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EXAMINATION OF TUBULIN-NUCLEOTIDE INTERACTIONS BY PROTEIN FLUORESCENCE QUENCHING MEASUREMENTS*

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Summary

The fluorescence emission spectrum of bovine brain tubulin is quenched upon binding of GTP, GMPP(NH)P, or GMPP(CH_2)P to the tubulin·GDP complex. At saturating levels of GTP or its nonhydrolyzable β-γ analogues, the partially quenched spectrum is virtually identical, suggesting that a similar conformational state is attained in each case. Titrations with each ligand yielded dissociation constants of 0.8, 3, and 3 μM for GTP, GMPP(NH)P, and GMPP(CH_2)P; in all cases the stoichiometry is essentially one molecule of nucleotide per dimer. It is concluded that GDP and GTP stabilize different conformations and that the nonhydrolyzable analogues mimic the binding of GTP. This may be related to the ability of GMPP(NH)P and GMPP(CH_2)P to maintain a pre-assembly conformation similar to tubulin·GTP.

Guanine nucleotides bind at two distinct sites on the bovine neurotubulin dimer (1). One site is exchangeable with added nucleotide, but the second site will not undergo exchange (2). Recent experiments in our laboratory (3) confirm the idea that GTP hydrolysis is an essential feature of the GTP-supported assembly of microtubules (4-6). Tubulin, charged with [γ-^{32}P]-GTP at the exchangeable site, undergoes assembly and concommitant hydrolysis. Removal of the so-called microtubule associated proteins (MAPs) fraction by phosphocellulose chromatography blocks both processes, and readdition of the MAPs restores both in a coordinated fashion. Nonetheless, the nonhydrolyzable analogues, GMPP(NH)P and GMPP(CH_2)P, will also support assembly (7-9), and the metabolic significance of hydrolysis is obscured by this observation. Tubules formed in the presence of these analogues have altered response to dilution and to calcium ion-induced depolymerization (9), and the nature of these changes in microtubule properties is a matter of considerable interest.

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To learn more about tubulin interactions with GTP and its nonhydrolyzable analogues, a study of attendant changes in the protein fluorescence emission spectrum upon ligand binding was undertaken. The observation that GTP binding to tubulin·GDP complex quenches the protein fluorescence by 25-28% permitted such an analysis. This report provides information that GTP (as well as GMPP(NH)P and GMPP(CH_2)P) and GDP stabilize different conformations. Data on the affinity and stoichiometry of GTP and analogue binding to tubulin was also obtained.

Experimental Procedures

Bovine brain tubulin was prepared by a modification of the Shelanski method (10) as described elsewhere (3). The purified microtubule protein was stored in 1-2 ml aliquots at -80°C. Immediately prior to use, the tubulin was purified by an additional polymerization/depolymerization cycle, followed by Sephadex G-25 filtration on a 1.5 x 50 cm column to remove uncomplexed guanine nucleotide. This procedure yields tubulin with GDP bound at the exchangeable nucleotide site (3). Protein concentration was determined by the method of Lowry et al. (11) with bovine serum albumin standards. All nucleotides and reagents were Sigma products except the GMPP(NH)P purchased from ICN, Inc.

The tubulin fluorescence (uncorrected) was measured in a thermally regulated chamber (37°) of a Hitachi-Perkin Elmer MPF-3 fluorescence spectrophotometer. Fluorescence titrations were performed by preparing 75 ml of a 1 x 10^{-7} M tubulin solution in GEMM buffer (100 mM glutamate, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8) at 4°. Additions of aliquots of 15.20 mM nucleotide solutions were made to the stock solution and 2.5 ml tubulin samples were withdrawn, warmed to 37°, and measured promptly (3-4 min.). Fluorescence quantum yields were recorded at excitation and emission wavelengths of 290 and 340 nm, respectively. During the course of each experiment, tubulin without added GTP or analogue was monitored to detect any time-dependent changes in tubulin stability, but none was detected.

Inner-filter effects, resulting from light absorption by guanine nucleotides, were minimized by exciting the protein at 290 nm, Corrections for the inner-filter effects were made by: 1. estimation of the fluorescence quenching upon addition of GTP to an L-tryptophan solution adjusted to yield a fluorescence intensity comparable to 10^{-7} M tubulin; 2. application of the method of Hélène (12) in which absorption data was applied; and 3. use of tubulin, boiled in 25% ethanol for 30 min. to achieve full denaturation, as in the first method. All three methods agreed to within ±5%, and it was observed that the inner-filtering by added nucleotide was never more than 10% under our experimental conditions.

