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Nuclear Relaxation Rates Study of GTP(γF)-tubulin Interaction Using ^{19}F-Nuclear Magnetic Resonance

Estudio de la interacción tubulina-GTP(γF) mediante velocidades de relajación utilizando ^{19}F-resonancia magnética nuclear

OCTAVIO MONASTERIO

Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

To study the relationship between the exchangeable GTP binding site (E-site) and the high affinity metal binding site we synthesided P^3-fluoro P^1-5'-guanosine triphosphate (GTP(γF), an analog of GTP. Our results show that this analog binds to the exchangeable GTP binding site of calf brain tubulin. The values of the dissociation constant and the stoichiometry of the GTP(γF)-Mn(II) complex as determined by EPR spectroscopy were 1.64×10^{-4} M and one mole of manganese per mole of nucleotide, respectively. The distance separating the high-affinity binding site for the divalent metal ion and the exchangeable nucleotide binding site was evaluated by using high-resolution ^{19}F-NMR. The ^{31}P- and ^{19}F-NMR spectra of GTP(γF) were studied, both the fluorine and the gamma-phosphate were split in a doublet with a coupling constant of 936 Hz. Tubulin purified by the method of Weisenberg (Weisenberg, R.C., and Timashef, S.N. (1970) *Biochemistry 9,* 4110-4116) was treated with colchicine to stabilize it, GTP(γF) was added and the 254.1 MHz ^{19}fluorine relaxation rates measured within the first four hours. Longitudinal and transversal relaxation rates were determined in the presence of colchicine-tubulin-Mn(II), (paramagnetic complex), or the ternary complex with magnesium (diamagnetic complex). The analysis of the temperature-dependent relaxation data indicates that the metal and the exchangeable nucleotide binding sites are separated by a maximal distance of 6 at 35°C, to 8.1 Å at 12°C.

Tubulin is a dimer of 110,000 daltons that self-assembles to form microtubules. Microtubules consist of 13 protofilaments of tubulin aligned longitudinally along the axis of the cylinder. Both magnesium and GTP play an important role in the process of polymerization of tubulin. It is well established that one mole of Mg^{2+} is tightly bound per mole of tubulin dimer and that extra Mg^{2+} ions are required for the polymerization process (Olmsted and Borisy, 1975; Himes *et al.*, 1977; Williams and Detrich, 1979). Other divalent cations which have been used to replace Mg^{2+} induce aberrant structures. Zn^{2+} (Larsson *et al.*, 1976; Gaskin and Kress, 1977; Eagle *et al.*, 1983) and Co^{2+} (Wallin *et al.*, 1977) promote the assembly of sheets consisting of up to 50-60 protofilaments. It has been reported however, that microtubules can be formed over a very narrow range of Zn^{2+} and Co^{2+} concentrations (Hakins *et al.*, 1980). Furthermore, Mn^{2+} can substitute for Mg^{2+} both at the high affinity divalent cation binding site and in the promotion of tubulin polymerization (Bluttalire *et al.*, 1980; Gaskin, 1981).

Ever since the isolation of tubulin from brain tissue by Weisenberg *et al.* (1968), it has been known that this protein contains two GTP binding sites per 110,000 daltons. One of these sites is exchangeable (E-site) with free GTP, the other is non-exchangeable. Guanosine 5'-(gamma-fluorotriphosphate) (GTP(γF)) behaves as a competitive inhibitor of GTP at the (E-site) (Monasterio and Timasheff, 1985; 1987). Removal of GTP from the E-site results in a loss of the high affinity metal binding site (Jemiolo and Grisham, 1982). This indicates that the exchangeable nucleotide binding site and the high-affinity metal ion binding site interact strongly but does not prove that these two sites are in fact topographicaly close to each other on the tubulin molecule. The results could be explained

whether the high affinity metal binding site is located at the E-site as a complex metal-nucleotide or a conformational change of tubulin release the metal from a site not essentially the same of the nucleotide.

