Equilibrium and Rapid Kinetic Studies on Nocodazole-Tubulin Interaction

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The interaction between nocodazole and calf brain tubulin in 10^{-2} \text{M} sodium phosphate, 10^{-4} \text{M} GTP, and 12\% (v/v) dimethyl sulfoxide at pH 7.0 was studied. The number of binding sites for nocodazole was shown to be one per tubulin monomer of 50,000 as a result of equilibrium binding studies by gel filtration and spectroscopic techniques. The presence of microtubule-associated proteins did not significantly affect the binding of nocodazole to tubulin. The apparent equilibrium constant measured at 25°C was (4 \pm 1) \times 10^{10} \text{M}^{-1}. Temperature does not significantly affect the apparent equilibrium constant; hence, the binding of nocodazole to tubulin is apparently entropy driven. Stopped flow spectroscopy was employed to monitor the rate of nocodazole binding under pseudo first order conditions. The effects of temperature and nocodazole concentration were studied. The apparent rate constants were dependent on the concentration of nocodazole in a nonlinear manner. In conjunction with results from structural and thermodynamic studies the kinetic results were interpreted to suggest a mechanism of $T + N \rightleftharpoons TN \rightleftharpoons T'N$, where $T$ and $N$ are tubulin and nocodazole, respectively. $T$ and $T'$ represent two conformational states of tubulin. Furthermore, the kinetic data are consistent with the thermodynamic data only if a model of two parallel similar reactions were considered, one rapid and the other slow. The initial binding step for both the rapid and slow phases was characterized by identical binding constants; however, there was a significant difference in the rates of isomerization. Hence, nocodazole is potentially a useful probe for amplifying differences in solution properties of tubulin subspecies.

Interactions between tubulin and drugs have been studied intensively by various laboratories with the aim of using drugs as perturbants of the microtubule system. To date, the drugs that are most intensely investigated include colchicine, vinblastine, and their respective derivatives. Results from these studies have proven to be quite useful in elucidating the assembly mechanism of tubulin, e.g. treadmilling (Margolis and Wilson, 1978), dissociation of $\alpha\beta$ dimers into $\alpha$ and $\beta$ monomers (Detrich et al., 1981), and the association of tubulin into larger aggregates with aberrant structures (Andreu and Timasheff, 1982a, 1982b, 1982c; Na and Timasheff, 1982; Andreu et al., 1984). Besides thermodynamic studies results from rapid kinetic experiments on the interaction between colchicine (Lambier and Engelborghs, 1981; Garland, 1978), colchicine analogue (Bane et al., 1984) and brain tubulin indicate complex mechanisms. The kinetic data are biphasic and may reflect the presence of two populations of tubulin interacting with these ligands. In an attempt to search for common factors governing tubulin-drug interactions, nocodazole is employed in studies from this laboratory.

The synthetic drug nocodazole (methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate) is reported to have antimitotic and antitumoral activity (DeBrabander et al., 1976; DeBrabander et al., 1975; Atassi and Tagon, 1975). The potential usefulness of this drug is due to its action being readily reversible, rapid, and specific toward malignant cells (DeBrabander et al., 1976). The target site of the drug is reported to be tubulin. In earlier studies it was shown that nocodazole inhibits the polymerization of brain tubulin in vitro (Hoebeke et al., 1976; Friedman and Platzer, 1978; Ireland et al., 1979; Lee et al., 1980), and the presence of microtubule-associated proteins does not amplify the inhibitory effect of the drug. Furthermore, results from structural studies show that the sulfhydryl residues become more accessible to chemical modification (Lee et al., 1980), indicating that binding of nocodazole induces significant structural changes in tubulin.

In spite of the potential clinical significance of nocodazole-tubulin interaction, the basic thermodynamic parameters which govern this interaction are still unknown. It was reported that nocodazole binds to purified brain tubulin dimer with a stoichiometry of one and the drug competitively inhibits colchicine binding to microtubule proteins even though there is no structural similarity between these two drugs (Hoebeke et al., 1976; Friedman and Platzer, 1978; Brodie et al., 1979). In view of the fact that colchicine binding to tubulin consists of a slow ligand-induced conformational change rendering the reaction essentially irreversible, the competition studies are not conducted at true equilibrium conditions. The results are much more difficult to analyze to reflect the thermodynamic interactions among these components. In view of the success of probing the solution properties of tubulin with drugs and the lack of thermodynamic and kinetic information on the interaction between nocodazole and tubulin, the aim of this study is to define the basic characteristics of nocodazole-tubulin interaction using both thermodynamic and kinetic approaches.