Binding parameters were analyzed by standard Scatchard analysis (13) with the degree of saturation, $\bar{\nu}$, defined as the quotient of the extent of fluorescence quenching at a particular nucleotide level and the maximal fluorescence quenching in the presence of excess nucleotide such that tubulin is fully complexed. All data were fit by standard linear least squares analysis with a Hewlett-Packard 9825A calculator and 9862 plotter.

Results and Discussion

Addition of GTP, GMPP(CH_2)P or GMPP(NH)P to tubulin·GDP complex leads to a consistent decrease in the intensity of the uncorrected tubulin fluorescence spectrum. This spectral change is illustrated in Fig. 1 where approximately 55% of the exchangeable nucleotide sites are occupied by GMPP(NH)P. There is no apparent shift in spectrum (emission maximum at 323 nm), but the spectral

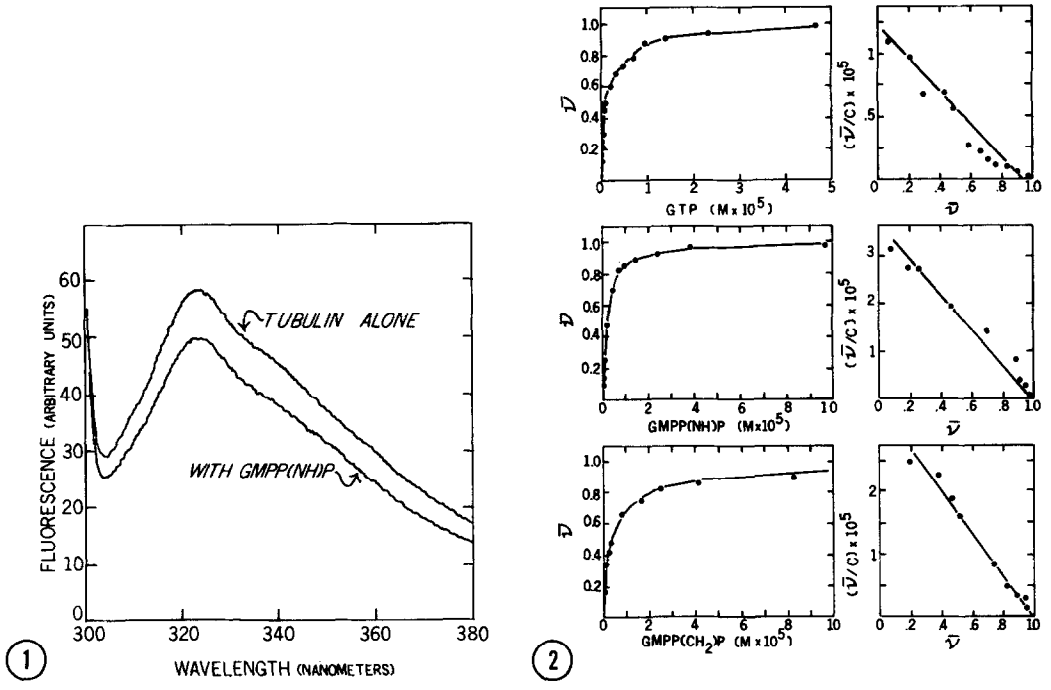

Figure 1 : Fluorescence spectra (uncorrected) of tubulin·guanine nucleotide complexes. Upper curve: 0.1 μM tubulin·GDP complex in GEMM buffer at 37°. Lower curve: same as above but with 3.5 μM GMPP(NH)P present.

Figure 2 : Plot of the increase in tubulin fluorescence quenching versus the total concentration of GTP, plot A; GMPP(NH)P, plot B; and GMPP(CH_2)P, plot C. At the right of each, the corresponding Scatchard type plot is presented, and the number of binding sites was estimated at 1.0, 0.93, and 1.03 for A, B, and C, respectively. Other details are given in the text.

intensity is quenched approximately 26-28% with GTP or its analogues. Addition of GDP (up to levels of 5 x 10^{-5} M) has essentially no effect on the fluorescence intensity, confirming that no conformational change occurs when added GDP merely exchanges with the tubulin·GDP complex. Likewise, in experiments not shown, it was possible to titrate the tubulin.GDP complex with GTP with attendant quenching and then to back titrate with GDP to partially reverse the fluorescence quenching. Inner-filtering effects at high GDP (> 5 x 10^{-5} M) precluded complete reversal of that quenching. It is noteworthy that similar results may be obtained with tubulin in the absence or presence of the MAPs fraction.

