*) was used to monitor the kinetics of S6-GDP exchange for tubulin-bound GDP and to examine how the kinetics were affected by the binding of stathmin to tubulin. GDP-tubulin 1:1 complex was first prepared by resuspending the pellet of precycled microtubules in ice-cold M buffer supplemented with 20 μM GDP. The solution was left on ice for 15 min and centrifuged at 400000*g*, 4 °C, for 10 min. The GDP-tubulin 1:1 complex was isolated by gel filtration over a Sephadex G25 column (PD-10, Pharmacia) equilibrated in P buffer (80 mM PIPES−KOH buffer, pH 6.8, with 0.5 mM $MgCl_2$ and 0.5 mM EGTA) containing no nucleotide. GTP-tubulin 1:1 complex was prepared similarly, except that the microtubule pellet was resuspended in M buffer containing 100 μM GTP. The exchange kinetics were monitored in P buffer at 25 °C on a stopped-flow apparatus (DX-17.MV, Applied Photophysics) working in fluorescence mode, with a slit of 0.5 mm and an excitation wavelength of 295 nm. An MTO 310a filter was placed on the emission beam to cut off the excitation light. The dead time was 1.2 ms. GDP-tubulin (1 μM final concentration) supplemented with stathmin at the desired concentration was mixed with 5 μM S6-GDP. Preliminary fluorescence titration curves of 1 μM GDP-tubulin by S6-GDP established that this concentration of S6-GDP was sufficient to obtain full replacement of bound GDP by S6-GDP, while the inner filter effect due to the absorbance of S6-GDP was kept minimal, i.e., it represented 5% of the total tubulin fluorescence. Kinetic studies also showed that superimposable time courses of fluorescence decrease were obtained with 5 and 10 μM S6-GDP, confirming that the recorded process truly represents the dissociation of GDP from tubulin, which kinetically limits the binding of S6-GDP at both concentrations.

In the presence of saturating concentrations of stathmin, the rate of nucleotide exchange was slow enough to be adequately monitored in a standard spectrofluorometer (Spex Fluorolog 2) with excitation and emission wavelengths of 295 and 340 nm, respectively. The value of the rate constant found in that instrument was identical to the one derived from the stopped-flow apparatus by using the 200 s time base of the instrument.

Simulation of Nucleotide Exchange on Tubulin in Equilibrium with Stathmin. Modeling the kinetics of tryptophan fluorescence change associated with nucleotide exchange was carried out with the KINSIM software and the following equations.

$$S + 2Tn \leftrightarrow Tn_2S \quad (k_+, k_-) \tag{1}$$

$$S + 2TN \leftrightarrow TN_2S \quad (k_+, k_-) \tag{2}$$

$$S + Tn + TN \leftrightarrow TnTNS \quad (k_+, k_-) \tag{3}$$

$$S + Tn + TN \leftrightarrow TNTnS \quad (k_+, k_-) \tag{4}$$

$$Tn + N \rightarrow TN + n \quad (k_{-1}) \tag{5}$$

$$Tn_2S + N \rightarrow TNTnS + n \quad (k_{-21}) \tag{6}$$

$$TNTnS + N \rightarrow TN_2S + n \quad (k_{-22}) \tag{7}$$

$$Tn_2S + N \rightarrow TnTNS + n \quad (k_{-22}) \tag{8}$$

$$TnTNS + N \rightarrow TN_2S + n \quad (k_{-21}) \tag{9}$$

$$F_{obs} = Tn\Phi_n + TN\Phi_N + (TnTNS + TNTnS)(\Phi_n + \Phi_N) + 2\Phi_N TN_2S + 2\Phi_n Tn_2S \tag{10}$$

In the above equations, n and N represent GDP and S6-GDP, respectively; accordingly, Tn and TN represent GDP-tubulin and S6-GDP-tubulin; S represents stathmin, and Φ_n and Φ_N are the specific fluorescences of Tn and TN. Equations 1−4 describe the equilibria between stathmin and the different tubulin species; note that due to the structural asymmetry of the T_2S complex, TNTnS and TnTNS are considered as two different species. For simplicity, formation of T_2S from one stathmin and two tubulin molecules is represented as a single trimolecular reaction (the rate constant is the same in eqs 1−4, independent of the bound nucleotide), most likely corresponding to a rapid equilibrium (T + S = TS) followed by a bimolecular reaction (TS + T ↔ T_2S). Similarly, dissociation of the complex is represented as a single monomolecular step (k_-), implying that following the slow dissociation of one molecule of tubulin (or stathmin) from T_2S, the TS (or T_2) complex falls apart very rapidly. Equations 5−9 describe exchange of S6-GDP (N) for bound GDP (n). The concentration of N being in large excess over that of n, eqs 5−9, as written, reduce to the rate-limiting monomolecular dissociation of GDP, and the reverse reaction (replacement of N by n) is negligible. The rate constant for dissociation of GDP from free tubulin is k_{-1}. The rate constants for dissociation of GDP from the first and the second tubulin molecules in the T_2S complex are k_{-21} and k_{-22}, respectively. Equation 10 describes the tryptophan fluorescence (F_{obs}) as a function of the concentrations of the different tubulin species. Φ_n and Φ_N represent the specific fluorescence of tubulin with bound GDP or S6-GDP, respectively. It is assumed that in the two tubulins of the T_2S complex the environment of the nucleotide is the same as in free tubulin; hence the values of Φ_N and Φ_n are conserved in the complex.

