# Kinetics of Interaction of 2-Amino-6-mercapto-9-β-ribofuranosylpurine 5′-Triphosphate with Bovine Brain Tubulin†

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ABSTRACT: The binding of the guanine nucleotide analogue 2-amino-6-mercapto-9-β-ribofuranosylpurine 5′-triphosphate ($S^6$-GTP) to tubulin from which the associated proteins and exchangeably bound nucleotide have been removed produces about a 15% decrease in intrinsic tubulin fluorescence. Using a fluorescence stopped-flow technique, we have examined the kinetics and mechanism of this process. Analysis of the data reveals that the binding is complex, involving at least one conformational change subsequent to nucleotide binding. The bimolecular association rate constant for binding of $S^6$-GTP to tubulin is approximately $6 \times 10^5$ $M^{-1}$ $s^{-1}$, suggesting that the orientation requirements are stringent. The kinetic parameters for dissociation of GDP, $S^6$-GTP, and $S^6$-GDP from the exchangeable nucleotide binding site have also been determined. $S^6$-GDP and GDP were found to have comparable rates of dissociation; $S^6$-GTP dissociated approximately twice as slowly as either GDP or $S^6$-GDP. Glycerol produces a significant decrease in the rates of nucleotide dissociation. The mechanism whereby glycerol produces such an effect is not known; however, it may involve slight changes in the conformation of the tubulin protomer.

Guanine nucleotides play a critical role in assembly of microtubules (Jacobs et al., 1974). A number of studies have shown that the microtubule protomer, a heterologous dimer consisting of two related subunits, termed α- and β-tubulin, has two binding sites for guanine nucleotides. Nucleotide bound to one site (E site)[1] exchanges readily with exogenous GTP; nucleotide bound to a second site (N site) does not exchange (Jacobs et al., 1974; Timasheff & Grisham, 1980). During or subsequent to assembly, nucleotide bound to the E site is hydrolyzed to GDP (Jacobs et al., 1974; David-Pfeuty et al., 1977, 1978; Margolis, 1981).

Some years ago, Lockwood et al. (1975) observed that the nonhydrolyzable analogues pNHppG and $pCH_2$ppG promoted assembly of tubulin. In addition, they found that microtubules assembled with these analogues had altered stability properties. These observations have since been confirmed in other laboratories (Sandoval et al., 1977; Karr et al., 1979; Sandoval & Weber, 1980; Terry & Purich, 1980; Kirsch & Yarbrough, 1981). To explain their observations, Lockwood et al. (1975) proposed that guanine nucleotides act as allosteric effectors to stabilize the conformation required for assembly.

To date, there has been no detailed study of the mechanism of tubulin–nucleotide interactions. However, there is evidence that suggests that the conformation of tubulin containing bound nucleotide may differ from that of tubulin from which the exchangeable nucleotide has been removed. Karr & Purich (1978) reported that nucleotide binding produces a quenching of intrinsic tubulin fluorescence and that GTP and GDP differ in their ability to quench this fluorescence. From these observations they concluded that GDP and GTP stabilized different tubulin conformations. Geahlen & Haley (1979) performed photoaffinity labeling studies of tubulin with the photoaffinity analogues 8-$N_3$GTP and 8-$N_3$GDP. They found that the efficiency of incorporation of labeled 8-$N_3$GTP was 4 times that of 8-$N_3$GDP and suggested that this could reflect differences in conformation of the tubulin–nucleotide complexes. Using a fluorescent GTP analogue containing an aminonaphthalenesulfonate moiety attached to the γ-phosphoryl group, Yarbrough & Kirsch (1981) demonstrated that binding of GTP to the E site was not of itself sufficient to induce assembly. However, the failure of this analogue to promote assembly could have been due to simple steric effects rather than the failure to induce the conformation required for assembly. More recently, Maccioni & Seeds (1983) have found that the rate of chymotryptic digestion of tubulin is considerably slowed by the presence of GTP. They concluded that nucleotide stabilizes an "active" tubulin conformation. In earlier studies, Maccioni & Seeds (1982) found no significant differences in the CD spectrum of tubulin when examined in the presence or absence of the nonhydrolyzable GTP analogue pNppG, suggesting that there is no gross rearrangement of conformation when nucleotide is bound.

Studies of transient kinetics of binding reactions, using stopped-flow or temperature-jump techniques, provide one of the best approaches to explore the kinetics and mechanisms of protein–ligand interactions (Kirschner et al., 1966; Hammes & Schimmel, 1970; Hammes & Wu, 1971). These techniques have been applied to many systems such as the lac repressor (Laiken et al., 1972), cyclic AMP receptor protein (Wu & Wu, 1974), RNA polymerase (Yarbrough et al., 1976; Wu

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[1] Abbreviations: PIPES, 1,4-piperazinediethanesulfonic acid; MAPs, microtubule-associated proteins; E site, guanine nucleotide binding site on tubulin at which exchange occurs; N site, guanine nucleotide binding site on tubulin at which exchange does not occur; Tu(−), tubulin from which the MAPs and exchangeable nucleotide have been removed; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $S^6$-GTP, 2-amino-6-mercapto-9-β-ribofuranosylpurine 5′-triphosphate; $S^6$-GDP, 2-amino-6-mercapto-9-β-ribofuranosylpurine 5′-diphosphate; p($CH_2$)ppG, guanosine 5′-(β,γ-methylenetriphosphate); p(NH)ppG, guanosine 5′-(β,γ-imidotriphosphate); R buffer, 0.1 M PIPES, pH 6.8, 1 mM $MgCl_2$, 0.1 mM DTT and EDTA, and 25% (w/v) glycerol; PM buffer, 0.02 M PIPES, pH 6.8, 1 mM $MgCl_2$, and 0.1 mM DTT and EDTA; PMG buffer, 0.02 M PIPES, pH 6.8, 1 mM $MgCl_2$, 0.1 mM DTT and EDTA, and 25% (w/v) glycerol; CD, circular dichroism.