The occurrence of this fluorescence quenching permitted the evaluation of apparent binding affinities of GTP and its analogues. Each was tested

separately, and the results of these experiments are shown in Fig. 2 (A-C). Each ligand has surprisingly similar dissociation constants: $K_{GTP} = 8 \times 10^{-7}$ M; $K_{GMPP(NH)P} = 3 \times 10^{-6}$ M; and $K_{GMPP(CH_2)P} = 3 \times 10^{-6}$ M. Linear Scatchard type plots were uniformly obtained. The stoichiometry is essentially one molecule of guanine nucleotide per tubulin dimer in the absence or presence of the MAPs fraction. For the latter, the amount of tubulin is taken to be 80-85% of the amount of total microtubule protein (14), and this suggests that the MAPs fraction does not significantly affect tubulin-nucleotide interactions at the exchangeable binding site. (Of course, any interactions that might occur at the higher concentrations required for assembly are probably unlikely under our experimental conditions. It should be noted we have confirmed that assembly of microtubules cannot interfere with our spectral measurements since the tubulin concentration ($\sim$ 0.01 mg/ml) in our work is significantly below the critical tubulin concentration of 0.2 mg/ml required for assembly.)

The results of the present report indicate that GDP and GTP stabilize a different conformation of the tubulin molecule. Secondly, tubulin does not show significant discrimination between GTP and its "β-γ analogues." In this respect, the ability of GTP, GMPP(NH)P, and GMPP(CH_2)P to induce assembly might be related to the stabilization of a required pre-assembly conformation. As suggested by Weisenberg et al. (9), hydrolysis of GTP might be more significant for the physiological requirement for microtubule disassembly and turnover.

It is also interesting to note that Lee et al. (15) have shown that 0.1 mM GTP causes no change in the ultraviolet circular dichroism spectrum of tubulin. Their results do not necessarily conflict with our findings since CD measurements often reflect changes in gross conformation and fluorescence spectroscopy may detect changes in microenvironments. The failure of CD measurements to detect such a change attests to our view that a small conformational change accompanies the replacement of tubulin bound GDP by GTP. It is not possible to distinguish the spatial location of the perturbed chromophore relative to the exchangeable nucleotide site, and we cannot support or exclude the speculation by Penningroth et al. (7) that an allosteric effect is involved. Such distinction is the aim of experiments now underway. In any case, the experiments presented here suggest that conformational changes required for insertion of dimers into a growing tubule are indeed rather small.

Interestingly, the value for the GTP dissociation constant at 37° is around ten-fold higher than at 0°C (16), but the significance of this change must await additional studies. Preliminary experiments in this laboratory indicate that this may reflect a temperature-dependent change in the off-rate constant for the tubulin·nucleotide complex, but additional work is clearly indicated.

In conclusion, our findings suggest that a pre-assembly conformation may be attained by GTP or its nonhydrolyzable analogues. It is also apparent that fluorescence measurements have great utility in probing tubulin/nucleotide interactions.

References

1. Stephens, R. E., Renaud, F.L., and Gibbons, I. R. (1967) Science, 156, 1606-1607.
2. Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968) Biochemistry, 7, 4446-4453; Weisenberg, R. C. (1972) Science, 177, 1104-1105.
3. MacNeal, R. K., and Purich, D. L. (1978) J. Biol. Chem., In Press.
4. Jacobs, M., Smith, H., and Taylor, E. W. (1974) J. Mol. Biol., 89, 455-468.
5. Maccioni, R., and Seeds, N. W. (1977) Proc. Natl. Acad. Sci., U.S.A., 74, 462-466.
6. Kobayashi, T. (1975) J. Biochem. (Tokyo) 77, 1193-1197; (1976) 79, 1357-1364.
7. Penningroth, S. M., Cleveland, D. W., and Kirschner, M. W. (1976) in Cell Motility (Goldman, R., Pollard, T., and Rosenbaum, J., eds.) pp. 1233-1258, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
8. Arai, T., and Kaziro, Y. (1976) Biochem. Biophys. Res. Commun., 69, 369-375.
9. Weisenberg, R. C., Deery, W. J., and Dickinson, P. (1976) Biochemistry, 15, 4248-4257.
10. Shelanski, M. L., Gaskin, F., and Cantor, C. R. (1973) Proc. Natl. Acad. Sci., U.S.A., 70, 765-768.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem., 193, 265-269.
12. Hélène, C. (1971) J. Mol. Biol., 58, 349-365.
13. Scatchard, G. (1946) J. Amer. Chem. Soc., 68, 2315-2317.
14. Sloboda, R. D., Dentler, W. L., and Rosenbaum, J. L. (1976) Biochemistry, 15, 4497-4505.
15. Lee, J. C., Corfman, D., Frigon, R. P., and Timasheff, S. G. (1978) Arch. Biochem. Biophys., 185, 4-14.
16. Jacobs, M., and Caplow, M. (1976) Biochem. Biophys. Res. Commun., 68, 127-135.