Analytical Ultracentrifugation Experiments. The hydrodynamic properties of the complexes of tubulin with stathmin and its 4E-mutated and phosphorylated variants were examined by sedimentation velocity in the analytical ultracentrifuge (Beckman Optima XL-A). Samples containing GDP-tubulin (5−10 μM) in P buffer and stathmin or its variants were centrifuged at 50 000 rpm at 20 °C. Radial scans were taken every 5 min at 278 nm. The data were analyzed (*28*, *29*) to determine the distribution of sedimentation coefficients and the weight-average sedimentation coefficient, by use of

$$S_w = \sum[S_i g(S_i)]/\sum[g(S_i)]$$

where $g(S_i)$ in an interacting system represents the concentration determined from absorbance measurement at the plateau of the scan at 278 nm. Since stathmin does not absorb at this wavelength, the concentration of tubulin−stathmin complexes is derived from measurement of tubulin absorbance exclusively, and stathmin is considered as a small ligand that has no mass. The error that results from neglecting the mass of stathmin is at most 8.5% when 100% of tubulin is in complex with stathmin.

RESULTS

Stathmin Slows Down GDP Dissociation from Tubulin by 20-fold. It has been shown by Yarbrough and Fishback (*26*, *27*) that the exchange of S6-guanine nucleotides for GDP or GTP at the E-site of β-tubulin was associated with a decrease in tryptophan fluorescence of tubulin, due to fluorescence resonance energy transfer from tryptophan to the bound thionucleotide. When GDP-tubulin 1:1 complex (1 μM) was mixed in the stopped-flow apparatus with a saturating amount of S6-GDP (5 μM), tubulin fluorescence decreased by 18% within a first-order process, representing dissociation of GDP from the E-site. The value of the rate constant (0.1 ± 0.02 s^{-1} in different experiments in P buffer at 25 °C) is in agreement with the one derived by Brylawski and Caplow (*30*) by use of an accessory enzyme system. The fluorescence method provides the advantage of continuous recording of the process, which allows a more accurate kinetic analysis. The rate of GTP dissociation from the 1:1 GTP-tubulin complex by excess S6-GTP was found to be 10-fold slower (0.012 s^{-1}) under the same solution conditions. The dependence of the rate of dissociation of GDP on solution variables showed that glycerol (4 M) caused a 10-fold decrease in the rate of GDP dissociation, in qualitative agreement with Yarbrough and Fishback (*27*). The rate of GDP dissociation increased ($k = 0.3$ s^{-1}) when the concentration of magnesium ions in P buffer was increased to 5 mM.

The method was used to characterize the nucleotide exchange properties of tubulin in complex with stathmin. In the presence of 1 μM tubulin and at a stathmin concentration of 0.5 μM (i.e., a stathmin:tubulin molar ratio of 0.5) and up to 10 μM stathmin, the process was much slower and was well accounted for by a single exponential of rate constant $k = 0.006 \pm 0.001$ s^{-1} (Figure 1a). The extent of fluorescence decrease was very slightly lower than in the absence of stathmin. The final level of tryptophan fluorescence was the same whether S6-GDP was first added to GDP-tubulin, followed by stathmin, or stathmin was first added and then S6-GDP. These results indicate that stathmin interacts with two tubulin molecules and slows down the dissociation of GDP bound to each of the two tubulins in the T_2S complex. When the molar ratio of stathmin to tubulin was lower than 0.5, the exchange kinetics were biphasic. An initial approximate analysis indicated that the overall process could be described by the sum of two exponential processes of rate constants 0.1 s^{-1} and 0.006 s^{-1}. The amplitude of the rapid phase decreased, while that of the slow phase increased, upon increasing the concentration of stathmin. These observations are consistent with the view that tubulin is in slow association−dissociation equilibrium with stathmin compared to the nucleotide exchange rate. Therefore, tubulin and tubulin−stathmin complexes exchange their bound nucleotides such as two independent, noninteracting species. If GDP was nonexchangeable in the T_2S complex, and dissociation of GDP from stathmin-bound tubulin occurred only via dissociation of the complex followed by GDP dissociation from tubulin, then the results would be different, i.e., the rate of exchange would tend toward zero upon increasing stathmin concentration, since the concentration of free tubulin able to release GDP would tend toward zero at high stathmin concentration. In conclusion, the slow rate of nucleotide exchange at saturation of tubulin by stathmin represents the rate of GDP dissociation from stathmin-bound tubulin, not the dissociation of the complex. The biphasic nature of the exchange kinetics at substoichiometric amounts of stathmin also argues for a slower dissociation of the T_2S complex. The slow dynamics of the T_2S complex is consistent with its high stability and is in agreement with analytical ultracentrifugation studies (*14*). Because the T_2S complex is likely to have an asym-