MATERIALS AND METHODS

The disodium salt of GTP was obtained from Boehringer Mannheim. Dimethyl sulfoxide was purchased from Sigma while nocodazole was from Aldrich. Extreme purity grade guanidine hydrochloride from Heico, Inc. was used after filtration through a sintered glass filter. Calf brain tubulin was purified by the Weisensberg procedure (Weisensberg et al., 1968; Weisensberg and Timasheff, 1970; Lee, 1982) and the polymerization and depolymerization method of Shalanski et al. (1973), as modified by Rung et al. (1979) with 90-min periods of centrifugation at 106,000 \times g and 4°C. These tubulin samples will be...
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referred to as W tubulin and C tubulin, respectively. Tubulin concentrations were determined spectrophotometrically in 6 mM guanidine HCl by using an absorbivity of 1.03 liters/(g·cm) at 275 nm (Na and Timasheff, 1981). All the binding studies were conducted in buffer consisting of 10 mM sodium phosphate, 10^{-4} M GTP, 12% (v/v) Me_{2}SO (PGD buffer) at 60,000 rpm.

Nocodazole binding was monitored by the method of Hirose and Kano (1971) at pH 7.0. Bio-Gel P-10 was washed, first with deionized distilled water, then with 95% ethanol, followed by drying of the gel at 80 °C for 12-16 h prior to the performance of the experiment in PGD buffer. 0.75 ml of buffer was added to 100 mg of Bio-Gel P-10 and allowed to equilibrate for 3 h, although control experiments have established that equilibrium is attained in about 1 h. Nocodazole and tubulin were then added, in a total volume of 0.45 ml, to the slurry and gently mixed by vortexing. The solutions were incubated for 20 min with five interruptions for mixing. Aliquots of the solution, free of gel particles, were withdrawn to determine the concentrations of nocodazole and tubulin. Procedures for data analysis are the same as that published by Oberfelder et al. (1984).

Binding of nocodazole to tubulin was also monitored by the gel filtration procedure of Hummel and Dreyer (1982) as modified by Fairclough and Fruton (1966). The experimental procedure was essentially the same as that published by Fairclough et al. (1975). The effect of temperature on binding of nocodazole to tubulin was monitored by an ultrafiltration technique using Centricron-30 microconcentrators from Amicon. 2-ml aliquots from solutions of tubulin at 1.0 mg/ml and varying concentrations of nocodazole were placed in the sample reservoirs of the microconcentrators, which were centrifuged for 5 min at 20,000 rpm in a Sorvall SS-34 rotor. The filtrate and retentate were collected and the concentrations of nocodazole in each fraction were monitored by UV absorbance using a molar extinction coefficient of 1.5 × 10^{4} at 320 nm.

Formation of tubulin-nocodazole complex can also be measured by spectrophotometry. Differences of spectra of tubulin in the presence of nocodazole were recorded on a Cary 118 spectrophotometer with water-jacketed square tandem cells (Hellma). The spectra were obtained with no observable differences between scans. The spectra were routinely recorded from 350 to 240 nm. Repeated scans were normally performed with no observable differences between scans.

The kinetics of nocodazole binding to tubulin were monitored by stopped flow measurements with a Durrum spectrophotometer in PGD buffer. The presence of dimethyl sulfoxide amplifies the necessity of very rigorous temperature control for the reagent reservoir and cell. Small differences in temperature would lead to artificial signals; hence, two water baths K-2/R water baths were employed to maintain uniform temperature control, one for the reagent reservoir and the other for the cell. Temperature settings on the two water baths were adjusted so that a flat baseline was observed when buffer solutions were mixed. Furthermore, it is essential to degas the buffer thoroughly to reduce other mixing artifacts. The reaction was monitored by a change in absorbance using a Cary 118 spectrophotometer with a Durrum spectrophotometer in PGD buffer. The presence of dimethyl sulfoxide amplifies the need for the use of water-jacketed square tandem cells (Hellma). The spectra were monitored by UV absorbance using a molar extinction coefficient of 10^{9} as used for the mean residue ellipticities of the tyrosine groups in a Cary model 118 instrument. The absorption of tubulin was monitored at room temperature as a function of pH, which was measured with a Radiometer PHM64 pH meter. The reference was a protein solution of identical concentrations at pH 7.0. The intrinsic dissociation constant, pK_{D}, of the tyrosine groups can be obtained by using the Linderstrøm-Lang equation.