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et al., 1976a), glutamine synthetase (Rhee & Chock, 1976), actin (Trybus & Taylor, 1982), myosin (Eccleston & Trentham, 1979), and the ribosomal elongation factor EF-Tu (Eccleston, 1981), to list but a few. Such an approach requires a change in some measurable physical parameter such as fluorescence or absorption be produced on binding of a ligand to a protein. The thioguanine analogues $S^6$-GTP and $S^6$-GDP have excellent biochemical and spectroscopic properties for such studies (Darlix et al., 1973; Eccleston & Trentham, 1977, 1979; Ecclestion & Bayley, 1980; Eccleston, 1981). They possess an intense absorption in the region of tryptophan fluorescence; hence, binding of these analogues often produces quenching of protein fluorescence due to resonance energy transfer (Eccleston & Trentham, 1977). In addition, the absorption properties of these analogues are very dependent on environment, so that absorption changes are often produced on binding to proteins. In order to more fully define the kinetics and mechanism of the tubulin–nucleotide interaction, we have synthesized the analogues $S^6$-GTP and $S^6$-GDP and used them to characterize the binding process. We have previously described the equilibrium aspects of this interaction (Fishback & Yarbrough, 1984). In this paper we describe the kinetic aspects of binding and exchange of these analogues. Fluorescence stopped-flow studies show two relaxations upon binding of $S^6$-GTP. Analysis of the data suggests that binding of $S^6$-GTP is followed by one or more unimolecular isomerizations of the tubulin–nucleotide complex. Binding of the thioguanine analogues to tubulin is accompanied by changes in the absorption spectrum of the nucleotide. By use of difference spectral techniques, the rate constants for dissociation of GDP, $S^6$-GTP, and $S^6$-GDP have been determined.

## Materials and Methods

*Chemicals.* $S^6$-GTP and $S^6$-GDP were synthesized as described previously (Fishback & Yarbrough, 1984). All other chemicals were of reagent grade.

*Preparation of Microtubular Protein and Tu(−).* Microtubular protein was prepared from bovine brain as described previously (Kirsch & Yarbrough, 1981). The molecular weight of the bovine tubulin dimer was taken to be approximately 100 000, assuming $\alpha$- and $\beta$-tubulins of 451 and 444–445 amino acids, respectively. Nucleotide bound to the exchangeable nucleotide binding site was removed by charcoal treatment of phosphocellulose-purified tubulin as described previously to yield Tu(−). This treatment has been shown to remove greater than 95% of exchangeably bound nucleotide (Kirsch & Yarbrough, 1981). Tu(−) is capable of reassembly in the presence of 10% dimethyl sulfoxide into microtubules that appear normal upon electron microscopy; these microtubules show 80–95% disassembly upon incubation at 3 °C (Kirsch & Yarbrough, 1981). Tu(−) contains slightly in excess of 1 mol (1–1.2) of guanine nucleotide/mol of tubulin dimer, presumably bound to the nonexchangeable nucleotide binding site since it is not removable by treatment with charcoal (M. Kirsch and L. Yarbrough, unpublished observations). Tu(−) is unstable. In the absence of glycerol, the ability to bind $S^6$-GTP is lost with a half-life of 1–2 h; in the presence of 25% glycerol, the half-life is increased to approximately 3–5 h. Thus, glycerol has a significant stabilizing effect on tubulin from which the exchangeable nucleotide has been removed. We have found Tu(−) to be unstable if stored at −80 °C; for this reason, it was prepared immediately before use in all experimental studies.

*Kinetic Studies of Nucleotide Displacement by Absorption Spectroscopy.* Following preparation of Tu(−), the binding of $S^6$-GTP was examined by difference spectroscopy to ensure that the tubulin was able to bind nucleotides. For studies of $S^6$-GTP displacement by GTP, a difference spectrum induced by binding of $S^6$-GTP was first generated by mixing two compartments of a tandem mix cell containing $S^6$-GTP in one compartment [approximately a 1.1-fold molar ratio to Tu(−)] and Tu(−) in the other. Once equilibrium had been reached [which occurred within the time for mixing when $S^6$-GTP was added to Tu(−)], a 4–8-fold molar excess of GTP (relative to tubulin) was added and the time-dependent change in the difference spectrum measured at 350 nm. Control experiments showed no difference in kinetic parameters when the change in absorbance was monitored at another wavelength in the difference spectrum. In experiments involving displacement of $S^6$-GDP, the difference spectrum was first generated by mixing Tu(−) with a 1.1-fold molar ratio of $S^6$-GDP. Displacement of GTP was then measured as described above. For measurements of the kinetics of displacement of GDP from tubulin, microtubules were reassembled and isolated by centrifugation, and the associated proteins were removed by phosphocellulose chromatography. Studies with microtubules reassembled with [$^3$H]GTP showed that tubulin purified in this manner contained 0.9–0.95 mol of guanine nucleotide/mol of tubulin as [$^3$H]GDP. Tubulin purified by the above procedure was used for studied of displacement of GDP by $S^6$-GTP or $S^6$-GDP. For these studies, the kinetics of formation of the difference spectrum generated on binding of $S^6$-GTP or $S^6$-GDP was monitored at 350 nm as described above. Kinetic data were analyzed by the nonlinear regression method described by Dell et al. (1973).

