FLUORESCENCE STOPPED-FLOW STUDY OF THE BINDING ØF S6-GTP TO TUBULIN

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1. Introduction

Microtubule assembly is accompanied by the hydrolysis of stoichiometric amounts of the nucleotide GTP [1,2]. Hydrolysis is therefore linked to the steady state rate of tubulin exchange. Only in conditions far from the steady state can the association step be faster than nucleotide hydrolysis [3]. The coupling of nucleotide hydrolysis to assembly allows a process of treadmilling to occur, as shown for actin [4] and microtubules [5]. In such a system at steady state, a net growth at one end is compensated by a net dissociation at the opposite end. The efficiency of this treadmilling depends on the nucleotide composition of at least one end [6,7].

The binding of the nucleotide to the exchangeable site on tubulin has been much studied (review [8]). The dissociation constant was found to be 6.1×10^{-8} M for GDP and 2.2×10^{-8} M for GTP, respectively [9]. Kinetics of nucleotide exchange have been studied at low temperature, where the rates are very slow: The ringlike oligomers under these conditions do not bind the nucleotide directly, but exchange dimers with bound nucleotide [10,11]. The estimated half-lives for exchange range from 20 min [10] to 90 min [11] at 0°C. At 35°C the value could not be determined, but was expected to be within a minute, on the basis of the extrapolation of the Arrhenius plot [11]. Using a coupled enzyme system, an off-rate constant of 0.01 s^{-1} at 40°C was determined [12].

Here, we report the results of a fluorescence stopped-flow study using the GTP analog S6-GTP. This nucleotide has an absorbance around 320–340 nm, depending on pH [13], which overlaps quite well with the fluorescence emission of the Trp residue in tubulin. A fluorescence decrease can therefore be expected when the nucleotide binds to tubulin, due to the possibility of radiationless energy transfer. Using the stopped-flow, it was possible to follow the fast reactions at 25 and 35°C. During the course of this study a dissociation constant of 1.7×10^{-8} M for S6-GTP and 8.3×10^{-8} M for S6-GDP was published in an abstract [14].

2. Materials and methods

Microtubules were purified from pig brain homogenates according to [15] as modified [16]. Glycerol was used only in the first polymerization to increase the yield. This preparation contained ~15% of microtubule-associated proteins. Pure tubulin was prepared by phosphocellulose chromatography [17]. Its quality was checked by the absence of polymerization in assembly buffer and the presence of a cold reversible polymerization in 10% dimethyl sulfoxide. Protein concentration was determined as in [18] using bovine serum albumin (Serva) as standard. All experiments were done in Mes buffer at pH 6.4, ionic strength 0.1 M with the following composition: 50 mM morpholinoethanesulfonic acid, 70 mM KCl, 1.0 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM NaN_3. Microtubule protein was stored in this buffer with 1 mM GTP in liquid nitrogen.

To remove the free nucleotides, microtubule protein was passed (eventually twice) down a Sephadex G-25 column, and the solution was again stored in liquid nitrogen. The tubulin–colchicine complex was made by incubating microtubule protein with 2 mM colchicine (Aldrich Chemicals) for 20 min at 30°C

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then passing the solution down a Sephadex G-25 column to remove the free colchicine. The solution to be used was thawed only just prior to the stopped flow experiments. In this way ageing of the sample was minimized. Mercaptoethanol was added up to 14 mM to prevent oxidation of the SH-groups. The ionic strength was eventually adjusted to 0.2 M with KCl to dissociated the rings [19].

Nucleotide S6-GTP was prepared as in [20].

Details about the stopped-flow instrument are in [21]. Excitation was done at 280 nm with a bandwidth of 10 nm, and emission was observed using a cut-off filter at 300 nm (Hoya, UV-30). The data are stored in a Gould transient recorder and transmitted to a PDP 11/34 computer via a simple interface [22].