Measurements of nuclear magnetic relaxation rates in the presence of paramagnetic probes lead to the determination of distances between individual atoms of a molecule in solutions and nearby paramagnetic reference point (Mildvan and Cohn, 1970). This makes it possible to determine distances $\leqslant$ 24 Å with a precision of better than 10% (Mildvan and Gupta, 1978). The objective of this work was to study the effect of temperature on the longitudinal relaxation rate to evaluate the distance between the divalent high affinity manganese site and the gamma-phosphate of GTP(γF), at the E-site, on the quaternary complex colchicine-tubulin-manganese-GTP(γF).

METHODS AND THEORY

Calf brain tubulin was prepared by the method of Weisenberg *et al.* (1968; 1970), modified by Lee *et al.* (1973), as well as by the recycling procedure of Shelanski *et al.* (1973), followed by phosphocellulose chromatography using the procedure of Himes *et al.* (1977).

Synthesis of Guanosine 5'-(gamma-fluorotriphosphate). The fluorinated analog of GTP was synthesized essentially according to the procedures described by Haley and Yount (1972) and Eckstein *et al.* (1975). This product was found to be pure by chromatography on DE-81 Whatman paper. The ^{31}P-NMR spectrum showed that the product synthesized was GTP(γF) which was confirmed by elemental analysis, ultraviolet absorption spectrum and the ratio of guanosine to acid-labile and to total phosphate.

Polymerization of tubulin. The self-assembly of tubulin into microtubules was monitored by turbidity measurements (Gaskin *et al.*, 1974). The composition of the assembly buffers and temperature conditions are described in the legends to the figures. Turbidity was monitored at 350 nm on Cary 14 or 118 recording spectrophotometers.

Exchange of magnesium per manganese. In order to replace magnesium per manganese at the high affinity binding site, tubulin was equilibrated with manganese(II) at the indicated concentrations, assembled at 37°C for 30 min, and filtered through a Sephadex G25 column (1 x 14 cm) equilibrated with the experimental buffer. The free and weakly bound manganese was removed by passing tubulin through a Chelex 100 column (packed in a pasteur pipet) equilibrated with the experimental buffer plus 10% D_2O. The sample used for the ^{19}F-NMR studies contained 0.6 mol of manganese per mol of tubulin and the total amount of divalent cation was 1.01 ± 0.05 mol per mol of tubulin (average of seven experiments). This is in agreement with the value of the displacement of magnesium by manganese reported by Bluttaire *et al.* (1980) for phosphocellulose-purified tubulin.

EPR Spectroscopy. The EPR spectra were recorded at 9.1 GHz on a Varian E-line EPR spectrometer equiped with a Varian E-257 variable temperature accessory at Boston Biomedical Research Institute. The following conditions were used: time constant 0.25 s; receiver gain 1.6 x 10^3; microwave power, 10 mW; modulation frequency, 100 KHz; microwave frequency 9.515 GHz. Free concentrations of $Mn(H_2O)_6^{2+}$ were determined from the relative height of the peaks to the total manganese concentration used.

Atomic Absorption Spectroscopy. Magnesium and manganese concentrations were determined on a Perkin-Elmer model 303 atomic absorption spectrometer using a specific lamp for each metal and an air-acetylene flame.

NMR Spectroscopy. ^{31}P-NMR spectra were recorded at 36.48 MHz on a Bruker WH-90 instrument operating in the Fourier-transform mode. Measurements of the ^{19}F relaxation rates at 254.1 MHz, were made by Fourier transform NMR on a homebuilt 270 MHz spectrometer, based on a 270 MHz magnet, and built by S. Kunz and A. Redfield at Brandeis University. Usually, the experiments were completed within four hours after the preparation of the tubulin sample to avoid protein denaturation. The lower field signal of the fluorine doublet was used for all the calculations. All experiments were performed in 5 mm NMR tubes. T_1 values were determined through the saturation-recovery-sequences method implemented in the spectrometer. The transverse relaxation time (T_2) was determined from the width of the signal peak at half-height (Δ) using the relationship given by People *et al.* (1959),