\[
\log \frac{\alpha}{1 - \alpha} = pK_{D} - 0.868[Z] = pK_{eq}
\]

where \(Z\) is the average net charge of the protein at any pH, \(w\) is the electrostatic interaction parameter, and \(\alpha\) is the degree of ionization calculated as \(\alpha = \Delta\text{of} / \Delta\text{max}\). \(\Delta\text{of}\) and \(\Delta\text{max}\) are the observed and maximal molar absorbance changes, respectively.

RESULTS

Nocodazole is not very soluble in aqueous solutions; hence, it is necessary to include 12% (v/v) Me_{2}SO to enhance the solubility of the drug in the buffer employed. Inclusion of such a high content of organic solvent may alter the structure of tubulin. Results obtained under these conditions may have no relevance to the normal behavior of tubulin in aqueous solution. Hence, a series of tests were conducted to compare the basic solution behavior of tubulin in 12% Me_{2}SO with those in aqueous solution. Tubulin structure was monitored by circular dichroism, and the results showed that there is little or no perturbation of the tertiary structure of tubulin by 12% Me_{2}SO. The same effect can be observed with C tubulin, although the basic circular dichroic spectrum of C tubulin in aqueous buffer is different from that of W tubulin.

The effect of 12% Me_{2}SO on the ionization of tyrosine residues was also studied. Results of this study were analyzed according to the Linderstrøm-Lang equation (Equation 4), and a straight line plot was obtained. The straight line plot implies that all the tyrosine groups are ionized as independent groups with an apparent pK_{D} of 11.3, which is 0.4 pH unit

\[
T = T_{o} = Ae^{\alpha t} + Be^{-\alpha t}
\]

where \(T\) is time, \(T_{o}\) and \(T\) are the transmittance at infinite time and \(t\), respectively, and \(A\) and \(B\) are the amplitudes, and \(\alpha\) and \(\beta\) are the observed rate constants.

Sedimentation velocity studies were carried out with a Beckman-Spinco model E analytical centrifuge equipped with a UV scanner. 10^{-4} M GTP and sodium double-sector centerpieces with sapphire windows were used in an An-D rotor. All of the experiments were conducted at 60,000 rpm, pH 7.0, and 23 °C in PGD buffer. Sedimentation coefficients were determined from the midpoints of the scanner-traced boundaries.

The observed sedimentation coefficients were normalized to standard conditions by correcting for solvent density, viscosity, and preferred solvent interactions in various concentrations of Me_{2}SO in accordance to Schachman (1959).

\[
S_{20,w} = S_{o} \frac{\rho_{obs} \rho_{sol}}{(1 + 0.01\varphi_{s})^{1/2}}
\]

where \(\varphi\) is viscosity, \(\rho_{obs}\) and \(\rho_{sol}\) are the densities of the water and the solvent, respectively, and \(\varphi_{s}\) and \(\varphi\) are partial specific volumes of the protein in water and in the presence of organic solvent, respectively.

Viscosity of the solvent was determined at 20.00 ± 0.01 °C with an Oswald-type semimicroviscometer with a flow time of 137.5 s for distilled deionized water.

The apparent partial specific volume, \(\phi\), of tubulin in PGD buffer was obtained by measuring the densities of protein solutions of varying concentrations (5-20 mg/ml) and those respective solvents against which they had been dialyzed. The densities were measured with a precision density meter (Mettler/Parra DMA 2D) at 20.00 ± 0.01 °C. The concentration and density data were combined to obtain the apparent values of \(\phi\) at each protein concentration, \(c\), according to

\[
\phi_{t} = \frac{1}{\rho_{w}} \left( \frac{\rho_{w} - \rho_{w} \rho_{sol}}{c} \right)
\]

where \(\rho_{w}\) is the solvent density, \(\rho\) is the protein solution density, and \(c\) is the protein concentration in g/ml. The true values of \(\phi\) were obtained by extrapolation of the apparent values to zero protein concentration.