3. Results

When pure tubulin is mixed with S6-GTP a first-order decrease of protein fluorescence is observed (fig.1). The rate constant is dependent on the concentration of S6-GTP in a rather unexpected way; it decreases with increasing nucleotide concentration and levels off at high concentrations (fig.2). This limiting rate constant depends on temperature (see table 1) and on the ionic strength.

With the microtubule protein mixture, an analogous behaviour is observed at an ionic strength of 0.1 M, except for a second and very slow phase. This was studied separately in the spectrofluorimeter.

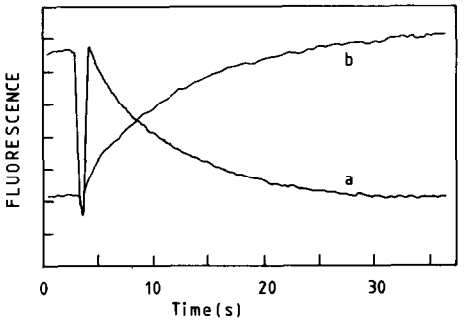

Fig.1. (a) Fluorescence decrease (arbitrary units) observed when microtubule protein is mixed with S6-GTP. Excitation was done at 280 nm, while emission was observed using a cut-off filter at 300 nm. (b) Fluorescence increase due to the displacement of bound S6-GTP by GTP. Both processes are single first-order processes. Of the 1000 words stored and used for calculations, only 200 (every 5th) are plotted in the graph.

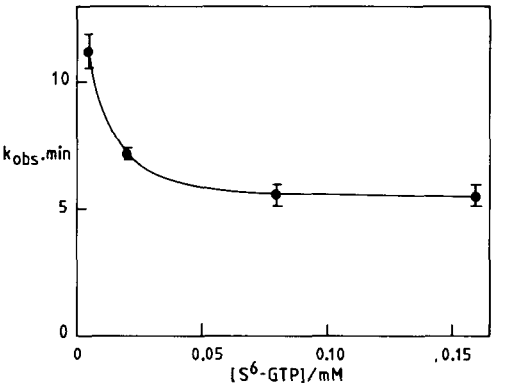

Fig.2. Dependence of the observed first-order rate constant on [S6-GTP]. At high [S6-GTP] the lower limiting rate constant (k_{+1}) is found. At lower concentrations the observed rate constant increases. This suggests a pre-existing conformational equilibrium (see text).

Table 1
Observed rate constants for binding and displacement reactions in the different conditions used

Protein	Nucleotide	k_{+1}[a]	k_{-2}[a]	T (°C)
Tubulin	S6-GTP	7.3	5.2	25
I = 0.1		13.8	10.0	35
	GTP		7.0	25
			9.0	35
MTP[b]	S6-GTP	8.8	5.7	25
I = 0.1		12.7	9.1	35
	GTP		3.0	25
			6.3	35
	GDP		5.2	25
			7.5	35
Tubulin	S6-GTP	5.0	4.4	25
I = 0.2		7.8	7.6	35
	GTP		6.0	25
MTP	S6-GTP	5.5	2.6	25
I = 0.2		11.1	5.6	35
	GTP		1.9	25
			6.0	35
MTP-Col	S6-GTP	14.4	6.8	25
I = 0.2		17.8	11.0	35
	GTP		5.1	25
			7.0	35

[a] In min^{-1}, with a standard deviation ~8%
[b] Polymerization prevented with 2 mM $CaCl_2$

Abbreviations: MTP, microtubule protein; MTP-Col, stable complex of microtubule protein and colchicine; I, ionic strength

When S6-GTP was mixed with the microtubule protein mixture at an ionic strength of 0.2 M, however, the second slow phase was not observed.

Similar results are obtained with tubulin–colchicine, although the observed rate constants are larger.

Displacement reactions normally allow the determination of the off-rate constants for bound ligands [23]. This can be applied to S6-GTP as well as GTP and GDP. The rate constants observed in displacement reactions are slower, than the association rate constants determined in the same conditions of temperature and ionic strength.