$$T_2 = 1/\pi\Delta \qquad (1)$$

Analysis of the relaxation data. The observed fluorine nuclear longitudinal relaxation rate ($1/T_1$), for GTP(γF) in the presence of the tubulin complexes, is the sum of at least three terms

$$1/T_1 = 1/T_{1p} + 1/T_{1d} + 1/T_{1o} \qquad (2)$$

where $1/T_{1p}$ and $1/T_{1d}$ are the paramagnetic and the diamagnetic contributions of the protein complex. $1/T_{1o}$ is the contribution to the relaxation rate from the solvent and the dissolved oxygen. $1/T_{1o}$ is independent of the protein concentration. Luz and Meiboom (1964) have

shown that in the temperature range where the relaxation rates of the coordinated ligands are greater than the difference in resonance frequencies between the free and the coordinated GTP(γF), $1/T_1$ for $Mn(H_2O)_6^{2+}$ is given by

$$1/T_{1p} = f/(\tau_M + T_{1M}) \quad (3)$$

where τ_M and T_{1M} are the residence time and the longitudinal relaxation time in the first coordination sphere of the metal ion, respectively, and f is the ratio of the number of GTP(γF) molecules in the first coordination sphere to the total number in solution. As discussed in detail by Mildvan and Cohn (1970) and Mildvan and Gupta (1978), the effect of the temperature on the longitudinal relaxation rate of fluorine at the γ-phosphate of the GTP(γF) may be used to determine whether the predominant contribution to the observed relaxation rate is from T_{1M} or τ_M.

The longitudinal relaxation rate of a nucleus in the coordination sphere of a paramagnetic ion, as formulated by Bloembergen (1957) and Solomon (1955) is given in terms of the dipolar and the scalar contributions to the longitudinal relaxation. The scalar contribution can be dropped when the paramagnetic probe is not forming a direct bond with the nucleus under observation and its contribution to the longitudinal relaxation rate is negligible. Furthermore, $1/T_{1M}$ must be corrected if the relaxation is exchange limited, permitting calculation of only an upper limit of the distance by the following equation:

$$r(\text{Å}) = C\left[(fT_{1p} - \tau_M) f(\tau_C)\right]^{1/6} \quad (4)$$

$$f(\tau_C) = \frac{3\tau_C}{1 + \omega_I^2 \tau_C^2} + \frac{7\tau_C}{1 + \omega_S^2 \tau_C^2} \quad (5)$$

In our study, r is the distance in angstroms, f is the fraction of GTP(γF) bound to the ternary complex colchicine-tubulin-manganese, C is a constant whose value is 795.76 (Mildvan and Gupta, 1978), and other symbols have their usual meaning.

RESULTS

The ^{31}P-NMR spectrum (Fig. 1) of the fluorinated analog of GTP, P^3-fluoro P^1-5' guanosine triphosphate, shows that the γ-phosphorus was shifted to upfield resonances and split in a doublet of doublet with a coupling constant (J_{FP}) of 935 Hz. This large coupling constant was produced by the direct coupling of the fluorine atom to the gamma-phosphorus. Fluorine substitution of the hydroxyl group on this

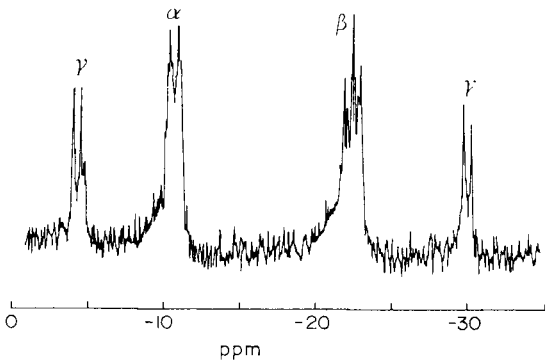

Fig. 1: ^{31}P-NMR spectrum of GTP(γF) at 36.48 MHz. The sample contained 17.6 mM GTP(γF) at pH 4.6 and 24.5 ± 1 °C. 14.850 scans were taken and 85% phosphoric acid was used as external reference. Peak assignments are indicated on the figure.

phosporus leads to an upfield shift of 12.7 ppm. The alfa-phosporus was not affected whereas the beta-phosporus was shifted to up field in 0.8 ppm. The effect of fluorine on the phosphate chain of the nucleotide is not affected by the nature of the base, since for ATP(γF) Vogel and Bridger (1982) have obtained the same chemical shifts for the substituted phosphorus resonances. These results confirm that the analog of GTP, that we have synthesized, is modified on the gamma-phosphate and the hydroxyl group with a pK_a around 6.8 has been replaced by fluorine.