The conformation of tubulin was monitored by circular dichroism using a Cary model 60 spectropolarimeter equipped with a model 60 attachment. The spectra were routinely recorded from 350 to 240 nm. Overlapping spectra were obtained with 0.2-, 0.1- and 0.01-cm fused silica cells. A value of 190 was used for the mean residue weight of tubulin in the calculation of ellipticities, \(\Theta\). All runs were performed at ~23°C.

Tyrosine titrations were performed spectrophotometrically in a Cary model 118 instrument. The absorption of tubulin was monitored at room temperature as a function of pH, which was measured with a Radiometer PHM64 pH meter. The reference was a protein solution of identical concentrations at pH 7.0. The intrinsic dissociation constant, pK_{D}, of the tyrosine groups can be obtained by using the Linderstrøm-Lang equation.

The abbreviation used is: Me_{2}SO, dimethyl sulfoxide.
higher than that obtained in aqueous buffer (Lee and Lee, 1979). This is at least in part due to alterations of dielectric constant in the solution. The slope of the plot is 1.04 ± 0.07, which is in agreement with that in aqueous buffer (Lee and Lee, 1979). Hence, the inclusion of 12% Me$_2$SO has not selectively perturbed the environment of tubulin tyrosine residues.

The quaternary structure of tubulin was monitored by sedimentation velocity, and the results are shown in Fig. 1. After correcting for solvent density, viscosity, and preferential solvent interaction there is no difference between the weight average sedimentation coefficients, $s_{20,w}$, of tubulin in the presence of 12% Me$_2$SO and that in aqueous buffer. The correction for preferential solvent interaction is significant and the values of $\varphi_i$ in 10 and 20% Me$_2$SO are summarized in Table I. Without considering preferential solvent interaction the observed values for $s_{20,w}$ would be lower and may lead to an erroneous conclusion that tubulin dissociates into its monomeric units in the presence of Me$_2$SO. The study on preferential solvent interaction between Me$_2$SO and tubulin can be further analyzed to yield information on the interaction at the protein-solvent interface, since

$$(1 - \varphi_i) = (1 - \varphi_i - (1 - \varphi_i)(\delta g_i/\delta g_0)$$

where $\delta g_i/\delta g_0$ is the preferential solvent interaction parameter and $\varphi_i$ is the partial specific volume of Me$_2$SO. A negative value for $\delta g_i/\delta g_0$ implies a preferential hydration at the protein surface whereas a positive value is a reflection of preferential interaction between Me$_2$SO and tubulin. As summarized in Table I, $\delta g_i/\delta g_0$ assumes negative values, i.e. preferential hydration. The magnitude of preferential hydration apparently increases with higher concentration of Me$_2$SO. A preferential hydration is usually associated with a solvent which stabilizes protein (Timasheff et al., 1976; Lee and Lee, 1981); hence, it may be concluded that the presence of Me$_2$SO should stabilize tubulin rather than destabilize the protein. Such a conclusion is in good agreement with the results obtained in structural studies presented in this report. In addition, Me$_2$SO has been employed as a solvent to enhance the reconstitution of microtubule (Himes et al., 1976, 1977; Robinson and Engelborghs, 1982). Hence, tubulin retains its native structure in 12% Me$_2$SO, and the results reported in this study should reflect qualitatively the intrinsic nature of the reaction, although it is understood that the actual values of equilibrium and rate constants may vary with a change in specific solvent composition.

Having established that 12% Me$_2$SO does not significantly alter the basic properties of tubulin, the interaction between nocodazole and tubulin is studied by the Hirose and Kano procedure. Fig. 2 presents a binding isotherm of nocodazole to tubulin monomer of 50,000 in molecular weight. It is evident that the binding is specific and saturable at about 1.0 site/tubulin monomer under these experimental conditions. Binding of nocodazole to W tubulin is indistinguishable from that to C tubulin as indicated by the overlapping of data

![Fig. 2. Binding isotherm of nocodazole to calf brain tubulin in 10^-8 M sodium phosphate, 10^-4 M GTP, and 12% (v/v) Me$_2$SO at pH 7.0, 25 °C. Symbols and techniques employed are: O, W tubulin with Hirose-Kano; □, C tubulin with Hirose-Kano; ▲, W tubulin with Hummel-Dryer; and ▲, W tubulin with difference spectroscopy. The line is the best fit from a nonlinear least squares analysis of all the data points.](image)