*Theory and Analysis of Kinetic Data.* Consider the simple, one-step binding mechanism

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}L$$

Under conditions in which $[L_0]$ (the initial ligand concentration) > $[P_0]$ (the initial protein concentration) $1/\tau$, the apparent first-order rate constant for the reaction, is given by the expression

$$1/\tau = k_1[L_0] + k_{-1}$$

Thus, for a simple bimolecular binding reaction, $1/\tau$ should show a linear dependence on ligand concentration with a slope equal to $k_1$ and an intercept equal to $k_{-1}$ (Hammes & Schimmel, 1970).

It is possible, however, that conformational changes occur either prior to or subsequent to ligand binding:

mechanism A

$$E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^* + L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^*{\cdot}L$$

mechanism B

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}L^*$$

If this occurs, the kinetics of the reaction becomes more complex [see Hammes & Schimmel (1970), Laiken et al. (1972), Wu & Wu (1974), Wu et al. (1976b), and Rhee & Chock (1976)]. If the rapid step is the preequilibrium isomerization (mechanism A), then the apparent first-order rate constant for the reaction is given by the expression

$$1/\tau = \frac{k_2[L_0]}{1 + k_{-1}/k_1} + k_{-2}$$

Plots of the apparent first-order rate constant vs. ligand concentration will be linear, as described for the simple binding reaction. However, the slope of the plot is equal to $k_2/(1 +$

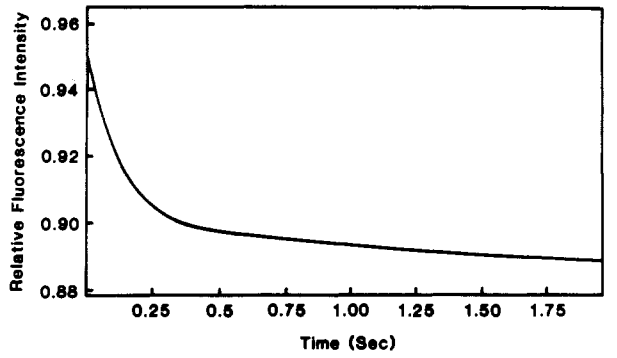


FIGURE 1: Kinetics of S$^6$-GTP binding as determined by fluorescence stopped-flow spectroscopy. Tu(−) (2 $\mu$M) was mixed with S$^6$-GTP by using stopped-flow technique, and the changes in intrinsic fluorescence were measured at 330 nm. The final S$^6$-GTP concentration was 10 $\mu$M. Measurements were performed in PMG buffer at 20 °C as described under Materials and Methods. The curve shown is a tracing of the computer plot of the data. The curve is well fit to a double-exponential decay curve: $F_t = 0.048e^{-8.28t} + 0.020e^{-0.59t}$.

$k_{-1}/k_1$). Thus, the effect of a rapid preequilibrium isomerization is to reduce the apparent rate of complex formation.

If there is a rapid bimolecular binding reaction followed by a slower unimolecular isomerization (mechanism B) and if there is a signal change associated with the bimolecular step, two first-order kinetic processes may be observed (Laiken et al., 1972; Wu et al., 1976a). The first-order rate constant for the first step ($1/\tau_1$) will show a linear dependence upon substrate concentration as described above for the simple bimolecular binding reaction. The first-order rate constant for the slower unimolecular isomerization ($1/\tau_2$) is given by the expression

$$1/\tau_2 = \frac{k_2}{1 + (k_{-1}/k_1)/[L_0]} + k_{-2}$$

At high ligand concentration, $1/\tau_2$ should tend toward a plateau with a value equal to $k_2 + k_{-2}$. If no signal is associated with the bimolecular binding reaction, only the unimolecular isomerization of the complex may be observed (Colen et al., 1974; Yarbrough et al., 1976; Rhee & Chock, 1976). Thus, by examining the dependence of rate constants on ligand concentration, information about reaction mechanisms can be obtained.

*Fluorescence Stopped-Flow Studies of S$^6$-GTP Binding.* For stopped-flow studies, Tu(−) was prepared and used within 3 h of preparation. This was necessary since Tu(−) slowly lost the ability to bind S$^6$-GTP as measured by difference spectroscopy. The loss of the ability to bind S$^6$-GTP was also correlated with the loss of ability to reassemble. Stopped-flow studies were performed on a Durrum-Gibson instrument interfaced to a Varian 620 digital computer as described by Colen et al. (1974). The light source was a 75-W xenon lamp, and the slit was approximately 0.3 mm. Excitation was performed at 290 nm, and emission was measured at 330 nm with a 10-nm narrow band-pass filter. Measurements were performed at 20 °C. Each data point is the average of three to five transients, which were stored, averaged, and then analyzed with a modification of the nonlinear regression method of Dell et al. (1973).