GTP(γF) behaves as a competitive inhibitor with respect to GTP in the colchicine-dependent GTPase activity of tubulin (Monasterio and Timasheff, 1985; 1987). To determine if GTP(γF) replaces GTP at the exchangeable site, tubulin-GDP was incubated with radioactive GTP and the excess of free nucleotide removed through gel filtration. The sample was divided in two halves. To one, GTP(γF) was added and to the other the same amount of GTP(γF)-free solvent was added. Fig. 2 shows the elution of tubulin-GTP in the presence and in the absence of GTP(γF). The amount of GTP bound to the exchangeable site decreased by 31.5% in the presence of the GTP(γF), indicating exchange between the nucleotides.

Measurements of the paramagnetic effect of manganese(II) on the longitudinal relaxation time (T_1) of the fluorine atom

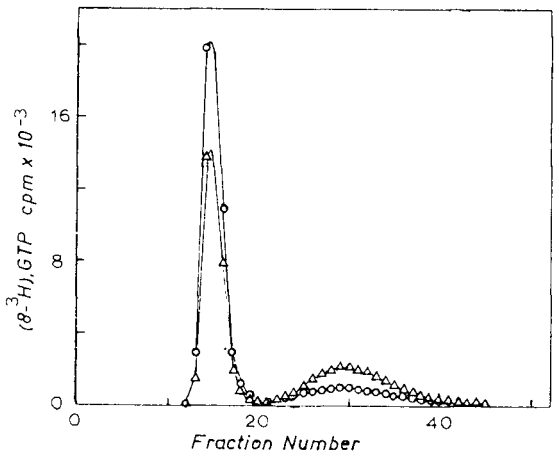

Fig. 2: Exchange of GTP by GTP(γF) at the exchangeable nucleotide site of tubulin. Gel filtration of (8-^{3}H)GTP-tubulin complex (8.52 mg/mL protein) with 0.9 mM (GTP(γF) (△) or the same volume of GTP(γF)-free solvent (○). Mixtures were filtered at 10°C on a 0.9 x 25 cm Sephadex G-25 column equilibrated with the same buffer (50 mM MES (4-morpholineethane sulfonic acid) and 3.4 x 10^3 mM glycerol at pH 7.0) used to exchange successively GTP by (8-^{3}H)GTP and this by GTP(γF) on the exchangeable nucleotide site of tubulin.

TABLE I

Temperature dependence of the apparent distance Mn(II)-fluorine in the quaternary complex colchicine-tubulin-Mn-GTP(γF)

Temperature °C	$1/fT_{1p}$ $s^{-1} \times 10^{-3}$	T_{1p}/T_{2p}	apparent distance (Å)	
			non-corrected	corrected[a]
12.4	0.582	10.4	8.43	8.13
15.8	0.844	11.9	7.93	7.49
20.7	0.998	22.1	7.71	7.20
25.7	1.082	49.1	7.61	7.05
30.5	1.641	53.0	7.10	6.20
35.4	1.771	–	7.01	6.01

[a]Distance was evaluated by using equation 4 (see text) and assuming that the chemical exchange rate ($1/\tau M$) was given at the lower temperature by $1/fT_2$ whose value was 2.95 x 10^3 S^{-1}.
The mixture in a volume of 0.5 mL contained 50 mM MES, pH 7.0; 3.4 x 10^3 mM glycerol; 0.3 mM colchicine; 0.18 mM tubulin and 0.78 mM GTP(γF). The relaxation rates were measured as indicated in Methods.