**Table I**

<table>
<thead>
<tr>
<th>Me$_2$SO (v/v)</th>
<th>$\varphi_i$</th>
<th>$\varphi_i$</th>
<th>$(\delta g_i/\delta g_0)_{T=25^\circ}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>ml/g</td>
<td>ml/g</td>
<td>g/g</td>
</tr>
<tr>
<td>0</td>
<td>0.736 ± 0.002</td>
<td>0.740 ± 0.002</td>
<td>0.736 ± 0.03 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>0.740 ± 0.002</td>
<td>0.736 ± 0.003</td>
<td>0.736 ± 0.08 ± 0.01</td>
</tr>
<tr>
<td>20</td>
<td>0.740 ± 0.002</td>
<td>0.736 ± 0.003</td>
<td>0.736 ± 0.08 ± 0.01</td>
</tr>
</tbody>
</table>

![Fig. 3. Difference spectra of nocodazole-tubulin complex as a function of nocodazole concentration in 10^-3 M sodium phosphate, 10^-4 M GTP, and 12% (v/v) Me$_2$SO at pH 7.0, 25 °C. Symbols and nocodazole concentrations in 10^-5 M are: 0, 0; 1, 2; 2, 25; 3, 3; 4, 3.5; 5, 4; 6, 4.5; and 3, 4.](image)
points for both sets of experiments. It suggests that the presence of microtubule-associated proteins does not significantly influence the binding of nocodazole to tubulin. Binding of nocodazole was also monitored by the Hummel-Dryer procedure, and the results are also shown in Fig. 2. Both procedures yield results that are very similar.

The binding of nocodazole to W tubulin was also monitored by difference spectroscopy. Fig. 3 shows a typical set of difference spectra of titrating tubulin with increasing concentrations of nocodazole. The absorbance at 325 nm increases with increasing amounts of nocodazole and approaches a maximum value. Assuming that the change in absorbance at 325 nm is directly proportional to the binding of the nocodazole to tubulin it is possible to obtain association constants, and the data are shown in Fig. 2. Since all of the data sets obtained by various methods are essentially within experimental uncertainties, the data as shown in Fig. 2 were analyzed by a nonlinear least squares fitting program. It is evident from the solid curve shown in Fig. 2 that there is 1.0 binding site/tubulin monomer with an apparent association constant of \( (4 \pm 1) \times 10^6 \text{ M}^{-1} \) at 25 °C. The effect of temperature on nocodazole-tubulin interaction was monitored by both difference spectroscopy and ultrafiltration. Studies were conducted within the range of 4–30 °C. The results were analyzed by the van’t Hoff plot, as shown by the inset in Fig. 2. There is only a very small dependence of association constant on temperature, and a value of 1.1 kcal/mol was calculated for \( \Delta H \). Hence, it may be concluded that the binding of nocodazole to tubulin is entropy driven.

The mechanism of nocodazole-tubulin interaction was studied by stopped flow spectroscopy. A typical tracing of time dependence voltage change is shown in Fig. 4. The data were analyzed in accordance with a simple pseudo first order reaction scheme; however, the data clearly show curvature and can only be analyzed with the consideration of two phases, as shown in Fig. 5. The apparent rate constant of the slow phase was calculated from the linear portion of data shown in Fig. 5. The contribution of the slow phase was then subtracted from the total voltage change as a function of time. The residual voltage changes were ascribed to the fast phase. The solid line in Fig. 4 represents the simulated curve using Equation 1 and the parameters resolved in Fig. 5. It is evident that a good fit can be achieved with the simplest model which includes two phases.

