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^{19}F Nuclear Magnetic Resonance Measurement of the Distance between the E-Site GTP and the High-Affinity Mg^{2+} in Tubulin†

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ABSTRACT: The distance separating the divalent metal ion high-affinity binding site and the exchangeable nucleotide binding site on tubulin was evaluated by using high-resolution ^{19}F NMR. The ^{31}P and ^{19}F NMR spectra of guanosine 5′-(γ-fluorotriphosphate) [GTP(γF)] were studied. Both the fluorine and the γ-phosphate were split into a doublet with a coupling constant of 936 Hz. Tubulin purified according to the method of Weisenberg [Weisenberg, R. C., & Timasheff, S. N. (1970) *Biochemistry 9*, 4110–4116] was incubated with 1 mM Mn^{2+}. After one cycle of assembly, Mn^{2+} replaced Mg^{2+} only partially, i.e., 60% at the high-affinity binding site. After colchicine treatment of tubulin to stabilize it, GTP(γF) was added, and the 254-MHz fluorine-19 relaxation rates were measured within the first 4 h. Longitudinal and transversal relaxation rates were determined at two concentrations of GTP(γF) and variable concentrations of colchicine–tubulin–Mn(II) (paramagnetic complex) or the ternary complex with magnesium (diamagnetic complex). The analysis of the relaxation data indicates that the rate of exchange of GTP(γF) from the exchangeable nucleotide site has a lower limit of 8.7×10^4 s^{-1} and the metal and exchangeable nucleotide binding sites are separated by an upper distance between 6 and 8 Å. These data confirm that the high-affinity divalent cation site is situated in the same locus as that of the exchangeable nucleotide, forming a metal–nucleotide complex.

The process of the self-assembly of microtubules from tubulin depends on magnesium ions and GTP. It has been well established that each tubulin α–β heterodimer contains one tightly bound Mg^{2+} ion and that extra Mg^{2+} ions are required for the polymerization process (Olmsted & Borisy, 1975; Lee & Timasheff, 1975; Himes et al., 1977; Williams & Detrich, 1979). When other divalent cations are used in place of Mg^{2+}, aberrant structures are formed. Zn^{2+} (Larsson et al., 1976; Gaskin & Kress, 1977; Eagle et al., 1983) and Co^{2+} (Wallin et al., 1977) promote the assembly of sheets consisting of as many as 50–60 protofilaments aligned in parallel. It has been reported, however, that microtubules can be formed over a very narrow range of Zn^{2+} and Co^{2+} concentrations (Haskins et

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al., 1980). On the other hand, Mn^{2+} can substitute for Mg^{2+} both in binding at the high-affinity site and in the promotion of tubulin polymerization (Buttlaire 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 M_r 110000. One of these sites is exchangeable with free GTP, and the other is nonexchangeable. Removal of GTP from the exchangeable nucleotide binding site results in a loss of the high-affinity metal binding site (Jemiolo & Grisham, 1982). This paper suggests either that the high-affinity metal binding site is located at the exchangeable nucleotide binding site as a metal–nucleotide complex or that removal of the nucleotide induces a conformational change in tubulin that releases the metal from its binding site, which is different from the nucleotide site. While this parallel dissociation of the divalent metal ion and of the GTP indicates that the exchangeable nucleotide binding site and the high-affinity metal ion binding site interact strongly, it does not prove that these two sites are, in fact, located close to each other on the tubulin molecule.

The distance between individual atoms of a molecule in solution and a nearby paramagnetic reference point can be measured by nuclear magnetic relaxation rates in the presence of paramagnetic probes (Mildvan & Cohn, 1970). This makes it possible to evaluate distances $\leq$24 Å with a precision of better than 10% (Mildvan & Gupta, 1978). The object of this present study was the determination of the distance between the high-affinity divalent cation site and the γ-phosphate of GTP at the exchangeable site, occupied by a manganese ion and guanosine 5′-(γ-fluorotriphosphate) [GTP(γF)], respectively, in order to establish whether the two sites are superimposed on each other through the formation of a metal–nucleotide complex. This study should also allow us to elucidate whether it is the inner or the second coordination sphere of the metal that is involved in the structure of the complex.

Materials and Methods

The sources of most of the chemicals were mentioned in the preceding paper (Monasterio & Timasheff, 1987). The deuteriated water was obtained from Bio-Rad Laboratories, and glycerol was obtained from Sigma Chemical Co. Tubulin was prepared according to the method of Weisenberg et al. (1968) (Weisenberg & Timasheff, 1970), modified by Lee et al. (1973), as well as according to the recycling procedure of Shelanski et al. (1973), followed by purification by chromatography on a phosphocellulose column according to the procedure of Himes et al. (1977).