in the complex tubulin-manganese-GTP (γF) (Fig. 3), show that at 20°C, in the presence of 25% glycerol, the value of T_1 decreased exponentially with time, following first-order kinetics with a half-life of 8.3 h. This could be the result of a slow exchange between GTP and GTP(γF) at the nucleotide exchangeable site. This possibility was ruled out because when manganese-tubulin was added to GTP(γF) its ^{19}F-NMR spectrum was only slightly shifted and no other absorption peaks were detected and because the value of the ratio T_{1p}/T_{2p} (Table I) was higher than 10 in several experiments with different concentrations of tubulin, manganese, and GTP(γF) (Monasterio and Timasheff, 1985). Rapid exchange was further supported by the fact that, the value of T_1 for the complex tubulin-Mg-GTP(γF), under the same experimental conditions, is five times higher than the value in the presence of tubulin-Mn-GTP(γF) complex after one hour of its preparation. This shows that most of the GTP at the exchangeable site has been replaced by its fluorinated analogue. Although these results support a rapid exchange, the temperature dependence of the relaxation rates indicates that the relaxation processes are mediated in part by the exchange (see below). Another explanation for the decay in T_1 of the manganese complex, could be that, under our experimental conditions, tubulin, which is not a stable protein (Prakash and Timasheff, 1982 and Croom *et al.*, 1985), undergoes a conformational change releasing manganese to the solvent where it interacts with free GTP(γF) in a different way than in the protein. Croom *et al.* (1985) have reported that phosphocellulose-purified tubulin denatures with a half-life of approximately 8 h, which is similar to the values indicated above. In order to check these possibilities, the values of T_1 were measured at a lower temperature (8°C). Under our experimental conditions the value of T_1 decreases in 56.2% from the value at zero minutes, after 18 h of measuring in the NMR spectrometer (Fig. 3). This result supports the hypothesis that the change in the value of T_1 is due to a releasing of manganese(II) from tubulin and tubulin is not stable under these experimental conditions. Andreu and Timasheff (1982) have shown that colchicine stabilizes tubulin. The values of T_1, for the quaternary complex, colchicine-tubulin-Mn-GTP(γF), did not change significantly in 24 hours at 8°C in

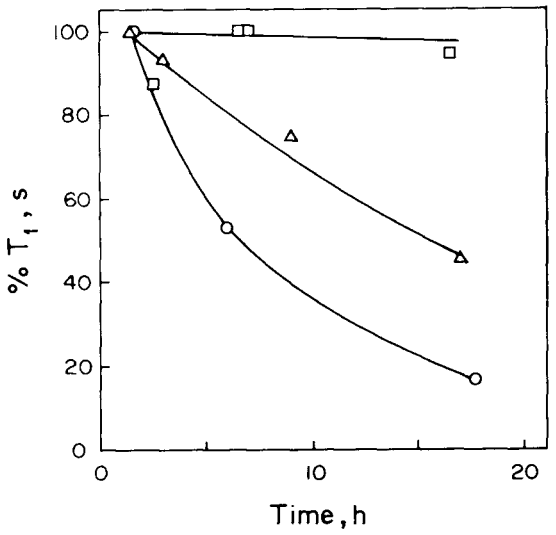

Fig. 3: Dependence of the longitudinal relaxation rate on the stability of tubulin. Values of the ^{19}F longitudinal relaxation rates, at 254.1 MHz, of GTP(γF) in the presence of the binary complex tubulin-Mn(II) at 19°C (○) and 8.3°C (△) and in the presence of the ternary complex colchicine-tubulin-Mn(II) at 9.5°C (□) were measured at the times indicated in the figure. The composition of the samples in a volume of 0.5 mL were: 50 mM MES, pH 7.0; 3.4x10^3 mM glycerol; 10% D_2O; 0.86 mM GTP(γF); 0.098 mM tubulin (Mn(II)/tubulin = 0.39) for the binary complex and 0.089 mM tubulin (Mn(II)/tubulin = 0.26), 0.31 mM colchicine for the ternary complex.

the presence of 25% glycerol (Fig. 3). As a result of the stability of tubulin all our experiments were conducted with the complex colchicine-tubulin-GTP(γF)-metal.