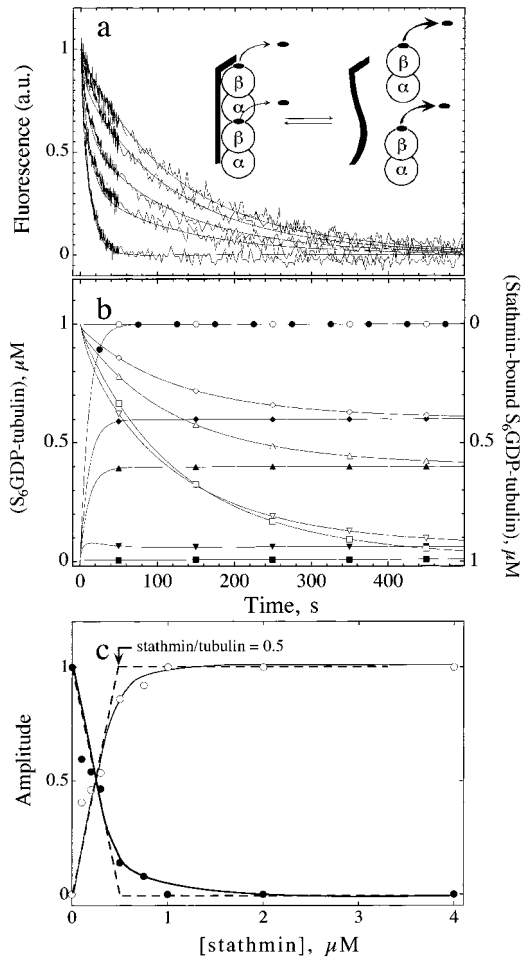


FIGURE 1: Stathmin slows down GDP dissociation from tubulin. A solution of GDP-tubulin 1:1 complex (1 μM final concentration) containing stathmin (at 2, 0.5, 0.3, 0.2, and 0 μM, top to bottom curves) in P buffer at 25 °C was mixed in the stopped-flow apparatus with S6-GDP (5 μM final concentration). (a) Noisy curves represent the recorded time courses of change in tryptophan fluorescence linked to GDP dissociation. The change in fluorescence is normalized to 1.0. Thin smooth curves represent the fitted curves from the model drawn in the figure for the T_2S complex (in which stathmin is represented as an elongated molecule), and the relevant equations described under Materials and Methods, with the following values of the rate parameters. $k_+ = 3\ \mu M^{-2}\cdot s^{-1}$, $k_- = 8 \times 10^{-4}\ s^{-1}$, $k_{-1} = 9 \times 10^{-2}\ s^{-1}$, $k_{-21} = 10^{-2}\ s^{-1}$, and $k_{-22} = 5 \times 10^{-3}\ s^{-1}$. (b) The model fitting the data shown in panel a was used to calculate the time dependence of the concentration of S6-GDP-bound free tubulin (TN, rapid phase, closed symbols, left ordinate scale) and of the stathmin-bound complexes of S6-GDP-tubulin ($TnTNS + TNTnS + 2TN_2S$, slow phase, open symbols, right ordinate scale) at the following concentrations of stathmin (micromolar): circles, 0; diamonds, 0.2; triangles, 0.3; upside-down triangles, 0.5; squares, 2. (c) Dependence of the amplitudes of the rapid phase, corresponding to GDP dissociation from free tubulin (●), and of the slow phase, representing GDP dissociation from stathmin-bound tubulin (○), derived from panel b.

metric structure (*16*), one can wonder how different the rate constants for GDP dissociation from each of the two tubulins can be and how large this potential difference should be to be measured in our assay. Simulation of the kinetics of nucleotide exchange (see Materials and Methods) allowed us to reject a few extreme cases. If only one of the two bound nucleotides in T_2S dissociated with rate constant k_{-21}, while the second would dissociate only via dissociation of the T_2S complex (i.e., $k_{-22} = 0$), then the kinetics would display half-of-the-site dissociation. This was not observed. On the other hand, if the rates of dissociation of the two GDP in T_2S differed by at least 5-fold while remaining both higher than the rate of dissociation of the complex, the exchange kinetics would remain biphasic at high stathmin:tubulin ratios. Clearly this does not correspond to the real case.

Table 1: Effect of Stathmin on Nucleotide Dissociation from Tubulin under Different Ionic Conditions[a]

ionic conditions	tubulin species	displaced nucleotide	rate constant (s^{-1})
P buffer	T	GDP	0.10 ± 0.02
P buffer	T_2S	GDP	0.006 ± 0.001
P buffer	T	GTP	0.012 ± 0.002
P buffer	T_2S	GTP	0.005 ± 0.001
P buffer + 5 mM $MgCl_2$	T	GDP	0.35 ± 0.05
P buffer + 5 mM $MgCl_2$	T_2S	GDP	0.02 ± 0.005

[a] Experiments were performed at 25 °C in P buffer modified as indicated. Tubulin (1 μM) was either free (T) or in complex with stathmin (T_2S). GTP was displaced from tubulin by S6-GTP, and GDP by S6-GDP.