Exchange of Magnesium by Manganese. Since manganese precipitates in the 1 M sucrose phosphate buffer, in which the stock protein was stored, the tubulin was equilibrated with 50 mM 4-morpholineethanesulfonic acid (MES), pH 7.0, and 3.4 M glycerol by a batch–dry column technique, as described by Na and Timasheff (1982). In order to replace magnesium by 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 G-25 column (1 × 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 amount of manganese bound to tubulin was found to depend both on the concentration of $MnCl_2$ used and on whether the exchangeable nucleotide site was occupied by GTP or GDP, as shown in Table I. The sample used for the ^{19}F NMR studies contained 0.6 mol of manganese/mol of tubulin, and the total amount of divalent

Table I: Exchange of Manganese(II) for Magnesium in Tubulin[a]

treatment	amount: mol of Mn/mol of tubulin	amount: mol of Mg + Mn/mol of tubulin
control		1.01 ± 0.04
1 mM $MnCl_2$	0.38 ± 0.02	
2 mM $MnCl_2$	0.22 ± 0.07	
one cycle of assembly + 1 or 2 mM $MnCl_2$	0.60 ± 0.07	1.01 ± 0.05

[a] Tubulin was equilibrated in 50 mM MES, pH 7.0, and 3.4 M glycerol with 1 or 2 mM $MnCl_2$ or assembled at 37 °C for 30 min in the same buffer plus 1 or 2 mM $MnCl_2$ and filtered through a Sephadex G-25 column equilibrated with the same solution at 10 °C. Free metal ion was removed by treatment with Chelex 100. The samples were analyzed for protein, and the amount of tubulin-bound metal ion was measured by atomic absorption spectroscopy.

cation was 1.01 ± 0.05 mol/mol of tubulin (average of seven experiments). This is in agreement with the value of the displacement of magnesium by manganese reported by Buttlaire et al. (1980) for phosphocellulose-purified tubulin.

Electron Paramagnetic Resonance (EPR) Spectroscopy. The EPR spectra were recorded at 9.1 GHz on a Varian E-line EPR spectrometer equipped with a Varian E-257 variable-temperature accessory at the Boston Biomedical Research Institute. The following conditions were used: time constant, 0.25 s; receiver gain, 1.6×10^3; microwave power, 10 mW; modulation frequency, 100 kHz; microwave frequency, 9.515 GHz.

Atomic Absorption Spectroscopy. Magnesium and manganese concentrations were determined on a Perkin-Elmer Model 303 atomic absorption spectrometer with a specific lamp for each metal and 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. The spectrometer was equipped with a temperature regulator system. The sample size was 1.0 mL in 10-mm Wildman precision tubes, fitted with Teflon vortex plugs. Ten percent D_2O was used as an internal lock. 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. The temperature of the probe was regulated to ±1 °C. Usually, the experiments were completed within 4 h after preparation of the tubulin sample to avoid protein denaturation. The following conditions were used: pulse width, 10 μs; spectral width, 4 kHz; delay time, at least $2T_1$. Generally 10000 scans were taken. The lower field signal of the fluorine doublet was used for all calculations. All experiments were performed in 5-mm NMR tubes. The longitudinal relaxation time (T_1) values were determined through the saturation–recovery sequence method implemented in the spectrometer. The transverse relaxation time (T_2) was determined from the width of the signal peak at half-height (Δ) according to the relationship given by Pople et al. (1959)

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

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

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

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 free and coordinated GTP(γF), $1/T_{1p}$ 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 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 comes from T_{1M} or from τ_M.

The longitudinal relaxation rate of a nucleus in the coordination sphere of a paramagnetic ion, as formulated by Bloembergen (1955) and Solomon (1957), is

$$1/T_{1M} = 1/r^6\{{}^2/_{15}S(S+1)\gamma_1{}^2g^2\beta^2[3\tau_c/(1+\omega_1{}^2\tau_c{}^2) + 7\tau_c/(1+\omega_s{}^2\tau_c{}^2)] + {}^2/_3S(S+1)(A^2/\hbar^2)[\tau_e/(1+\omega_s{}^2\tau_e{}^2)]\} \quad (4)$$

The definitions of the symbols and the numerical values of the constants have been given by Mildvan et al. (1967). The first and the second terms of eq 4 are the dipolar and the scalar contributions to the relaxation rate, respectively. In this equation, τ_c is the correlation time for the dipolar interaction and τ_e that for the scalar interaction. τ_c is given by

$$1/\tau_c = 1/\tau_r + 1/\tau_M + 1/\tau_s \quad (5)$$

where τ_r is the rotational correlation time for the tubulin complex, $1/\tau_M$ is the dissociation rate of the complex, and τ_s is the electron spin relaxation time. The correlation times, τ_r and τ_M, may be described (Bloembergen & Morgan, 1961) in terms of the activation energies, $(E_a)_i$:

$$\tau_i = \tau_i \exp[(E_a)_i/RT] \quad (6)$$

As the temperature decreases, τ_r and τ_M increase but τ_s may either increase or decrease (Garrett & Morgan, 1966). Usually τ_r increases with increasing temperature for small complexes and decreases with increasing temperature for enzyme complexes (Reed et al., 1971; Reed & Ray, 1971). In general, the exchange time (τ_M) has larger E_a values ($\geq$7 kcal/mol) than τ_r or τ_s ($\leq$3 kcal/mol) (Eigen & Wilkins, 1965; Mildvan & Cohn, 1970).