Control experiments show that no signal is obtained when S6-GTP is mixed with tubulin in the presence of a large excess of GTP.

4. Discussion

The fact that the observed rate constant upon binding of S6-GTP to tubulin, does not increase with the concentration of S6-GTP proves that the fluorescence decrease is linked to a conformational change. It is clear that the displacement of GTP or GDP show rate constants that differ from the association reactions, so that insufficient removal of bound nucleotides cannot be considered as the cause of the limited concentration dependence observed. Even a second passage of the protein down a G-25 column did not change the concentration dependence. This phenomenon can, however, be explained with a mechanism based on a pre-existing conformational equilibrium [24]:

$$T \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} T^* + \text{S6-GTP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} T^*\text{–S6-GTP}$$

For such a mechanism a first fast process is predicted for direct binding of S6-GTP to the tubulin initially present in the T* conformation. The following rate constant applies:

$$k_{obs} = k_{+2}\ [\text{S6-GTP}] + k_{-2} \qquad (1)$$

Indications for a contribution of such a phase were present, but due to a very small amplitude and a high rate, the rate constants could not be determined.

A second process is predicted for re-equilibration of the conformational equilibrium, with a rate constant:

$$k_{obs} = k_{-1}\ (1 + K_2\ [\text{S6-GTP}])^{-1} + k_{+1} \qquad (2)$$

Therefore at high [S6-GTP], the observed rate constant is essentially k_{+1}, while at lower concentrations, relation (2) holds and a higher rate constant can be found. In our solutions of low [S6-GTP], the pseudo first-order condition breaks down and the simple equation (2) can no longer be used. An increase is, however, observed. For microtubule protein at 25°C and 0.2 M ionic strength, the value of $k_{+1} = 5.5\ \text{min}^{-1}$ is deduced from the limiting rate at high [S6-GTP]. At the [S6-GTP] concentration used, $k_{obs} = 12\ \text{min}^{-1}$ was found. Therefore, $k_{-1} \gg 6.5\ \text{min}^{-1}$ and $K_1 \ll 1$.

Displacement reactions allow the determination of k_{-2}: $2.6\ \text{min}^{-1}$ is found for S6-GTP. Using the relation for the overall affinity (K_{app}):

$$K_{app} = K_2/(1 + K_1^{-1}) = 5.9 \times 10^7\ \text{M}$$

together with the value of k_{-2}, an estimation can be made of $k_{+2} \gg 2.5 \times 10^6\ \text{M}^{-1}\ \text{s}^{-1}$. Such a high rate constant is not unreasonable for the binding of small molecules to proteins and explains why a fast phase is not observed.

With pure tubulin similar results are obtained both at an ionic strength of 0.1 and 0.2 M.

That the fast step found when S6-GTP binds to the microtubule protein mixture has the same rate constant as in the case of pure tubulin, indicates that it is due to the binding of S6-GTP to the tubulin dimers. The subsequent slow step found in these conditions, is interpreted as due to the slow exchange of the rings. Indeed, the slow phase is not observed at 0.2 M ionic strength where the rings are broken down. However, it is surprising that the slow phase is much slower (half-life ~ 5 min) than expected on the basis of the protein exchange rates observed at 35°C, using radioactive tubulin [11].

The conformational change upon nucleotide binding is sped up by the presence of colchicine bound to the protein, showing that tubulin is in a different conformational state after colchicine binding, as concluded from binding kinetics [21,25]. Colchicine binding strengthens the intersubunit bond of tubulin [26], increases the affinity of the complex for the microtubule end as compared to tubulin [27] and induces GTPase activity [28].

The displacement experiments allow the determination of the off-rate k_{-2} constants for all the differ-

ent nucleotides. These rate constants show that nucleotide exchange can be relatively slow even in tubulin dimers. Displacement reactions with the microtubule protein mixture at an ionic strength of 0.1 M also show a slow phase, attributed to protein exchange with the rings.