With the model described under Materials and Methods, the experimental curves were well accounted for (see Figure 1a), with the same value of $7 \times 10^{-3}\ s^{-1}$ for k_{-21} and k_{-22} and a 10-fold lower value of the rate of T_2S complex dissociation ($k_- = 8 \times 10^{-4}\ s^{-1}$). However, simulations showed that the accuracy of our data would well accommodate values of k_{-21} and k_{-22} differing by 3-fold at most, provided that $(k_{-21} + k_{-22})/2 = 7 \times 10^{-3}\ s^{-1}$. If the dissociation rate constant of the T_2S complex is of the order of $10^{-3}\ s^{-1}$, then the value of k_+, the global association rate constant for formation of the T_2S complex, is determined by adjusting the span of the exchange curves in the range of concentrations of stathmin covering formation of T_2S complex. Values of k_+ of $3 \pm 1\ \mu M^{-2}\ s^{-1}$ provided a good fit to the ensemble of the curves. The model and the values of parameters used in the fit were used to calculate the change in the concentrations of free and stathmin-liganded tubulin in the S6-GDP bound form during the exchange reaction (Figure 1b). Nucleotide exchange appears to take place at very different rates on these two species, accounting for the biphasic nature of the process (Figure 1c).

In conclusion, the rate of GDP dissociation from the two tubulins bound to stathmin is lowered to comparable extents despite the structural asymmetry of the complex, and dissociation of the T_2S complex is slower than nucleotide dissociation from the complex.

The dissociation of GTP from tubulin in the absence and presence of stathmin was studied in similar experiments. Bound GTP was displaced by S6-GTP. The rate of GTP dissociation was also slowed by stathmin, to the same value as the one found for GDP dissociation from T_2S, $0.005\ s^{-1}$. Hence the effect of stathmin was less pronounced on GTP than on GDP dissociation. Finally, stathmin also slowed GDP dissociation about 20-fold when the concentration of magnesium ions was increased. Values of the dissociation rate constant of GDP from tubulin and from T_2S complex under a variety of conditions are summarized in Table 1.

Regulation of Stathmin Interaction with Tubulin by Phosphorylation. The fact that stathmin slows down nucleotide exchange on tubulin can be used as a probe to investigate how phosphorylation of stathmin affects its interaction with tubulin.

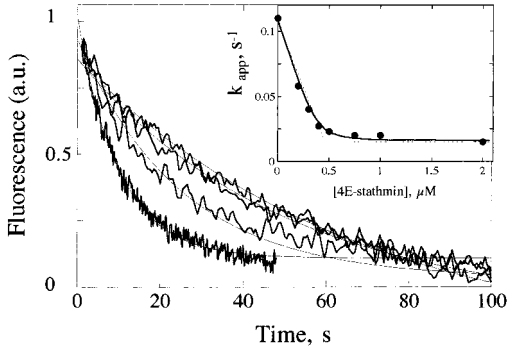


FIGURE 2: Effect of 4E-stathmin on GDP dissociation from tubulin. Experimental conditions are as described for Figure 1. The concentrations of 4E-stathmin are (bottom to top) 0, 0.3, 1, and 2 μM. Smooth lines represent the best fit to a single first-order process, whose rate constant k_{app} varies with stathmin concentration (inset). The dashed line in the inset represents the high-affinity titration curve.

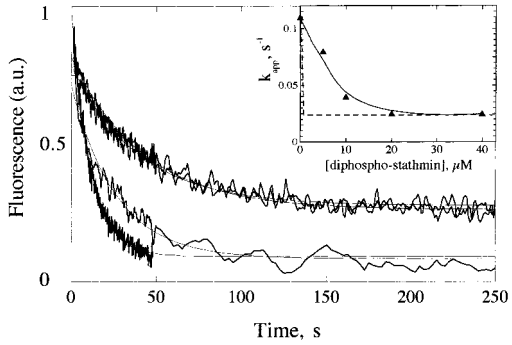


FIGURE 3: Effect of diphosphostathmin on GDP dissociation from tubulin. The experiment was conducted and analyzed as described under Figure 2. The concentrations of diphosphostathmin are (bottom to top) 0, 10, 20, and 40 μM. (Inset) Dependence of the apparent dissociation constant k_{app} on the concentration of diphosphostathmin. The dashed line represents the titration curve expected for a high-affinity T_2S complex.

The rate of GDP dissociation from tubulin was assayed in the presence of the 4E mutant of stathmin (S16/25/38/63E) and of the diphosphostathmin (phosphorylated on serines 16 and 63 by PKA, see Materials and Methods). The 4E-stathmin slowed GDP dissociation from tubulin less than wild type stathmin (Figure 2). A 7-fold decrease in GDP dissociation rate was reached at saturation by the mutated stathmin. Seventy percent of the maximum effect was reached at a 0.5 molar ratio of 4E-stathmin to tubulin. At molar ratios lower than 0.5, the time courses did not display a clear biphasic behavior and were simply accounted for by a monoexponential process.

Diphosphostathmin slowed GDP dissociation from tubulin only 4-fold at saturation (superimposable exchange kinetics were obtained at 20 and 40 μM diphosphostathmin). At all stathmin:tubulin molar ratios, the exchange kinetics were analyzed in terms of monoexponentials. The half-effect on the exchange rate was observed at 8.5 μM diphosphostathmin (Figure 3).