Results

The ^{31}P NMR spectra of GTP and its fluorinated analogue, GTP(γF), are shown in Figure 1. The spectra were obtained at pH 4.6 in H_2O with 10% D_2O. No corrections were made in the pH values for the presence of D_2O. The γ-phosphorus of the analogue was shifted upfield and split into a doublet with a coupling constant (J_{FP}) of 935 Hz, generated by the direct coupling of the fluorine atom to the γ-phosphorus. The fluorine substitution of the hydroxyl group on this phosphorus led to an upfield shift of 12.7 ppm. The α-phosphorus was not affected whereas the β-phosphorus was shifted upfield by 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 adenosine 5′-(γ-fluorotriphosphate) Vogel and Bridger (1982) have obtained the same chemical shifts for the substituted phosphorus resonances. These results confirm that the analogue of GTP, which we have synthesized, is modified on the γ-phosphate and that the group that has been replaced is the hydroxyl with a pK of ~6.8 on the γ-phosphorus of GTP. The titration curve of this analogue did not have an inflection between pH 4.0 and pH 8.0.

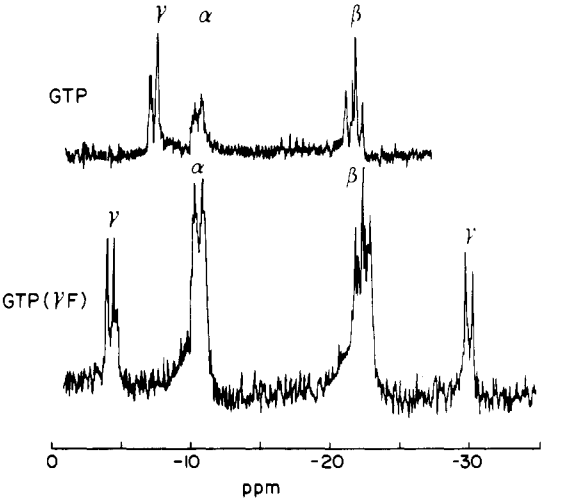

FIGURE 1: Phosphorus-31 nuclear magnetic resonance spectra at 36.48 MHz of GTP (sodium salt) and GTP(γF) at pH 4.6, 24.5 ± 1 °C: GTP 20 mM, 1041 scans; GTP(γF), 17.6 mM, 14 850 scans. The external reference for both spectra was 85% phosphoric acid. Peak assignments are indicated on the figure.

Under our experimental conditions, tubulin polymerizes to form microtubules in the presence of manganese ions (Monasterio and Timasheff, unpublished experiments), indicating that manganese replaces magnesium in the active conformation of tubulin independently of the procedure used to purify the protein (Buttlaire et al., 1980).

In the preceding paper it was shown that tubulin in the absence of free Mg^{2+} and GTP is unstable. Because the NMR experiments required at least 2 h to have a working signal at the low concentrations used of both tubulin and GTP(γF), it was necessary to determine the experimental conditions to measure the paramagnetic effect of manganese on the fluorine atom of GTP(γF). To carry out these experiments, the samples were filtered through a Chelex 100 column to eliminate free and weakly bound divalent cations and the relaxation rates were measured at different times. The value of T_1 for the tubulin–Mg–GTP(γF) complex, at 20 °C, in the presence of 25% glycerol decreased exponentially with time, following first-order kinetics with a half-life of 8.3 h. Such a decrease could be the result of a slow exchange between GTP and GTP(γF) at the nucleotide exchangeable site. This possibility was ruled out by the presence of two peaks of absorption produced by the ternary complex which is characteristic of a rapid exchange and because the value of the ratio T_1/T_2 was higher than 10 in several experiments with different concentrations of tubulin, manganese, and GTP(γF) (Monasterio & Timasheff, 1985). Rapid exchange was further supported by the fact that, under the same experimental conditions, the value of T_1 for the tubulin–Mn–GTP(γF) complex was 5 times higher than that for tubulin–Mg–GTP(γF) 1 h after 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 between tubulin and the nucleotide, 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 could be that, under our experimental conditions, tubulin, which is not a stable protein (Prakash & Timasheff, 1982; Croom et al., 1985), undergoes

Table II: Coupling Constant and Chemical Shift from Sodium Fluoride as External Standard for the Interaction of GTP(γF) with Tubulin under Different Experimental Conditions

condition	J_{PF} (Hz)	chemical shift[a] (ppm)
tubulin treated with alkaline phosphatase, pH 7.0	936 ± 1	51.1
tubulin + 1 M sucrose, pH 7.0	936 ± 1	51.2
tubulin + colchicine, pH 6.8	936 ± 1	51.2
tubulin + colchicine, pH 7.7	935 ± 2	51.1
tubulin + colchicine + glycerol, pH 7.0	936 ± 1	51.1

[a] Only the resonance peak at the lower field was considered.

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 in 0.1 M 1,4-piperazinediethanesulfonic acid, pH 6.9, 2 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, and 2 mM dithioerythritol denatures with a half-life of approximately 8 h, which is similar to the values indicated above. In order to check the influence of tubulin stability on the T_1 values, these values were measured at lower temperature (8 °C), showing that the value of the half-life increased to 14.7 h. 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 h at 8 °C in the presence of 25% glycerol. These results support the hypothesis that the change in the value of T_1 in the absence of colchicine was produced by a conformational change of tubulin under our experimental conditions. As a result, all our experiments were conducted with the quaternary complex, i.e., colchicine–tubulin–GTP(γF)–metal.