The ^{19}F-NMR spectrum of 0.78 mM GTP(γF) in the presence of colchicine-tubulin-magnesium complex is shown in Figure 4A. The narrow fluorine lines produced by GTP(γF) are separated by 936 Hz, value of the coupling constant, and they are located at 47.4 ppm and 51.1 ppm downfield from sodium fluoride, used as an external standard. Addition of the complex colchicine-tubulin-manganese (II) (Figure 4B) produces a change in both the longitudinal and the transversal relaxation times evidenced by the broading of the line. There were no significant changes in either the position of the lines or in the coupling constant. These results demonstrate that the paramagnetic effect of manganese on the relaxation rate of the fluorine nuclei at the γ-phosphate is mediated by the interaction of the analog at the exchangeable nucleotide site on tubulin.

In order to test if the effect of manganese, tightly bound to tubulin, was produced only when the analog was forming the quaternary complex, 10 mM GTP was added to the mixture. The paramagnetic effect of manganese on both relaxation times of the fluorine signal was abolished. These results confirm that GTP replaces GTP(γF) at the exchangeable nucleotide site. This experiment, however, did not eliminate the possibility that manganese could be released from tubulin and to form a binary complex GTP(γF)-Mn contributing to the relaxation times. With this possibility in mind we carried experiments with fluorine phosphate which does not interact with the exchangeable nucleotide site (it has not effect on the assembly of microtubules at concentrations of 0.1 M, unpublished results) and its ^{19}F-NMR spectrum is shifted from the ^{19}F-NMR spectrum of GTP(γF). Manganese should affect the relaxation times of fluorine phosphate if the sample contains free manganese in equilibrium with the quaternary and the binary (GTP(γF)-Mn) complexes. In order to calculate the relative concentrations of nucleotide and FPO_3 for the displacement experiments,

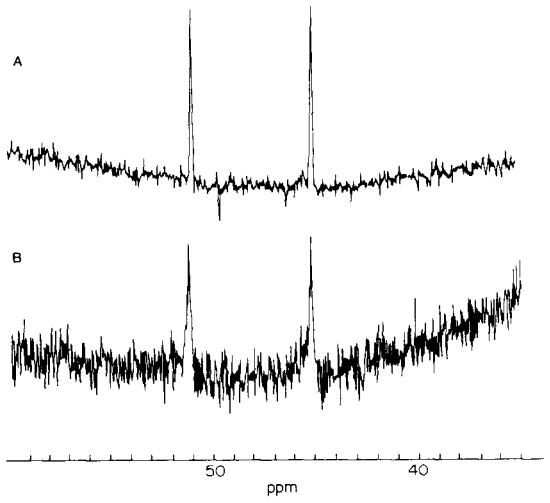

Fig. 4: ^{19}F-NMR spectra at 254.1 MHz of a mixture containing: 50 mM MES, pH 7.0; 3.4x10^3 mM glycerol; 0.31 mM colchicine; 0.78 mM GTP(γF) and 0.16 mM tubulin (A) Mg(II)/tubulin = 1; (B) Mn(II)/tubulin = 0.28. The chemical shifts were measured relative to sodium fluoride as external standard.

the dissociation constant of the GTP(γF)-Mn complex was measured by EPR spectroscopy following the method described by Cohn and Townsed (1954). The Scatchard plot of the binding of Mn(II) to GTP(γF) (Fig. 5) shows that, in the range of GTP(γF) used, the stoichiometry was 1 mol of manganese per mol of nucleotide with a dissociation constant of 1.64×10^{-4} M. The dissociation constant for the FPO_3-Mn complex is 1.8×10^{-3} M (Mildvan *et al.*, 1967). With these values of dissociation constants, 5 mM FPO_3 was chosen to study its effect on the longitudinal relaxation time of 0.8 mM GTP(γF) in the presence of colchicine-tubulin-Mn complex. When FPO_3 was added to a solution containing the complex colchicine-tubulin-Mn-GTP(γF), T_1 of GTP (γF) changed from 0.09 to 0.18 s and when GTP(γF) was added to a solution containing the complex colchicine-tubulin-Mn plus FPO_3, T_1 of FPO_3 changed from 0.39 to 0.45 s. These results can be explained in terms of an exchange of manganese between the complex GTP(γF) and FPO_3. Hence, exchange between the tubulin quaternary complex (colchicine-tubulin-GTP(γF)-Mn) and the binary complex (GTP(γF)-Mn) cannot be ruled out.