In conclusion, 4E-stathmin and diphosphostathmin interact with tubulin more weakly than unphosphorylated stathmin. The stability of the complex is lowered to a greater extent by the phosphorylation of serines 16 and 63 than by the serine to glutamate mutation. The fact that exchange kinetics are simple exponentials, the rate constant of which varies with saturation by modified stathmin, indicates that, in contrast to stathmin, mutated and phosphorylated stathmin are in rapid equilibrium with tubulin. Second, the structural/biochemical properties of the tubulin−stathmin complex are affected by either phosphorylation or serine to glutamate mutation of the regulatory serines.

Effect of Phosphorylation on the Hydrodynamic Properties of Stathmin. To get a deeper insight into the effect of phosphorylation on the structure and activity of stathmin, the hydrodynamic properties and the tubulin-sequestering activity of the different stathmin variants were examined. In the presence of substoichiometric amounts of stathmin (stathmin:tubulin < 0.5), the sedimentation velocity of tubulin displayed bimodal distributions of sedimentation coefficients (*14*). This behavior can arise either in the case of high-affinity complexes in rapid equilibrium or in the case of kinetically controlled ligand-mediated dimerization reactions (*31*). In contrast, the sedimentation coefficients of tubulin in the presence of 4E-stathmin or diphosphostathmin displayed a monomodal distribution (Figure 4a,b). In addition, tubulin samples sedimenting in parallel in the presence of a saturating concentration of stathmin or 4E-stathmin exhibited detectably different sedimentation coefficients. With wild-type stathmin, the sedimentation coefficient of the T_2S complex was 7.7 S even at concentrations as high as 50 μM stathmin, while a higher limit of 7.3 S was observed at 30 and 50 μM 4E-stathmin. The 4E-stathmin concentration dependence of the sedimentation coefficient was consistent with the formation of a ternary T_2S complex.

In the presence of diphosphostathmin, the apparent sedimentation coefficient of tubulin increased with the concentration of diphosphostathmin in a saturating fashion, and the value of the sedimentation coefficient at saturation was not reached at 50 μM diphosphostathmin (Figure 4c). However, the same limit value of 7.3 S was derived from double-reciprocal plots of the data (not shown).

In conclusion, ternary T_2S complexes are formed between tubulin and all stathmin variants, but the structures of the T_2S complexes are somewhat different, probably less compact, when stathmin is mutated or phosphorylated. The T_2S complexes formed with 4E- or diphosphostathmin are in rapid equilibrium, which suggests that the affinity of stathmin for tubulin is lowered by phosphorylation or by the serine to glutamate mutation. Figure 4c shows that half-saturation of 5 μM tubulin was reached at 1.5 μM 4E-stathmin (corresponding to 0.25 μM free 4E-stathmin) and at 10−15 μM diphosphostathmin, which suggests, in agreement with Figure 3, that diphosphostathmin binds tubulin 2 orders of magnitude more weakly than 4E-stathmin. Substituting glutamate for serine therefore mimics phosphorylation qualitatively but not quantitatively.

In conclusion, the results of the hydrodynamic studies correlate well with the nucleotide exchange data. It was reported (*12*) that at pH 7.5 stathmin did not interact with tubulin and only induced catastrophes, presumably by interacting only with microtubule ends. The hydrodynamic properties of tubulin and its complexes with stathmin and diphosphostathmin were examined comparatively at pH 7.5. The sedimentation coefficient of tubulin alone was unchanged upon increasing pH from 6.8 to 7.5. Upon addition of 5 μM stathmin to 10 μM tubulin at pH 7.5, 80% of the material sedimented as a 7.6 S species, indicating that