The ^{19}F NMR spectra of GTP(γF) in the presence of tubulin showed only one signal split by 936 Hz under several experimental conditions, as shown in Table II. Chemical shifts and coupling constants were affected neither by the presence of GDP at the exchangeable nucleotide site nor by stabilizing agents, such as sucrose, glycerol, or colchicine added to the solvent. A change in pH from 6.8 to 7.7 also had no effect. The absence of other peaks in the presence of tubulin could be due either to a strong interaction between tubulin and the analogue resulting in very broad peaks, or to the interaction between tubulin and the analogue being in fast exchange relative to the chemical shift produced by the GTP(γF)–tubulin interaction or to the value of the coupling constant. The NMR spectrum of 0.78 mM GTP(γF) in the presence of the colchicine–tubulin–magnesium complex is shown in Figure 2a. The narrow fluorine lines produced by the γ-phosphate of GTP(γF) are separated by 936 Hz, i.e., the same value of the coupling constant, and they are located at 47.4 and 51.1 ppm downfield from sodium fluoride, which was used as the external standard. Replacement of this complex by colchicine–tubulin–manganese(II) produced a change in both the longitudinal and the transversal relaxation times, as evidenced by the broadening of the line (Figure 2b). There were no changes either in the position of the lines in the spectrum 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 interaction with the analogue at the exchangeable nucleotide site on tubulin [see preceding paper for the interaction of GTP(γF) with the exchangeable site (Monasterio & Timasheff, 1987)]. Such a paramagnetic effect could occur whether the ternary complex [colchicine–tubulin–manganese(II)] remained stable in solution after the interaction with GTP(γF) or the divalent cation

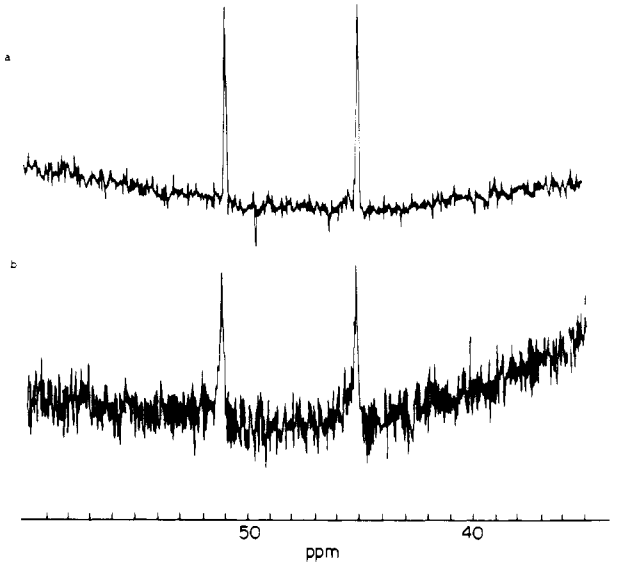

FIGURE 2: Fluorine-19 nuclear magnetic resonance spectra at 254.1 MHz of a mixture containing 50 mM MES, pH 7.0, 3.4 M glycerol, 0.31 mM colchicine, 0.78 mM GTP(γF), and 0.16 mM tubulin. (a) Mg^{2+}/tubulin = 1; (b) Mn^{2+}/tubulin = 0.28. The chemical shifts were measured relative to sodium fluoride as external standard.

was released due to a conformational change at the nucleotide exchangeable site induced by the interaction with the analogue. In the latter case, the cation would exchange freely with the free nucleotide in the solution.

In order to test if the effect of the tightly bound manganese appeared only when the analogue was forming the quaternary complex, 10 mM GTP was added to the mixture. This abolished the paramagnetic effect of manganese on both relaxation rates of the fluorine signal, confirming that GTP was replacing GTP(γF) at the nucleotide exchangeable site. This experiment, however, did not eliminate the possibility of a contribution of the GTP(γF)–Mn complex to the relaxation rates of the fluorine signals. Although this type of displacement experiments are usually done to demonstrate the specificity of interaction of nucleotide analogues with the nucleotide binding site where the paramagnetic probe has an effect, in general the following possibility has not been considered: If the substrate is in fast exchange (with respect to the parameter measured), and the paramagnetic probe forms a complex with both the protein and the nucleotide apparently without a chemical shift between them, it is not simple to differentiate by the displacement experiments the two interactions of the probe. With this possibility in mind, experiments were carried out with fluorophosphate, which forms a complex with manganese and has no effect on the assembly of microtubules at concentrations of 0.1 M (Monasterio and Timasheff, unpublished results). Figure 3 shows the ^{19}F NMR spectrum of fluorine phosphate, which has two peaks of higher absorption than GTP(γF) with a coupling constant (J_{FP}) of 870 ± 2 Hz, shifted upfield from that of GTP(γF) in 0.56 ppm.