The effect of nocodazole concentration on the apparent rate constants of the fast and slow phase at 25 °C is shown in Fig. 6. The range of nocodazole concentration available to this study is limited due to the low solubility of the ligand under these experimental conditions. The higher nocodazole concentrations employed in this study represent the upper limits of solubility. Within this narrow range of ligand concentration the apparent rate constants could be analyzed as a linear function of ligand concentration. The simplest mechanism to fit the data would be \( T + N \rightleftharpoons TN \). However, there is supporting evidence to indicate that tubulin undergoes a conformational change, e.g. nocodazole induces GTPase activity and increases accessibility of sulfhydryl groups (Lin and Hamel, 1981; Lee et al., 1980). Hence, the data were further analyzed for the mechanism of

\[
T + N \rightleftharpoons k_1 TN \rightleftharpoons k_3 T^*N
\]

where \( k_1 \) and \( k_2 \) are the forward and reverse rate constants of the binding reaction and \( k_3/k_1 \) equals \( K_d \), the dissociation constant for the initial formation of the \( TN \) complex, and \( k_3 \) and \( k_4 \) are the forward and reverse constants of the ligand-induced conformational change in tubulin. Adopting such a mechanism, the data can be further analyzed to obtain \( K_d \), \( k_3 \), and \( k_4 \), since (Strickland et al., 1976)

\[
K_d = \frac{k_3}{k_1 + k_4} \tag{7}
\]

In this analysis, the fast and slow phases are treated as independent parallel reactions, each of which involves a sim-
two-step mechanism. The rationale for such a decision will be elaborated under “Discussion.” The results are summarized in Table II and the curves generated with these parameters are shown in Fig. 6. Similar studies were conducted at 30 °C. Results were analyzed in a similar fashion and are summarized in Table II and Fig. 7. The binding of nocodazole is apparently favored at 25 °C; however, the equilibrium constant for tubulin isomerization, k3/k4, is favored at 30 °C. The same trend is observed for both fast and slow phase. The affinity of nocodazole for tubulin, as revealed by $K_d$, is essentially identical in the fast and slow phase under the same experimental condition. The equilibrium constants for tubulin isomerization are also identical between the two phases, although the rates of the fast phase are 3-4-fold greater. The ratio of the amplitudes of the slow and fast phases remains essentially constant as a function of nocodazole concentration and temperature and assumes a value of $2 \pm 0.4$.

The two phases may represent different reactivities of various tubulin oligomers. Since Mg$^{2+}$ is known to induce tubulin aggregation, the effect of Mg$^{2+}$ on the kinetics of nocodazole-tubulin interaction was also studied. Within experimental uncertainties, there is no observable difference between the data set obtained with or without 2 x 10$^{-5}$ M Mg$^{2+}$. $K_d$, k3, and k4 are essentially identical and the ratio of the amplitudes remains unchanged.

The physical state of tubulin under these experimental conditions was probed by sedimentation velocity. In PGD buffer and at 200 μg/ml to 2 mg/ml of tubulin, the protein sediments as a single component with a sedimentation coefficient $s_{20,w}$ of about 5.8 ± 0.5 S after correcting for preferential solvent interaction as shown in Fig. 1. Addition of 5 x 10$^{-5}$ M nocodazole does not induce any observable change in the value of $s_{20,w}$ in PGD buffer. However, in the presence of both 2 x 10$^{-3}$ M Mg$^{2+}$ and 5 x 10$^{-5}$ M nocodazole the value of $s_{20,w}$ increased to 6.2 S at 200 μg/ml of tubulin. The increase in $s_{20,w}$ implies the presence of tubulin aggregates larger than tubulin dimers. Yet, under these exact experimental conditions the kinetics of nocodazole-tubulin interaction remain unchanged suggesting that the aggregation of tubulin has no effect on the kinetics of this interaction or that the effect is small and cannot be detected presently.

**DISCUSSION**

Results from equilibrium ligand-binding studies showed that there are two binding sites/tubulin dimer. In these measurements the concentration of ligand-tubulin complex was determined by absorbance using the extinction coefficient of free nocodazole. However, difference spectroscopy yielded results indicating a change in absorptivity for complexed nocodazole; thus, it is essential to assess the effect of this change on the accuracy of the measurements. Under the experimental conditions at 5 x 10$^{-5}$ M nocodazole (i.e. at saturating concentrations) a maximum change in absorbance of 0.05 at 325 nm can be expected. This magnitude of change can only lead to a 7% overestimation, an uncertainty within the experimental error involved in this study. Hence, it is reasonable to conclude that there are two binding sites and not one/tubulin dimer. The latter value was reported by Hoebbeke et al. (1976). The source of the difference in stoichiometry is not clear, although there are quite significant differences in the experimental conditions employed. The binding study of Hoebbeke et al. was conducted at pH 6.4 and in the presence of 5 x 10$^{-5}$ M Mg$^{2+}$ and 5 mg/ml of bovine serum albumin. The low concentration of Mg$^{2+}$ has been shown in this report not to affect the kinetic measurements; hence, it may be concluded that it should not be a major factor in causing a change in stoichiometry. The effect of pH and high concentration of bovine serum albumin on the binding of nocodazole to tubulin is not known, although it has been reported that tubulin is less stable at low pH (Gaskin et al., 1974; Lee and Timasheff, 1977).