The temperature dependence of both $1/fT_{1p}$ and $1/fT_{2p}$ between 12 and 35°C indicates that the relaxation rates of GTP(γF) in the quaternary complex can be dominated in part by $1/\tau_M$, the reciprocal of the residence time of GTP(γF) in the coordination sphere of manganese (Table I). To calculate the distance we assume two extreme cases: 1) Longitudinal relaxation rate is dominated by $1/T_{1M}$. In this case the value of τ_M was delete from the equation (4) (non corrected apparent distances, Table I). 2) Longitudinal relaxation rate is dominated by τ_M (corrected apparent distances, Table I). The difference in distances evaluated following the assumptions indicated above shows that the contribution of the chemical exchange rate to the longitudinal relaxation rate was small. However, in order to be rigorous, the small contribution of τ_M must be considered in the evaluation of distances between Mn(II) and fluorine

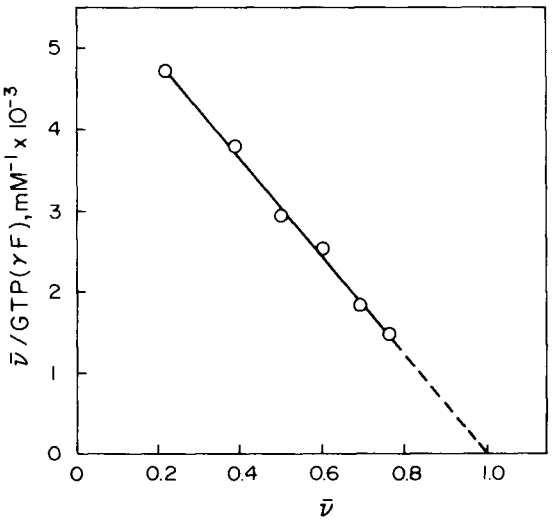

Fig. 5: Scatchard plot for the binding of Mn(II) to GTP (γF). Solutions containing 50 mM MES, pH 7.0; 3.4 M glycerol and 0.1 mM $MnCl_2.6H_2O$ and different concentrations (68-580 μM) of GTP(γF) (determined at 260 nm with a extinction coefficient of 11800 at pH 5) were measured through EPR. The concentration of free $Mn(H_2O)_6^{2+}$ was determined at 25°C, as indicated under Methods with samples of 50 μL.

nuclei at the γ-phosphate of GTP(γF) in the quaternary complex.

The effective correlation time (τ_c) for the colchicine-tubulin-Mn-GTP(γF) interaction was evaluated by measuring the longitudinal relaxation time at two different frequencies, assuming that the correlation time was not frequency dependent. By measuring the same sample at 254.1 and 84.7 MHz in the presence of both magnesium and manganese, the value of the correlation time was 3.72×10^{-10} s. As indicated by Sloan and Mildvan (1976), this type of calculation introduces an error in the absolute distance of 4%.

DISCUSSION

The results presented in this paper show that the high affinity cation binding site is located at an upper distance of 6 to 8 Å from the gamma-phosphate of the GTP at the exchangeable site. This value of the distance indicates that the phosphate moiety at the exchangeable nucleotide site and the metal at the high affinity site are sharing the same locus on tubulin. This can be explained by the formation of a