Whether the sample contains the quaternary and the binary [GTP(γF)–Mn] complexes in equilibrium with free manganese ion, fluorophosphate (FPO_3) should increase the relaxation times of the fluorine signal. Since FPO_3 competes only with the binary complex for manganese, an increase of the relaxation times should demonstrate the presence of the complex GTP(γF)–Mn. The lack of interaction of FPO_3 with tubulin was confirmed by the following results (Table III): The complex colchicine–tubulin–Mg had no significant effect on the longitudinal relaxation time (T_1) of FPO_3, T_1 changed from 0.67 s without the complex to 0.74 s, and the effect of the

Table III: Effect of the Complex Colchicine–Tubulin–Metal (Mg^{2+} or Mn^{2+}) on the ^{19}F NMR Signal of Fluorophosphate (FPO_3) and GTP(γF) at pH 7.0[a]

conditions	J_{FP} (Hz)		T_1 (s)		Mn/Tub ratio	tubulin (mM)
	FPO_3	GTP(γF)	FPO_3	GTP(γF)		
FPO_3[b]	868		0.67			
FPO_3[b] + complex–Mg	872		0.74		0.00	0.31
FPO_3[c] + complex–Mn	868		0.39		0.11	0.20
GTP(γF) + complex–Mg		936		0.55	0.00	0.23
GTP(γF) + complex–Mn		936		0.09	0.12	0.18
FPO_3[c] + complex–Mg + GTP(γF)	872	936	0.91	0.49	0.00	0.18
FPO_3[c] + complex–Mn + GTP(γF)	868	936	0.37	0.08	0.11	0.20
FPO_3[d] + complex–Mn + GTP(γF)	872	936	0.45	0.18	0.12	0.18

[a] All the experiments were done at 10 ± 2 °C except for the first and second ones, which were done at 19 and 15 °C, respectively. The complex colchicine–tubulin–metal was prepared as described under Materials and Methods. Footnotes *b*, *c*, and *d* are 0.1, 1 × 10^{-3}, and 5 × 10^{-3} M FPO_3, respectively. The concentration of GTP(γF) was 0.8 mM.

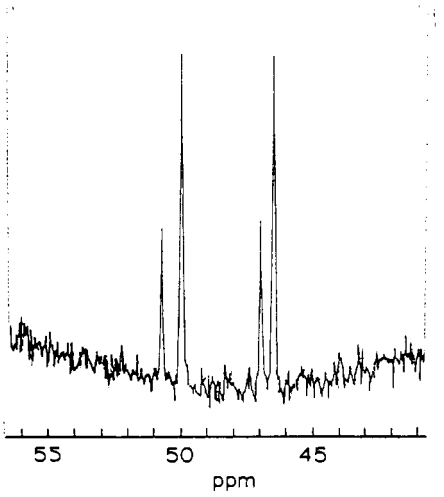

FIGURE 3: Fluorine-19 nuclear magnetic resonance spectra at 254.1 MHz of fluorophosphate and 1 mM GTP(γF), 0.2 mM tubulin–Mg^{2+} complex, 0.3 mM colchicine, 50 mM MES, pH 7.0, and 3.4 M glycerol in a final volume of 0.5 mL at 10 ± 1 °C. Sodium fluoride was used as external standard.

complex colchicine–tubulin–Mn on FPO_3 T_1 was similar. Thus, the paramagnetic effect of the colchicine–tubulin–manganese complex on the FPO_3 signal was negligible relative to its effect on GTP(γF) (T_1 changed from 0.55 to 0.09 s). These results indicate that FPO_3 was unable to release tubulin–tightly bound manganese from the protein.

In order to calculate the relative concentrations of nucleotide and FPO_3 for the displacement experiments, the dissociation constant of the GTP(γF)–Mn complex was measured by EPR spectroscopy following the method described by Cohn and Townsend (1954). Solutions containing 50 mM MES, pH 7.0, 3.4 M glycerol, 0.1 mM $MnCl_2 \cdot 6H_2O$, and different concentrations (68–580 μM) of GTP(γF) (determined at 260 nm with a extinction coefficient of 11 800 at pH 5) were measured for free manganese through EPR. The concentration of free $Mn(H_2O)_6^{2+}$ was determined at 25 °C, as indicated under Material and Methods with samples of 50 μL. The Scatchard plot of the binding of Mn(II) to GTP(γF) (not shown) shows that, in the range of GTP(γF) used, the stoichiometry was 1 mol of manganese/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, whose values were evaluated from the slopes of the lines shown in Figure 4. As shown in Table III, this effect was small: the longitudinal relaxation time for GTP(γF) changed from 0.9 to 0.18 s and for FPO_3 from 0.39 to 0.45 s. These