## RESULTS

*Fluorescence Stopped-Flow Studies of Binding of S$^6$-GTP to Tu(−).* Binding of S$^6$-GTP to Tu(−) is accompanied by an approximately 15% quenching of intrinsic tubulin fluorescence (Fishback & Yarbrough, 1984). Using fluorescence stopped-flow technique, we have examined the binding of S$^6$-GTP

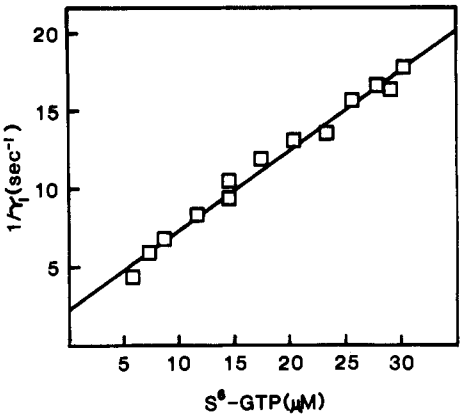


FIGURE 2: Dependence of $1/\tau_1$ on S$^6$-GTP concentration. The final concentration of tubulin in all reactions was 1 $\mu$M. $1/\tau_1$, the rate constant for the rapid bimolecular step of the binding reaction, was obtained by computer analysis of the data as described under Materials and Methods. $1/\tau_1$ was plotted against the S$^6$-GTP concentration, and the data were fit by linear regression analysis. This analysis yielded a correlation coefficient of 0.993. For a bimolecular binding reaction, $1/\tau_1 = k_1[S^6\text{-GTP}] + k_{-1}$. From the intercept on the $y$ axis, $k_{-1}$ was found to be 2.27 s$^{-1}$. $k_1$ was calculated from the slope and found to be $5.94 \times 10^5$ M$^{-1}$ s$^{-1}$. From the ratio $k_{-1}/k_1$, the equilibrium constant for the bimolecular step was calculated to be 3.82 $\mu$M.

to Tu(−) in buffer containing 25% (w/v) glycerol (PMG buffer). Figure 1 shows the kinetics of the change in tubulin intrinsic fluorescence when 2 $\mu$M Tu(−) was mixed with 20 $\mu$M S$^6$-GTP. The change in fluorescence was biphasic and could not be fit to an equation describing a single exponential decay. However, the observed decay curve was readily fit to an equation describing a two-exponential decay process:

$$\Delta F_t = a_1e^{-t/\tau_1} + a_2e^{-t/\tau_2}$$

Thus, binding is a complex process involving at least two sequential steps (Wu et al., 1976a).

In Figure 1, the relative fluorescence intensity resulting when Tu(−) was mixed with buffer lacking S$^6$-GTP was taken as the reference point with a value of 1.0. The fluorescence intensity at the earliest observable time following mixing with S$^6$-GTP had a value of approximately 0.95. Thus, there is a significant decrease in fluorescence intensity that occurs too quickly to be observed by fluorescence stopped-flow technique. This most probably is due largely to inner filter effects since $10^{-5}$ M S$^6$-GTP has significant absorption in the regions used for both excitation and emission of fluorescence. It is also possible that this decrease may be due in part to some aspect of binding that occurs too rapidly to be resolved by stopped-flow technique. If such events occur they could only be resolved by further studies involving temperature-jump technique.

As described under Materials and Methods, analysis of the concentration dependence of the rate constants for the observed relaxations provides information about the binding mechanism. Figure 2 shows the effect of increasing S$^6$-GTP concentration on the rate constant ($1/\tau_1$) for the faster reaction. As can be seen from the figure, $1/\tau_1$ shows a linear dependence on S$^6$-GTP concentration over the range of concentrations examined (5–32 $\mu$M) (the strong absorbance of S$^6$-GTP precluded experiments at higher concentrations due to inner filter effects). Thus, the faster process appears to correspond to the rapid bimolecular step of the binding reaction. A linear least-squares fit of the data yielded a value for $k_1$ of $5.94 \times 10^5$ M$^{-1}$ s$^{-1}$ and a value for $k_{-1}$ of 2.27 s$^{-1}$ according to

$$1/\tau_1 = k_{-1} + k_1[S^6\text{-GTP}]$$

The correlation coefficient for a linear least-squares fit to the

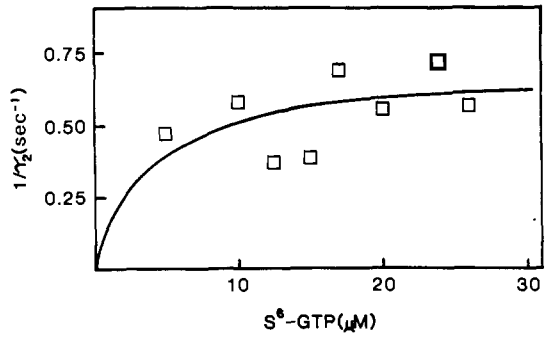


FIGURE 3: Dependence of $1/\tau_2$ on S$^6$-GTP concentration. $1/\tau_2$, the rate constant for the slower step in the binding reaction, was obtained from computer analysis of the data. The values obtained are plotted against S$^6$-GTP concentration. The solid liine is a theoretical curve fit to the observed data by assuming a rapid bimolecular association of tubulin with S$^6$-GTP followed by a slower unimolecular isomerization of the complex. For such a reaction, $1/\tau_2 = k_{-2} + k_2/(1 + K_1/[S^6\text{-GTP}])$. The value of the kinetic constants used for calculation of the theoretical curve were $k_{-2} = 0.006$ s$^{-1}$, $k_2 = 0.7$ s$^{-1}$, and $K_1 = 3.82$ μM.

data was 0.993. From the ratio of these constants, $k_{-1}/k_1$, a dissociation constant ($K_1$) of 3.82 μM was obtained for the first step of the binding reaction. This analysis assumes that no rapid preequilibrium step is involved. This assumption appears reasonable in view of the value obtained for $k_1$ ($5.9 \times 10^5$ M$^{-1}$ s$^{-1}$); however, further studies such as temperature-jump experiments would be required to demonstrate conclusively that there is no rapid preequilibrium of two conformational states of tubulin in the absence of bound nucleotide.