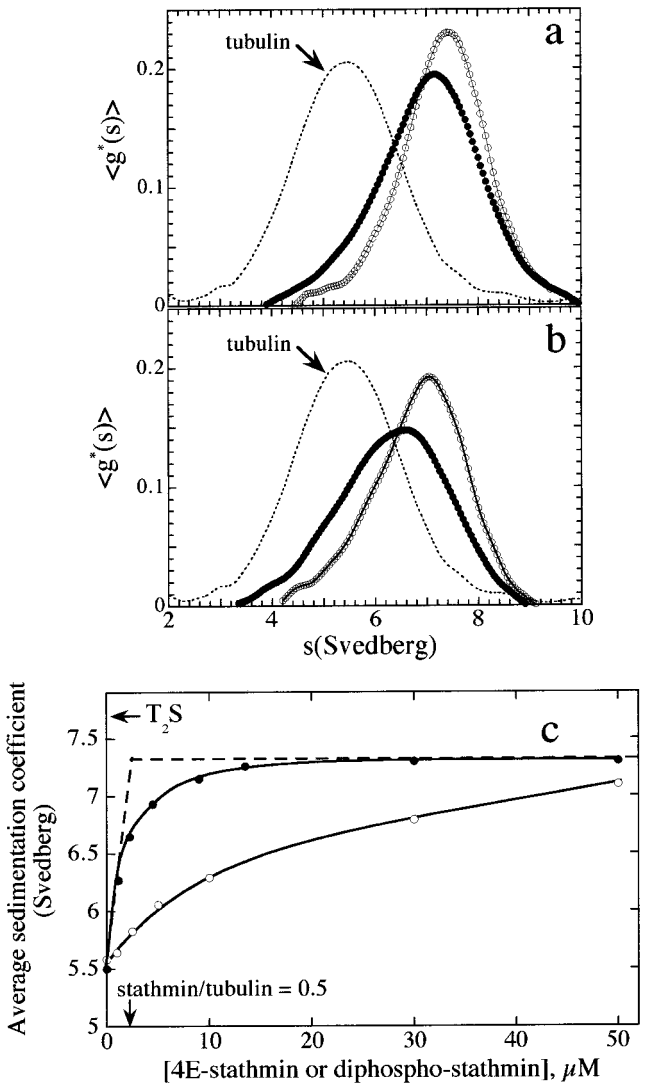


FIGURE 4: Hydrodynamic properties of the complexes of tubulin with 4E-stathmin and diphosphostathmin. The distributions of the sedimentation coefficients of tubulin (5 μM) in the absence (dashed line) and in the presence of 10 μM (●) or 30 μM (○) of 4E-stathmin (panel a) or diphosphostathmin (panel b). The experiment was carried out at 20 °C in P buffer. The weight-average sedimentation coefficient of the tubulin-containing species (panel c) was measured in the presence of 4E-stathmin (●) or diphosphostathmin (○) at the indicated concentrations. The dashed line is the titration curve that is expected for a high affinity complex of 4E-stathmin and tubulin.

stathmin formed the same high-affinity T_2S complex at pH 7.5 as at pH 6.8 (*14*). Because of the very high stability of the T_2S complex, this technique, which is used in the 10^{-5} M concentration range, fails to detect a change in stability upon raising the pH (see Discussion). On the other hand, upon addition of 5 μM diphosphostathmin to 10 μM tubulin, the sedimentation coefficient of tubulin remained unchanged at pH 7.5, while it increased to a value of 6.4 at pH 6.8, consistent with the data obtained at 5 μM tubulin shown in Figure 4. These results indicate that the affinity of diphosphostathmin for tubulin, which is already low at pH 6.8, is further lowered upon increasing pH.

DISCUSSION

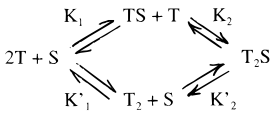
The kinetics of nucleotide exchange on tubulin have been monitored by the tryptophan fluorescence change associated with the replacement of bound GDP or GTP by S6-GDP or S6-GTP (*26*, *27*). The value of 0.1 ± 0.02 s^{-1} found here for the dissociation of GDP from dimeric tubulin at 25 °C in P buffer is in agreement with earlier data obtained by Engelborghs and Eccleston (*32*) using the same method; however, in those times the observed reaction had been attributed to the association of S6-GTP with tubulin. The result also agrees with the value of 0.14 s^{-1} reported by Brylawski and Caplow (*30*), who used an independent method. On the other hand, a 10-fold lower rate constant (0.015 s^{-1}) was found by Yarbrough and Fishback (*27*), in similar conditions except for the presence of 25% glycerol in the buffer. We checked (data not shown) that the presence of glycerol slows down nucleotide dissociation from tubulin. We find that GTP dissociates from tubulin at a 10-fold slower rate than GDP, consistent with the reported difference in affinity of GTP and GDP for tubulin exchangeable site in the presence of 1 mM magnesium ions (*33*).

In vitro, stathmin interacts with two molecules of $\alpha\beta$-tubulin in a highly stable ternary complex, T_2S. The rate of dissociation of GDP bound to each of the two β-tubulins is decreased 20-fold in the T_2S complex. Although the individual rates of GDP dissociation from each of the two tubulins in T_2S cannot be distinguished, modeling of the data indicates that the two rate constants k_{-21} and k_{-22} could possibly differ by up to 3-fold. This result is consistent with the available hydrodynamic and structural studies of the T_2S complex. Electron microscopy (*16*) shows that stathmin binds along a polarized dimer of two $\alpha\beta$-tubulins linked by the conventional longitudinal $\alpha\beta$ bond and caps one end of this $\alpha\beta\alpha\beta$ dimer, presumably the β end, thus preventing GDP dissociation from the two tubulins. Our data are more supportive of this model than of the one recently proposed on the basis of the cross-linking of stathmin to the α subunit, which places the two $\alpha\beta$-tubulins of the complex in a head-to-head configuration, the two β subunits being at the two ends of the dimer, in a position where nucleotide dissociation would not be hampered (*34*). The change in the effect on nucleotide dissociation associated with phosphorylation or mutation of the serines suggests that it is the N-terminal region of stathmin, which contains the regulated serines, that caps the β-end of the complex. One should note that although the two $\alpha\beta$-tubulin heterodimers in T_2S interact head-to-tail, in a fashion reminiscent of the interaction of tubulin subunits in a microtubule protofilament or a ring, the exchange of nucleotide in the stathmin-induced $\alpha\beta-\alpha\beta$ dimer is less hampered than in those more associated forms of tubulin, maybe because the interface between the two tubulins in the dimer is less constrained than in a more rigid protofilament or ring. Accordingly, stathmin enhances GTP hydrolysis on tubulin−colchicine (Carlier, unpublished observations), which implies the possibility of turnover of the nucleotide in the site when stathmin is bound.