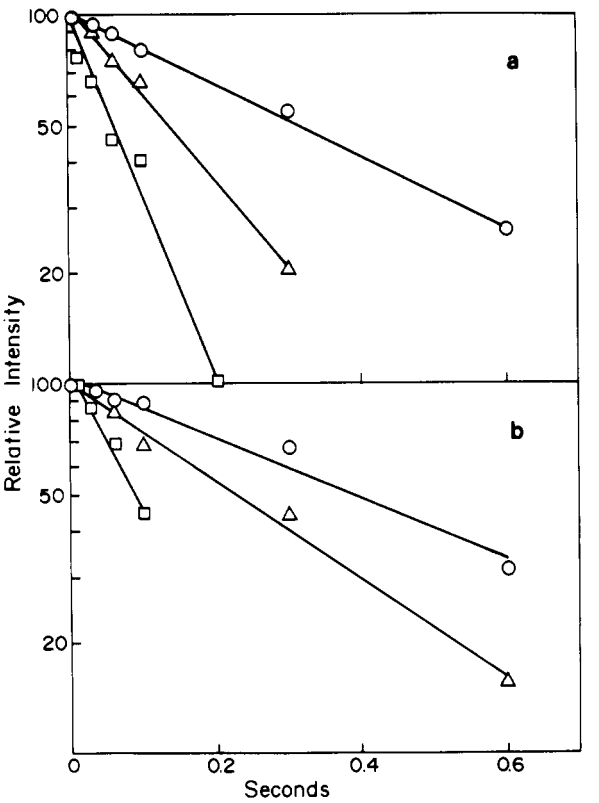

FIGURE 4: Semilogarithmic plot of ^{19}F NMR signal intensity vs. time. The intensity of the signal was the difference between the signal obtained with an infinite delay time after a 180° pulse and the signal with a delay time in the range of T_1. The mixture in a volume of 0.5 mL contained 50 mM MES, pH 7.0, 3.4 M glycerol, 0.3 mM colchicine, 0.18 mM tubulin, and 0.8 mM GTP(γF) (□) or 0.8 mM GTP(γF) + 5 mM FPO_3 [(○) relaxation time of FPO_3; (△) relaxation time of GTP(γF)]. The upper lines correspond to the peak at the higher magnetic field used in the determination of the distance.

results can be explained in terms of an exchange of manganese between the complex GTP(γF)–Mn and FPO_3. The treatment of the complex colchicine–tubulin–Mn–GTP(γF) with 5 mM ethylenediaminetetraacetic acid abolished the paramagnetic effect of manganese on the fluorine nuclei of GTP(γF), supporting the results with FPO_3. All of these facts indicate that the interaction of GTP(γF) with the exchangeable nucleotide site releases the metal, at least in part, from its high-affinity binding site. Hence, exchange between the tubulin quaternary complex and the GTP(γF)–Mn complex is possible.

The effect of temperature between 12 and 35 °C on the paramagnetic contribution to the relaxation rates ($1/fT_{1p}$ and $1/fT_{2p}$) of GTP(γF) in the quaternary complex is shown in Figure 5. From the positive slopes of the Arrhenius plot of $1/fT_{2p}$ vs. temperature, it follows that the transversal relaxation

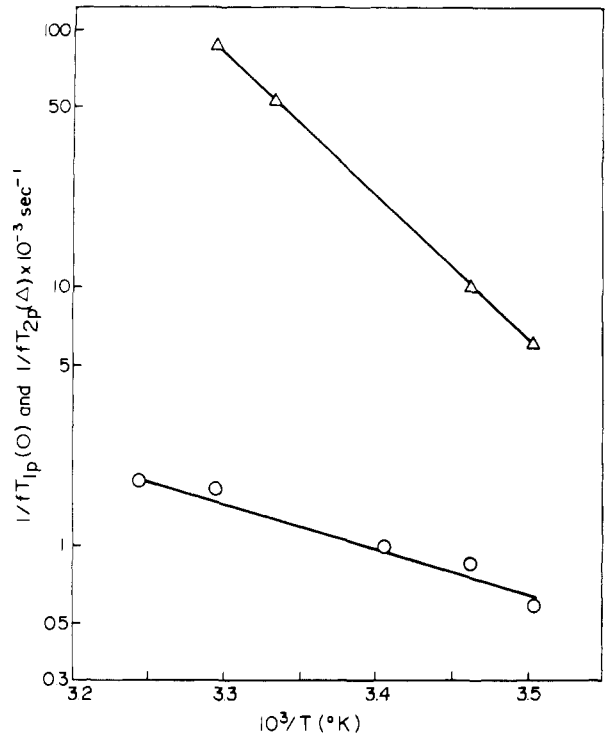

FIGURE 5: Arrhenius plot of the temperature dependence of the paramagnetic contribution to the relaxation rates of fluorine in the quaternary complex, colchicine–tubulin–Mn–GTP(γF). The composition of the buffer was the same as in Figure 4.

rate of the complex colchicine–tubulin–Mn–GTP(γF) is governed by $1/\tau_M$. The Arrhenius plots of both $1/fT_{1p}$ and $1/fT_{2p}$ were found to have positive slopes. The energy of activation calculated from the slopes gave values of 26.12 and 8.23 kcal/mol for the transversal and longitudinal relaxation rates, respectively. The temperature dependence of both $1/fT_{1p}$ and $1/fT_{2p}$ indicates that the relaxation rates are dominated by $1/\tau_M$, the reciprocal of the residence time of GTP(γF) in the coordination sphere of manganese.