If the faster process represents the bimolecular binding reaction, the slower process must represent a step subsequent to binding. As described under Materials and Methods, the rate constant for a slower unimolecular isomerization step that occurs subsequent to binding will show a concentration dependence of the following form:

$$1/\tau_2 = \frac{k_2}{1 + (k_{-1}/k_1)/[L_0]} + k_{-2}$$

Thus, $1/\tau_2$ should tend toward a plateau at concentrations of $L_0$ much greater than $K_1$ with $1/\tau_2 \simeq k_{-2} + k_2$.

The effect of increasing concentrations of S$^6$-GTP upon the rate constant for the slower reaction is shown in Figure 3. Considerable scatter is observed in the data, but the rate constant for the slower reaction does appear to plateau: this is consistent with a reaction involving the rapid formation of a bimolecular complex and a slower unimolecular isomerization. We attempted to fit the data to an exponential of the type shown above with a nonlinear least-squares fitting procedure; however, due to the relatively large experimental variation in values for $1/\tau_2$, we were unable to achieve an adequate computer fit. Nevertheless, inspection of the data suggests that $1/\tau_2$ is tending toward a plateau of about 0.7 s$^{-1}$, consistent with a reaction involving formation of a bimolecular complex followed by a slower unimolecular isomerization.

Further information about the mechanism of binding was obtained by comparison of kinetic and equilibrium binding data. The equilibrium constant ($K_1$) calculated from the kinetic data was 3.82 μM. This value is almost a 100-fold larger than the overall apparent dissociation constant ($K_d = 3 \times 10^{-8}$ M) determined for the Tu·S$^6$-GTP complex by equilibrium fluorescence titrations (Fishback & Yarbrough, 1984). The overall apparent dissociation constant for a reaction involving rapid formation of a bimolecular complex followed by a slower unimolecular isomerization of the complex is given by the expression (Yarbrough et al., 1976)

$$K_d = \frac{K_1 K_2}{1 + K_2}$$

where $K_1 = k_{-1}/k_1$ and $K_2 = k_{-2}/k_2$. Thus, given $K_d = 3 \times 10^{-8}$ M and $K_1 = 3.82 \times 10^{-6}$ M, one can calculate a value for $K_2$ of $8 \times 10^{-3}$; $k_2$ must, therefore, be much greater than $k_{-2}$, and the equilibrium lies far toward the isomerized form of the complex. Consequently, the value of $1/\tau_2$ at the plateau in Figure 3 is approximately equal to $k_2$ (0.7 s$^{-1}$). On the basis of the values obtained for $K_1$, $K_d$, and $K_2$, one can then calculate that $k_{-2}$ is approximately equal to $5.5 \times 10^{-3}$ s$^{-1}$. This value is in reasonable agreement with the value estimated for $k_{-2}$ from the nucleotide exchange data described below.

*Kinetics of Displacement of GDP from 6S Tubulin by S$^6$-GTP.* The binding of S$^6$-GTP (or S$^6$-GDP) to tubulin produces a characteristic difference spectrum with a maximum at 350 nm and minima at 328 and 338 nm (Fishback & Yarbrough, 1984). By using this signal, it is possible to define the kinetic parameters for nucleotide exchange, thereby providing additional information about the protein–nucleotide interaction (Waechter & Engel, 1975, 1977; Neidl & Engel, 1979; Eccleston, 1981). We have used this approach to define the kinetic parameters associated with exchange of GTP, GDP, and other nucleotide analogues bound to tubulin.

The relevant equilibria for a simple displacement reaction are

$$\text{Tu·GXP} \underset{k'_1}{\overset{k'_{-1}}{\rightleftharpoons}} \text{GXP} + \text{Tu(-)} \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} \text{Tu·S}^6\text{—GTP} \qquad (\text{S}^6\text{—GTP})$$

If the concentration of displacing nucleotide is sufficiently high, if the equilibrium constant is sufficiently small so that the back-reaction can be neglected, and if essentially all tubulin exists initially as the Tu·GXP complex, the following condition is obtained:

$$\frac{\partial[\text{Tu·GXP}]}{\partial t} = k'_{-1}[\text{Tu·GXP}]$$

In other words, if $k'_2[S^6\text{-GTP}]$ is much greater than $k'_{-1}$, then the appearance of the difference spectrum associated with S$^6$-GTP binding should follow pseudo-first-order kinetics and the first-order rate constant obtained should be approximately equal to $k'_{-1}$. For a more complex reaction, such as the one proposed above for binding of S$^6$-GTP to tubulin, the apparent first-order rate constant for exchange is a function of the kinetic parameters for the two steps of the binding reaction, as will be described below.