If the pre-existing conformational equilibrium is due to the protein, it is to be expected that GTP binding itself would also change some conformational parameters. In fact, a small fluorescence decrease was observed with GTP binding, but it was rather difficult to measure.

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References

[1] Weisenberg, R. C. and Deery, W. J. (1976) Nature 263, 792–793.
[2] David-Pfeuty, T., Laporte, J. and Pantaloni, D. (1978) Nature 272, 282–284.
[3] Carlier, M. F. and Pantaloni, D. (1981) Biochemistry 20, 1918–1924.
[4] Wegner, A. (1976) J. Mol. Biol. 108, 139–150.
[5] Margolis, R. L. and Wilson, L. (1978) Cell 13, 1–8.
[6] Engelborghs, Y. and Van Houtte, A. (1981) Biophys. Chem. 14, 195–202.
[7] Carlier, M. F. and Pantaloni, D. (1982) Biochemistry in press.
[8] Jacobs, M. (1979) in: Microtubules (Roberts, R. and Hyams, J. eds) pp. 225–278, Academic Press, London, New York.
[9] Jameson, L. and Caplow, M. (1980) J. Biol. Chem. 255, 2284–2292.
[10] Zeeberg, B., Cheek, J. and Caplow, M. (1980) Biochemistry 19, 5078–5086.
[11] Pantaloni, D., Carlier, M. F., Simon, C. and Batelier, G. (1981) Biochemistry 20, 4709–4716.
[12] Jacobs, M., Smith, H. and Taylor, E. W. (1974) J. Mol. Biol. 89, 455–468.
[13] Trentham, D., Eccleston, J. F. and Bagshaw, C. R. (1976) Quart. Rev. Biophys. 9, 217–282.
[14] Yarbrough, L. R., Fishback, J. and Colen, A. H. (1981) Fed. Proc. FASEB 40, 1548.
[15] Shelanski, M. L., Gaskin, F. and Cantor, C. R. (1973) Proc. Natl. Acad. Sci. USA 70, 765–768.
[16] Engelborghs, Y., De Maeyer, L. C. M. and Overbergh, N. (1977) FEBS Lett. 80, 81–85.
[17] Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y. and Kirschner, M. W. (1975) Proc. Natl. Acad. Sci. USA 72, 1858–1862.
[18] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[19] Marcum, J. M. and Borisy, G. G. (1978) J. Biol. Chem. 253, 2825–2833.
[20] Eccleston, J. and Trentham, D. R. (1977) Biochem. J. 163, 15–19.
[21] Lambeir, A. and Engelborghs, Y. (1981) J. Biol. Chem. 256, 3279–3282.
[22] Hennau, F. and Ceuterick, F. (1981) Rev. Sci. Instrum. 52, 771–772.
[23] Gutfreund, H. (1974) in: Chemistry of Macromolecules, MTP Intl. Rev. Sci. Biochemistry ser. 1 (Gutfreund, H. ed) pp. 261–286, Butterworths, London; University Park Press, Baltimore MD.
[24] Pecht, I. and Lancet, D. (1977) in: Chemical Relaxation in Molecular Biology (Pecht, I. and Rigler, R. eds) Springer-Verlag, pp. 306–338, Berlin, New York.
[25] Garland, D. L. (1978) Biochemistry 17, 4266–4272.
[26] Detrich, H. W. iii, Williams, R. C. jr, Macdonald, T. L., Wilson, L. and Puett, D. (1981) Biochemistry 20, 5999–6005.
[27] Lambeir, A. and Engelborghs, Y. (1980) Eur. J. Biochem. 109, 619–624.
[28] David-Pfeuty, T., Simon, C. and Pantaloni, D. (1979) J. Biol. Chem. 254, 11696–11702.