The effect of GTP(γF) concentration on the transversal relaxation rate shows that at 0.31 and 0.78 mM nucleotide the values of T_2 were 5.6 and 5.3 ms, respectively. The lack of dependence of T_2 on ligand concentration suggested that the process is kinetically first order, the most likely reaction being

$$\text{colchicine–Tub–Mn–GTP}(\gamma\text{F}) \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{colchicine–Tub–Mn} + \text{GTP}(\gamma\text{F}) \quad (7)$$

If the simplest kinetic assumption is made, i.e., the exchange rate is equal to k_{off} in the reaction, the value of $1/fT_{2p}$ can be used to set the lower limit of k_{off} at 8.7×10^4 s^{-1} at 30 °C. The second-order rate constant (k_{on}) for the binding of GTP(γF) to the complex colchicine–tubulin–Mn can be calculated from this k_{off} value and the dissociation constant, 1.8×10^{-4} M^{-1}, for GTP(γF) from the quaternary complex of tubulin (see preceding paper) (Monasterio & Timasheff, 1987) with the assumption that the dissociation constant for the tubulin complex with manganese is not very different from that with magnesium.

The effect of the concentration of the ternary complex colchicine–tubulin–Mn on the longitudinal relaxation rate of the fluorine nuclei of GTP(γF) is shown in Figure 6. The concentration of the nucleotide was 0.8 mM, and variable concentrations of the ternary complex were added to different samples, resulting in the fractional values of bound nucleotide indicated in the figure. The dependence of the longitudinal

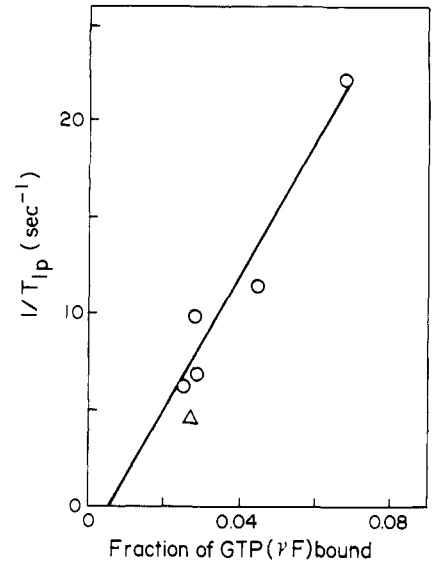

FIGURE 6: Effect of the ternary complex, colchicine–tubulin–Mn, on the longitudinal relaxation rate of the fluorine nucleus of the GTP analogue. Relaxation times (T_1) were measured in a mixture containing 50 mM MES, pH 7.0, and 3.4 M glycerol at 10 ± 1 °C. The fraction of GTP(γF) bound corresponds to the ratio between the quaternary complex and total GTP(γF).

relaxation rate was linear with the fraction of bound GTP(γF) and, within experimental error, independent of the method used to prepare the tubulin. These data were used to calculate the value of $1/fT_{1p}$ used in the determination of the distance between the paramagnetic probe and the fluorine nucleus. The results of the temperature dependence of $1/fT_{1p}$ have shown that this relaxation rate is exchange limited, permitting calculation of only an upper limit of the distance and making it necessary to correct the relaxation data by the exchange rate. The distance was calculated from

$$r\,(\text{Å}) = C\left\{(fT_{1p} - \tau_M)\left[\frac{3\tau_c}{1 + \omega_1^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2}\right]\right\}^{1/6} \quad (8)$$

which is a reduced version of eq 4. Here, r is the distance in angstroms, f is the fraction of GTP(γF) bound to the complex colchicine–tubulin–Mn, C is a constant whose value is 795.76 (Mildvan & Gupta, 1978), and other symbols have their usual meaning. The scalar term of eq 4 has been dropped from this equation because the paramagnetic probe is not forming a direct bond with the fluorine moiety and its contribution to the longitudinal relaxation rate is negligible.

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 magnesium or manganese, the value of the correlation time was found to be 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%. The value of the apparent T_{1M} for fluorine at the phosphate in the quaternary tubulin complex was estimated from the data of Figure 6 to be 2.8×10^{-3} s. Introduction of these parameters into eq 8 gave values of r, the Mn–F distance, of ≤7.84 Å at 10 °C and ≤6.12 Å at 35 °C.

Discussion

The results presented in this paper show that the high-affinity cation site is located at a maximal distance of 6–8 Å

from the γ-phosphate of the exchangeable site GTP. This value of the distance indicates that the phosphate moiety of the exchangeable site nucleotide 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 in either 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 a complex with the γ-phosphate because the results of small-molecule crystallography and 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 & Grisham, 1974). The results support a complex formed between phosphate and the second coordination sphere of Mn^{2+} with an intervening water molecule, similar to the case of pyruvate kinase (Sloan & 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 we used GTP(γF), which has less affinity for manganese than GTP. This could be the result of the substitution of the hydroxyl group with pK ~6.8 at the γ-phosphate by fluorine. The fact that fluorine is more electronegative than the hydroxyl group should reduce the electronic density of the oxygen atom of the γ-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 α- and β-phosphates, increasing the distance between the metal and the γ-phosphate. Thus, the formation of a complex 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 this study to calculate the distance between manganese and the γ-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 negligibly small.