Figure 4 shows the kinetics of the change in absorbance at 350 nm when S$^6$-GTP is added to 6S tubulin (10 μM in R buffer containing GDP at the exchangeable nucleotide binding site) to give a final S$^6$-GTP concentration of 40 μM. The kinetics of this process were not altered by the prior addition of stoichiometric amounts of GDP, consistent with our and others finding that nucleotide bound to the exchangeable nucleotide binding site following assembly is predominantly GDP. The data are well fit to a single-exponential decay curve with a first-order rate constant of 0.015 s$^{-1}$. To ensure that we were observing a true first-order process associated with dissociation of GDP, the final S$^6$-GTP concentration was raised to 80 μM. No significant differences in the kinetic parameters were observed. Moreover, the kinetics of displacement of GDP were comparable when monitored by fluorescence quenching associated with S$^6$-GTP binding. When S$^6$-GTP was added

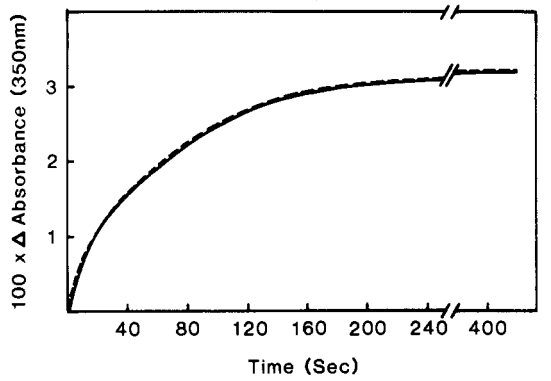


FIGURE 4: Kinetics of displacement of GDP from 6S tubulin by S$^6$-GTP in PMG buffer. S$^6$-GTP was added to a solution of 6S tubulin (10 $\mu$M) containing 0.9–0.95 mol of GDP/mol of tubulin, and the change in absorbance was measured at 350 nm. The final S$^6$-GTP concentration was 40 $\mu$M; temperature was maintained at 20 °C. (—) Experimental data; (--) theoretical curve calculated with the experimentally determined kinetic parameters. The first-order rate constant for the exchange reaction was 0.015 s$^{-1}$.

Table I: Kinetics of Dissociation of Tubulin–Nucleotide Complexes in Buffer Containing 25% Glycerol

| complex | displacing nucleotide | $10^3 k_e$ (s$^{-1}$)[a] |
|---|---|---|
| Tu·S$^6$-GTP[b] | GTP | 6 ± 0.6 |
| Tu·S$^6$-GDP[b] | GTP | 20 ± 0.5 |
| Tu·GDP[b,c] | S$^6$-GTP | 16 ± 3.0 |
| Tu·S$^6$-GTP[c] | GTP | 8 ± 1.3 |
| Tu·GDP[c] | S$^6$-GTP | 15 ± 3.5 |

[a] $k_e$ is the apparent first-order rate constant obtained from computer analysis of the data as described under Materials and Methods. Values given are the means and standard deviations of three to six samples. [b] Measurements were performed at 20 °C in PMG buffer. At least a 5-fold excess of displacing nucleotide was added and the change in absorbance at 350 nm monitored. [c] Measurements were performed in R buffer.

to solutions of Tu(−), 80–90% of the signal change occurred within the time for mixing. Thus binding in the absence of exchangeably bound nucleotide occurs very rapidly as indicated by the fluorescence stopped-flow studies. Therefore, we conclude that such an approach can be used to provide information about the kinetics of dissociation of nucleotides bound to the exchangeable nucleotide binding site of tubulin.

One possible complication of these studies could result if there is significant hydrolysis of added nucleotide. This is unlikely for several reasons. First, we have found that chromatography of microtubular protein on phosphocellulose removes more than 90% of nucleosidediphosphate kinase activity. Second, experiments were performed at 20 °C, which prevents assembly and thus assembly linked nucleotide hydrolysis. Third, experiments were usually completed within 5 min or less. Thus, we feel there is no significant nucleotide hydrolysis occurring during these experiments.

*Kinetic Parameters of Dissociation of Nucleotides from 6S Tubulin in Buffers Containing Glycerol.* Using the approach described above, we have determined the kinetic parameters for displacement of GDP, S$^6$-GDP, and S$^6$-GTP from 6S tubulin in buffers containing 25% (w/v) glycerol. The results are presented in Table I. In all cases, we find that the changes in absorbance follow apparent first-order kinetics. In both PMG and R buffers, the rate constant for dissociation of S$^6$-GTP is about (6–8) × 10$^{-3}$ s$^{-1}$ (Table I). Under the same conditions GDP has a dissociation rate constant of (15–16) × 10$^{-3}$ s$^{-1}$. A similar value of 20 × 10$^{-3}$ s$^{-1}$ is obtained for dissociation of S$^6$-GDP. Thus, both GDP and S$^6$-GDP exhibit comparable kinetics of dissociation, and dissociate 2–3 times as rapidly as S$^6$-GTP. Similar experiments were not performed to determine the kinetics of GTP release since the addition of GTP alone to solutions of Tu(−) produced a small time-dependent increase in absorbance, most likely due to formation of some aggregates. This effect was not seen when S$^6$-GDP or S$^6$-GTP was added to Tu(−). As a control in all experiments, we obtained the difference spectrum over the region 500–300 nm prior to undertaking kinetic studies. Any significant aggregation produced an increase in absorbance outside the thioguanine absorption band as a result of increased light scattering.

Table II: Kinetics of Dissociation of Tubulin–Nucleotide Complexes in Buffer Lacking Glycerol[a]

| complex | displacing nucleotide | $10^3 k_e$ (s$^{-1}$)[a] |
|---|---|---|
| Tu·S$^6$-GTP | GTP | 39 ± 6.3 |
| Tu·S$^6$-GDP | GTP | 80 ± 2.0 |
| Tu·GDP | S$^6$-GTP | 79 ± 13.5 |

[a] Measurements were performed at 20 °C in PM buffer and the data analyzed as described in Table I.