metal-nucleotide complex either in the inner or the second coordination sphere of the metal. Considering the value of the distance, it is not possible to have the first coordination sphere of the metal involved in the complex with the gamma-phosphate, because the results of small-molecule crystallography molecular model studies show that the Mn(II) to phosphorus distance is 2.8-3.0 Å for an inner sphere complex of tetrahedral phosphate (Mildvan and Grisham, 1974). The results support a complex formed between phosphate and the second coordination sphere complex of Mn^{2+} with an intervening water molecule, similar to the case of pyruvate kinase (Sloan and Mildvan, 1976). However, the formation of the complex with the first coordination sphere of the metal cannot be eliminated because exchange limitation may conceal slowly exchanging inner sphere GTP(γF) by rapidly exchanging second sphere nucleotide on tubulin-bound manganese. In our studies GTP(γF), which has less affinity for manganese than GTP, was used. This could be the result of the substitution of the hydroxyl group with pK ~ 6.8, at the gamma-phosphate by fluorine. The fact that fluorine is more electronegative than the hydroxyl group should reduce the electronic density on the oxygen atom of the gamma-phosphate which forms the bond with the metal resulting in the lower affinity of the metal for the nucleotide. Therefore, it is quite possible that the complex is formed with the oxygens of the alfa and beta-phosphates, increasing the distance between the metal and the gamma-phosphate. Thus, the formation of a complex GTP-Mg^{2+} at the first coordination sphere cannot be disregarded. Considering both possible modes of coordination between manganese and the γ-phosphate of GTP(γF), these results demonstrate that the complex Mn(II)-GTP(γF) is located at the exchangeable nucleotide site of tubulin.

The distance-dependent paramagnetic part of the longitudinal relaxation rate described by the Solomon-Bloembergen equation used in the present study to calculate the distance between manganese and the gamma-phosphate of GTP(γF) involves the following assumptions: 1) the dipolar correlation time is frequency independent; 2) the longitudinal relaxation rate ($1/T_{1p}$) is not exchange-limited; 3) the outer sphere contribution to $1/T_{1p}$) is small; 4) the hyperfine contact contribution to $1/T_{1p}$ should be negligible small. Only assumption 3 will be discussed here, the others were discussed by Monasterio (1987).

The use of equation (4) to calculate the distance between the fluorine nuclei at the gamma-phosphate of GTP(γF) and the paramagnetic center given by Mn(II) in the complex colchicine-tubulin-GTP(γF)-Mn, requires a high rate of exchange of GTP(γF) out of the quaternary paramagnetic complex, compared to the $1/fT_{1p}$ values. As indicated by Mildvan and Cohn (1970), when the value of $\omega_I^2\ \tau_c^2 < 1$ and the longitudinal relaxation rate is dominated by T_{1M}, the temperature dependence of T_{1p}, given by τ_s should be positive or negative. Also, the ratio T_{1p}/T_{2p} should be greater than one. If the relaxation process is dominated by the residence time (τ_M), the temperature dependence should be positive and the ratio T_{1p}/T_{2p} equal to one. As shown in Table I, the longitudinal relaxation rate has a positive coefficient of temperature and the ratio of T_{1p}/T_{2p} is higher than one. This results indicate that the process is dominated by T_{1M} and the chemical exchange rate cannot be the rate limiting the process. Hence, the following assumption could be valid: the change of distance between the paramagnetic center given by manganese and the gamma-phosphate of GTP(γF) induced by temperature could be due to a conformational change at the tubulin E-site.

The inhibitory effect of GTP(γF) on tubulin polymerization (Monasterio and Timasheff, 1985; 1987) could be the result of the failure of this analog of GTP to form the appropriate complex with the γ-phosphate at the exchangeable nucleotide site and the divalent metal. ATP(γF) binds Mg^{2+} poorly, supporting the idea that the terminal phosphate is important in metal binding (Vogel and Bridger, 1982). This confirms the conclusion that the gamma-phosphate of GTP forms a metal complex

which is essential to stimulate tubulin polymerization. Gaskin (1981) found that Zn^{2+}-induced sheets are not dependent of a Zn-GTP complex supporting the thermodynamic studies of Lee and Timasheff (1975) who found that at least one additional mole of magnesium is added per mole of tubulin in microtubules. An understanding of the role of the added magnesium in the polymerization of tubulin will require the characterization of the low affinity magnesium binding sites.

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