It is not known whether in vivo stathmin is bound to GDP-tubulin; however, if this is the case, the present work indicates that the exchange of GTP for bound GDP, which is a required step for tubulin polymerization, would be greatly delayed unless the tubulin−stathmin complex dissociates rapidly, presumably upon phosphorylation of stathmin.

Nucleotide exchange can be used as a probe of the stability of the complexes of tubulin with stathmin and its derivatives. The biphasic kinetics of dissociation of GDP at stathmin:

tubulin ratios lower than 0.5 indicates that the exchange rate between stathmin and tubulin in the complex is slower than the dissociation of GDP from the complex, which implies that the rate constant for dissociation of T_2S has a value of 10^{-3} s^{-1} or less. The slow dynamics of this complex is consistent with its sedimentation behavior in the analytical ultracentrifuge (*14*). Modeling of the data provides a rough estimate of the thermodynamic parameters for stathmin interaction with tubulin. The equilibrium between stathmin and tubulin can be described by the following scheme within which two tubulin (T) molecules bind to stathmin (S):



The system is described by the following equilibrium dissociation constants:

$$K_1 = [T][S]/[TS]$$

$$K_1' = [T]^2/[T_2]$$

$$K_2 = [TS][T]/[T_2S]$$

$$K_2' = [T_2][S]/[T_2S]$$

Two isoenergetic pathways ($K_1K_2 = K_1'K_2'$) are defined by this square model. The TS or T_2 intermediate complexes are assumed to have a low stability and to be in rapid equilibrium with T + S or T + T, respectively. The T_2S complex is the only highly stable complex, i.e., $K_2 \ll K_1$ and $K_2' \ll K_1'$. The slow step in the dissociation of T_2S may be either dissociation of tubulin (k_{-2}) or dissociation of stathmin (k_{-2}') from T_2S, followed by the very rapid dissociation of the TS or T_2 intermediate. Whichever rate, k_{-2} or k_{-2}', is predominant in the overall process must be slow (<0.001 s^{-1}) for unmodified stathmin and faster (>0.1 s^{-1}) for mutated or phosphorylated stathmin. For unmodified stathmin, the good fit of the model implies that the global association rate constant for T_2S formation, k_{+2}/K_1, is in the range of 1−4 $\mu M^{-2}\cdot s^{-1}$. Hence the value of the global equilibrium dissociation constant of the T_2S complex, $K_1K_2 = [T]^2[S]/[T_2S]$, lies in the range of $(2-5) \times 10^{-4}$ μM^2 according to the present data. For 4E-stathmin, at 1 μM tubulin and 0.5 μM 4E-stathmin, 70% of the tubulin is in complex, which implies a value of K_1K_2 of about 0.1 μM^2, 3 orders of magnitude higher than for unmodified stathmin. For diphosphostathmin, 50% of tubulin is in complex at 8.5 μM diphosphostathmin, corresponding to a value of K_1K_2 of 16 μM^2, 5 orders of magnitude higher than for unmodified stathmin. The analytical ultracentrifugation experiments are carried out in a range of higher tubulin and stathmin concentrations, which of course is less suitable for evaluating the different binding parameters of the stathmin variants; however, the results qualitatively confirm that 4E-stathmin and diphosphostathmin interact with tubulin more weakly than stathmin, and the effect of phosphorylation on stathmin−tubulin interaction is more severe than the effect of the serine to glutamate mutation. The rate of GDP dissociation is decreased to a lower extent in those complexes, suggesting that they do not have the exact same conformation as the high-affinity T_2S complex, and their sedimentation coefficient is lower (7.3 S) than the one measured for the T_2S complex (7.7 S), consistent with a less compact structure.

Adequate methods are being developed to quantitate the tubulin−tubulin and tubulin−stathmin interactions in the T_2S complex (Amayed et al., manuscript in preparation). Remarkably, the stability of the T_2S complex that emerges from the present solution studies is much higher than estimates provided by other methods in which stathmin was bound to a bead as a GST fusion protein (*35*) or to a sensor chip (*15*). The reason for these discrepancies remains unclear. It is possible that in those methods a single 1:1 tubulin−stathmin complex was formed, and the second tubulin binding site was hindered due to the attachment of stathmin to a solid matrix. The appreciable differences in stabilities of the different complexes of tubulin with wild-type or mutated or phosphorylated stathmin found here correlate well with their reported different activities in vivo and are consistent with their in vitro tubulin sequestering activities, which are measured under conditions where the different stathmin species are saturated by tubulin. It has been argued (*20*) that the tubulin binding activities of the 4E mutant and a coiled-coil mutant of stathmin were comparable while their abilities to destabilize microtubules were strikingly different, leading to the conclusion that the function of stathmin is not linked to its ability to bind tubulin in a phosphorylation-regulated fashion. However, those binding studies were conducted at a high concentration of free tubulin (10−20 μM). It is not surprising that, at such a high concentration of free tubulin, a potential difference in stability of the different complexes could not be detected. Moreover, correlating the binding data with differences in in vivo activities of the different stathmin variants appears inappropriate since in vivo the complexes are at equilibrium with an unknown but probably different concentration of free tubulin.