The principal uncertainty in the calculated distance arises from the uncertainty in the correlation time. However, since the distance varies as the one-sixth power of the correlation time, fairly large changes in the correlation time would not alter markedly the calculated distance. The temperature dependence of the relaxation rates, shown in Figure 5, displayed a negative value for the slope given by d(ln $1/T_1$ or T_2)/d($1/T$). This was interpreted as an exchange-limited interaction of GTP(γF) with tubulin. This interpretation was supported by the high activation energy of the longitudinal relaxation rate. In general, the exchange time (τ_M) has larger activation energy values [≥7 kcal/mol (Eigen & Wilkens, 1965)] than τ_r or τ_s [≤3 kcal/mol (Mildvan & Cohn, 1970)]. This result, however, is not congruent with the presence of the same number of peaks of absorption and chemical shift, whether tubulin was present or not, as well as with a value of the ratio T_1/T_2 greater than 10. Mildvan and Cohn (1970) have shown that the Arrhenius plot of the relaxation rates can have a negative slope, even though fast exchange is occurring, if T_{1M} is dominated by τ_s. The value of the correlation time (τ_c), 0.37×10^{-9} s, is in the range of the τ_s value for various pyruvate kinase–Mn(II) complexes (Reed & Cohn, 1973). In the present case, τ_M is greater than 10^{-6} s. A theoretical calculation of τ_r for the rotation of a prolate ellipsoid of the size of tubulin was carried out, by using the Hu–Zwanzig equation (Boere & Kidd, 1982):

$$\tau_r = \epsilon^* a_{max}^3 \eta / 6kT \qquad (9)$$

where ϵ^* is the rotational frictional coefficient calculated by Hu and Zwanzig (1974) for the slip boundary condition, η is the viscosity coefficient (0.0159 g s^{-1} cm^{-1} for a 3.4 M glycerol solution at 30 °C (*Handbook of Chemistry and Physics*, 1945), a is the major axis of the tubulin dimer [40 Å (Timasheff, 1981)], k is the Boltzmann constant, and T is the Kelvin temperature. From this equation, the theoretical rotation time was calculated to be 9.2 ns. The theoretical calculation of τ_r for the nucleoside triphosphate–metal complex, assuming a rigid sphere with radius equal to the longer semiaxis of the spheroid of 7 Å (Tanswell et al., 1975), gives a value of 0.036 ns. The experimental value of τ_c is between the two calculated theoretical values, proving that the correlation time is not given by the free complex Mn(II)–GTP(γF) in solution. The calculated and experimental values for the rotational, residence, and electron spin relaxation times suggest that the correlation time may be determined principally by the electron spin relaxation time. If the correlation time is dominated by the electron spin relaxation time, T_{1e} of the manganese ion is given by (Bloembergen & Morgan, 1961)

$$1/T_{1e} = B[\tau_v/(1 + \omega_s^2\tau_v^2) + 4\tau_v/(1 + 4\omega_s^2\tau_v^2)] \qquad (10)$$

where B is a constant containing the electronic spin S and the zero-field splitting of manganese and τ_v is a time constant for transient symmetry distortions of the manganese complex. This correlation time has two limiting cases. In the first case, where $\omega_s^2\tau_v^2 \gg 1$, T_{1e} displays maximum frequency dependence, and in the second case, where $\omega_s^2\tau_v^2 \ll 1$, it is independent of the frequency. From the ratio of $1/fT_{1p}$ at two frequencies and with the conditions described above, limits for the τ_c values can be estimated. Assuming that τ_c is either frequency independent or dependent gives values for the correlation time of 7.86×10^{-10} and 2.75×10^{-9} s at 254.1 MHz.

The outer-sphere contribution to the longitudinal and transversal relaxation rates is small because the stable complex of tubulin with manganese at the high-affinity site was used (Buttlaire et al., 1980) and free manganese was negligible in the sample (see Table III). The inequality of the observed $1/T_{1p}$ and $1/T_{2p}$ argue against the presence of an appreciable outer-sphere contribution. The hyperfine contact contribution to $1/T_{1M}$ is small because the distance between the manganese and the γ-phosphate of GTP(γF) does not allow a direct coordination between the ligand and the paramagnetic center.

Since all enzyme-catalyzed reactions of nucleoside triphosphates require divalent cations, such as Mg^{2+} or Mn^{2+}, and the role of this activator in catalysis is to assist the departure of the leaving phosphate group and to facilitate nucleophilic attack on the γ-phosphate, by water in the case of tubulin, it is reasonable that the high-affinity cation site must be located at the exchangeable nucleotide site. Also, it is interesting to speculate about the possible role of the water molecule in the second coordination sphere of the metal at the exchangeable site. The nucleotide at this site becomes nonexchangeable after tubulin polymerization, and the water bound to the phosphate moiety in the complex could be the nucleophilic agent that produces the hydrolysis of the γ-phosphate. On the other hand, the absence of metal at the

nonexchangeable nucleotide site contributes to the stability of the GTP molecule at this site.

The inhibitory effect of GTP(γF) on tubulin polymerization could be the result of the failure of this analogue of GTP to form the appropriate complex of the divalent metal with the γ-phosphate of the exchangeable site nucleotide because of manganese complexation with the α- and β-phosphates or mediation by a water molecule. ATP(γF) binds Mg^{2+} poorly, supporting the idea that the terminal phosphate is important in metal binding (Vogel & Bridger, 1982). This confirms the conclusion that the γ-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 on a Zn–GTP complex, supporting the thermodynamic studies of Lee and Timasheff (1975), who found that at least an additional 1 mol 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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Registry No. GTP, 86-01-1; GTP(γF), 57817-57-9; Mg, 7439-95-4.

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