The fluorescence stopped-flow data presented above demonstrated that binding of S$^6$-GTP is a complex process involving at least one intermediate. The data are consistent with the rapid formation of a tubulin–nucleotide complex followed by a slower unimolecular isomerization of that complex. For such a reaction, the apparent first-order rate constant for the nucleotide exchange reaction ($k_e$) can be shown, from the method of Hammes & Schimmel (1970), to be given by the expression

$$k_e = \frac{k_{-1} + k_{-2}}{k_{-1} + k_2 + k_{-2}}$$

under conditions where the back-reaction can be neglected. Thus, since both $k_2$ and $k_{-1}$ are much greater than $k_{-2}$

$$k_e \approx \frac{k_{-1} + k_{-2}}{k_{-1} + k_2}$$

Given values for $k_{-1}$ and $k_2$ of 2.2 s$^{-1}$ and 0.7 s$^{-1}$, one can calculate that $k_{-2}$ is approximately equal to $1.3k_e$. The $k_e$ for dissociation of Tu·S$^6$-GTP was found to be (6–8) × 10$^{-3}$ s$^{-1}$ in buffer containing 25% (w/v) glycerol. This corresponds to a value for $k_{-2}$ of from 0.008 to 0.010 s$^{-1}$, in reasonable agreement with the value 5.5 × 10$^{-3}$ s$^{-1}$ estimated from the overall dissociation constant and the rapid kinetic data. In summary, both the rapid kinetic and nucleotide exchange kinetic data are consistent with a two-step mechanism for binding of S$^6$-GTP to tubulin, which involves the rapid formation of a bimolecular complex and at least one slower unimolecular isomerization of the complex.

*Kinetics of Nucleotide Displacement from Tubulin in Buffers Lacking Glycerol.* We have previously found that fluorescent analogues of GTP bind considerably more avidly to tubulin in buffers containing glycerol (Yarbrough & Kirsch, 1981). For example, the $K_d$ of the Tu·γ-AmNS-GTP complex is decreased from 23 $\mu$M to 3.2 $\mu$M by the addition of 25% (w/v) glycerol. We have therefore examined the kinetics of nucleotide dissociation in buffer lacking glycerol. Since tubulin from which the exchangeable nucleotide has been removed is unstable in the absence of glycerol and loses the ability to bind nucleotide or reassemble, experiments were performed within 1 h of preparation of Tu(−). The results are summarized in Table II. They show that dissociation of GDP, S$^6$-GDP, and S$^6$-GTP occurs much more rapidly in the absence of glycerol. For example, the apparent rate constant for dissociation of both GDP and S$^6$-GDP is about 0.08 s$^{-1}$, approximately 4–5 times

greater than is observed in the presence of 25% glycerol. However, in the presence or absence of glycerol, the nucleoside diphosphates dissociate about twice as rapidly as the nucleoside triphosphates.

## Discussion

Values for the bimolecular association rate constants for protein–ligand associations can vary over several orders of magnitude ranging from about $10^6$ to $10^9$ $M^{-1}$ $s^{-1}$ (Hammes & Schimmel, 1970). The value of $5.9 \times 10^5$ $M^{-1}$ $s^{-1}$ that we obtained for the bimolecular association rate constant of $S^6$-GTP with tubulin is much less than that calculated for a diffusion-controlled reaction. It is generally assumed that such a low value is due in part to stringent steric and orientation influences upon binding. The low value could also reflect the presence of a rapid preequilibrium, as noted earlier. For example, Laiken et al. (1972) observed two relaxations during fluorescence stopped-flow studies of the binding of inducer to the lac repressor. One relaxation was linearly dependent upon ligand concentration. Upon analysis of this relaxation, they obtained an apparent bimolecular rate constant for the binding reaction of only $6 \times 10^4$ $M^{-1}$ $s^{-1}$, which led them to suggest that a rapid preequilibrium might be occurring. This was subsequently confirmed by the temperature-jump experiments of Wu et al. (1976b). However, it should be noted that the apparent bimolecular rate constant we have obtained for tubulin–nucleotide binding is 1 order of magnitude larger than that observed for lac repressor–inducer binding.

Using a fluorescence stopped-flow technique, we have obtained data that suggest that binding of guanine nucleotides to tubulin is followed by changes in tubulin conformation that occur within a time scale of several seconds. Engelborghs & Eccleston (1982) have also performed more limited studies of the binding of $S^6$-GTP to tubulin by using fluorescence stopped-flow technique. They observed that the rate constant for the binding reaction decreased as the $S^6$-GTP concentration was increased and proposed a mechanism involving a rate-limiting conformational change prior to nucleotide binding. The limiting rate constant that they obtain is remarkably similar to the value we have obtained for dissociation of GDP from tubulin in the absence of glycerol if allowance is made for a small temperature effect on the rate of dissociation (our value of 0.08 $s^{-1}$ at 20 °C vs. their value of 0.12 $s^{-1}$ at 25 °C). In their experiments, they used gel chromatography to attempt to remove exchangeably bound nucleotide. We have found that gel chromatography does not completely remove bound nucleotide; following gel chromatography tubulin will usually contain 0.2–0.5 mol of GDP. Moreover, we found such tubulin to be incapable of assembly (Kirsch, 1979). Consequently, we have developed methods for nucleotide removal using charcoal that has been pretreated with serum albumin (Sandoval et al., 1977; Yarbrough & Kirsch, 1981). Second, their experiments were most likely complicated by inner filter effects. $S^6$-GTP has strong absorption bands both at 280 nm, which they used for excitation, and at the wavelength that they observed fluorescence (330 nm). For these reasons, we have not made measurements at $S^6$-GTP concentrations above 30 $\mu$M; in contrast, they have performed measurements at concentrations exceeding 150 $\mu$M. For these reasons, we believe that our data are somewhat more reliable and that proof of an equilibrium prior to binding will require temperature-jump measurements.