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REFERENCES

1. Mitchison, T. J., and Kirschner, M. W. (1984) *Nature 312*, 237−242.
2. Carlier, M.-F., Hill, T. L., and Chen, Y. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 771−775.
3. Carlier, M.-F., Melki, R., Pantaloni, D., Hill, T. L., and Chen, Y. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5257−5261.
4. Melki, R., Carlier, M.-F., and Pantaloni, D. (1988) *EMBO J. 7*, 2653−2659.
5. Walczak, C. E. (2000) *Curr. Opin. Cell Biol. 12*, 52−56.
6. Tournebize, R., Popov, A., Kinoshita, K., Ashford, A. J., Rybina, S., Pozniakovsky, A., Mayer, T. U., Walczak, C. E., Karsenti, E., and Hyman, A. A. (2000) *Nat. Cell Biol. 2*, 13−19.
7. Sobel, A. (1991) *Trends Biochem. Sci. 16*, 301−305.
8. Belmont, L. D., and Mitchison, T. J. (1996) *Cell 84*, 37−47.
9. Marklund, U., Larsson, N., Melander Gradin, H., Brattsand, G., and Gullberg, M. (1996) *EMBO J. 15*, 5290−5298.
10. Horwitz, S. B., Shen, H.-J., He, L., Dittmar, P., Neef, R., Chen, J., and Schubart, U. K. (1997) *J. Biol. Chem. 272*, 8129−8132.
11. Larsson, N., Marklund, U., Melander Gradin, H., Brattsand, G., and Gullberg, M. (1997) *Mol. Cell Biol. 17*, 5530−5539.
12. Howell, B., Larsson, N., Gullberg, M., and Cassimeris, L. (1999) *Mol. Biol. Cell 10*, 105−118.

13. Andersen, S. S. L., Ashford, A. J., Tournebize, R., Gavet, O., Sobel, A., Hyman, A. A., and Karsenti, E. (1997) *Nature 389*, 640−643.
14. Jourdain, L., Curmi, P., Sobel, A., Pantaloni, D., and Carlier, M.-F. (1997) *Biochemistry 36*, 10817−10821.
15. Curmi, P., Andersen, S. S. L., Lachkar, S., Gavet, O., Karsenti, E., Knossow, M., and Sobel, A. (1997) *J. Biol. Chem. 272*, 25029−25036.
16. Steinmetz, M. O., Kammerer, M. A., Jahnke, W., Goldie, K. N., Lustig, A., and Van Oostrum, J. (2000) *EMBO J. 19*, 572−580.
17. Nogales, E., Whittaker, M., Milligan, R. A., and Downing, K. H. (1999) *Cell 96*, 79−88.
18. Erickson, H. P., and Pantaloni, D. (1981) *Biophys. J. 34*, 293−309.
19. Lawler, S. (1998) *Curr. Biol. 8*, R212−R214.
20. Larsson, N., Segerman, B., Melander Gradin, H., Wandzioch, E., Cassimeris, L., and Gullberg, M. (1999) *Mol. Cell. Biol. 19*, 2242−2250.
21. Amayed, P., Curmi, P., Sobel, A., Pantaloni, D., and Carlier, M.-F. (1999) *Biophys. J. 76*, 42a.
22. Curmi, P., Maucuer, A., Asselin, S., Lecourtois, M., Chaffotte, A., Schmitter, J.-M., and Sobel, A. (1994) *Biochem. J. 300*, 331−338.
23. Melander Gradin, H., Larsson, N., Marklund, U., and Gullberg, M. (1998) *J. Cell Biol. 140*, 131−141.
24. Eccleston, J. F., and Trentham, D. R. (1977) *Biochem. J. 163*, 15−29.
25. Hoard, D. E., and Ott, D. G. (1965) *J. Am. Chem. Soc. 87*, 1785−1788.
26. Fishback, J. L., and Yarbrough, L. R. (1984) *J. Biol. Chem. 259*, 1968−1973.
27. Yarbrough, L. R., and Fishback, J. L. (1985) *Biochemistry 24*, 1708−1714.
28. Stafford, W. F., III (1992) *Anal. Biochem. 203*, 295−301.
29. Gilbert, L. M., and Gilbert, G. A. (1973) *Methods Enzymol. 27*, 273−296.
30. Brylawski, B. P., and Caplow, M. (1983) *J. Biol. Chem. 258*, 760−763.
31. Cann, J. R., and Kegeles, G. (1974) *Biochemistry 13*, 1868−1874.
32. Engelborghs, Y., and Eccleston, J. (1982) *FEBS Lett. 141*, 78−81.
33. Correia, J. J., Baty, L. T., and Williams, R. C. (1987) *J. Biol. Chem. 262*, 17278−17284.
34. Wallon, G., Rappsilber, J., Mann, M., and Serrano, L. (2000) *EMBO J. 19*, 213−222.
35. Larsson, N., Segerman, B., Howell, B., Fridell, K., Cassimeris, L., and Gullberg, M. (1999) *J. Cell Biol. 146*, 1289−1302.

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