Whether the conformational change we have observed is related to the ability of tubulin to reassemble is not known. It is known, however, that many ligands modulate the ability of tubulin to form microtubules; these include colchicine, vinblastine, nocodazole, taxol, and others (Timasheff & Grisham, 1980). Garland (1978) has studied the kinetics of interaction of colchcine with tubulin and concluded that binding of this ligand occurs by a mechanism involving rapid bimolecular binding followed by a slower unimolecular isomerization of the complex. Taxol also alters the assembly and stability properties of tubulin, although the mechanism has not been clearly defined (Schiff et al., 1979; Schiff & Horowitz, 1981; Hamel et al., 1981). Since taxol eliminates the requirement for GTP in forming microtubules, it may also act to alter the conformation of tubulin. Thus, tubulins may be proteins with structures that are easily altered by ligands. Such a property could be instrumental in enabling tubulin to perform its many functions in cells.

Other data also suggest that tubulin may have a different conformation when GTP is bound. For example, Maccioni & Seeds (1983) have recently found that GTP alters the rate at which tubulin is digested by chymotrypsin. We have also obtained additional data suggesting that tubulin containing bound nucleotide has a different conformation. For example, we have found that the quenching of intrinsic tubulin fluorescence by acrylamide and iodide is altered by GTP. In addition, we have found that the rate of reaction of tubulin sulfhydryls with iodoacetic acid is decreased by about 50% in the presence of GTP (J. L. Fishback and L. R. Yarbrough, unpublished results). Thus, there is now considerable evidence that indicates a difference in tubulin conformation when GTP is bound. However, it appears that the conformational changes produced by nucleotide binding do not involve large changes in the amounts of $\alpha$ helix and $\beta$ structure present in tubulin. Maccioni & Seeds (1982) have reported that there is little or no alteration in the far-UV CD spectrum of tubulin when GTP is bound. We have confirmed these findings.

In our studies we have used the GTP analogue $S^6$-GTP. Can one draw conclusions about the mechanism of interaction of tubulin with GTP from studies with this analogue? We feel that one can for the following reasons: first, $S^6$-GTP is quite effective in promoting assembly of tubulin; second, microtubules formed with this analogue have normal stability properties; third, GTP and $S^6$-GTP bind to tubulin with very comparable affinities. Thus, in most ways that we have examined $S^6$-GTP behaves very similar to GTP. This is not unexpected since $S^6$-GTP has been found to replace GTP in several systems requiring GTP (Darlix et al., 1973; Eccleston & Trentham, 1977; Eccleston & Bayley, 1980; Eccleston, 1981).

We have previously demonstrated that glycerol causes a significant increase in the affinity of tubulin for nucleotides (Yarbrough & Kirsch, 1981). The present studies reveal that this increased affinity is largely due to a decreased rate of dissociation in the presence of this agent, in agreement with less quantitative observations made earlier by Arai et al. (1975). The mechanism whereby glycerol exerts such effects is not known, but it could involve changes in tubulin conformation. Timasheff and co-workers (Lee & Timasheff, 1977; Na & Timasheff, 1981) have found that glycerol does not promote changes in tubulin conformation detectable by CD measurements. However, glycerol could induce conformational changes not detectable by CD measurements.

Engelborghs & Eccleston (1982) have examined the kinetics of displacement of $S^6$-GTP from phosphocellulose-purified tubulin in buffer lacking glycerol by using stopped-flow techniques, at both 25 and 35 °C. They observed a first-order process and obtained rate constants of 5.2 $min^{-1}$ and 10 $min^{-1}$ at 25 and 35 °C, respectively. This corresponds to rate con-

stants of 0.087 $s^{-1}$ and 0.167 $s^{-1}$. Thus, the value of 0.039 $s^{-1}$ that we have obtained for the rate constant of $S^6$-GTP dissociation at 20 °C is in reasonable agreement with the value obtained by these workers considering differences in temperature and buffer conditions. Recently, Brylawski & Caplow (1983) have used an indirect enzymatic method to measure the rate of GDP release from tubulin. They calculated that the lower limit of the first-order rate constant for dissociation was 0.14 $s^{-1}$. Their experiments appear to have been performed at 37 °C. The rate constant we have obtained for displacement of GDP and $S^6$-GDP in the absence of glycerol at 20 °C (0.08 $s^{-1}$) is in agreement with the results obtained by these workers in view of the increased rates of GDP dissociation that have been observed at higher temperatures (Engelborghs & Eccleston, 1982; Arai et al., 1975).

In summary, we have been able to use the thioguanine nucleotide to effectively explore the thermodynamic, kinetic, and mechanistic aspects of the tubulin–nucleotide interactions. These analogues should prove useful for further studies required to understand the molecular mechanisms involved in promotion of tubulin assembly by guanine nucleotides.

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**Registry No.** $S^6$-GTP, 17670-19-8; $S^6$-GDP, 16541-19-8; GTP, 86-01-1; GDP, 146-91-8; glycerol, 56-81-5.

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