Results from equilibrium ligand-binding studies showed that these sites are independent and identical. Yet the kinetic data imply that there are two parallel reactions, each of which consists of a simple two-step mechanism. Are these observations consistent? Let us examine these data together.

The equilibrium binding data were employed to establish the limits within which the kinetic data were interpreted to provide a reasonable mechanism for tubulin-nocodazole interaction. The kinetic study has resolved the overall binding reaction into two exponential terms, implying that at least two sequential steps must be involved. The simplest two-step mechanism probably includes a rapid bimolecular nocodazole-tubulin binding step and a slow isomerization step, which can be associated with a conformational change in either nocodazole or tubulin. It is unlikely that the source of the slow phase is an isomerization of nocodazole since a biphasic observation was also reported for the interaction between tubulin and colchicine (Lambier and Engelborghs, 1981) or colchicine analog (Bane et al., 1984). There is very little structural resemblance between nocodazole and colchicine.

### Summary of kinetic parameters for nocodazole-tubulin interaction

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$K_d$ (Fast phase)</th>
<th>k3</th>
<th>k4</th>
<th>$K_{d(emp)}$ (Equation 9)</th>
<th>$K_d$ (Slow phase)</th>
<th>k3</th>
<th>k4</th>
<th>$K_{d(emp)}$ (Equation 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>M</td>
<td>s$^{-1}$</td>
<td>s$^{-1}$</td>
<td>M</td>
<td>M</td>
<td>s$^{-1}$</td>
<td>s$^{-1}$</td>
<td>M</td>
</tr>
<tr>
<td>25</td>
<td>0.9 ± 0.1 x 10$^{-4}$</td>
<td>1.7 ± 0.05</td>
<td>0.16 ± 0.05</td>
<td>0.9 ± 0.4 x 10$^{-3}$</td>
<td>0.7 ± 0.1 x 10$^{-4}$</td>
<td>0.6 ± 0.05</td>
<td>0.05 ± 0.03</td>
<td>0.7 ± 0.4 x 10$^{-3}$</td>
</tr>
<tr>
<td>30</td>
<td>3.2 ± 0.1 x 10$^{-4}$</td>
<td>10 ± 0.05</td>
<td>0.15 ± 0.05</td>
<td>0.5 ± 0.1 x 10$^{-3}$</td>
<td>2.5 ± 0.1 x 10$^{-4}$</td>
<td>2.5 ± 0.05</td>
<td>0.06 ± 0.03</td>
<td>0.6 ± 0.3 x 10$^{-3}$</td>
</tr>
</tbody>
</table>
Furthermore, they have different binding sites. On the other hand, there is direct evidence to implicate a conformational change in tubulin as a consequence of tubulin-nocodazole complex formation (Lee et al., 1980; Lin and Hamel, 1981). Hence, the slow isomerization step most likely reflects a conformational change in tubulin. If one accepts this assignment of the fast and slow phases to the binding and isomerization steps, respectively, the observed rate constants can be further analyzed to obtain values for $k_1$, $k_2$, $k_3$, and $k_4$ in Equation 6. The bimolecular step becomes pseudo first order with respect to nocodazole at high concentration of the ligand, then (Amdur and Hammes, 1966; Wu et al., 1976)

$$k_{obs} = k_0 + k_1 [N].$$

Analyzing the data obtained at 25°C, values of $k_1 = (1.6 \pm 0.2) \times 10^7$ M$^{-1}$ s$^{-1}$ and $k_2 = (0.18 \pm 0.03)$ s$^{-1}$ can be obtained. These rate constants lead to a $K_d$ of $1.1 \times 10^{-6}$ M since $K_d = k_2/k_1$. Under these same experimental conditions, the observed rate constants for the isomerization step is related to the bimolecular step as expressed in Equation 7. At infinite dilution of nocodazole, $k_{obs}$ approaches $k_s$ with a value of 0.01 s$^{-1}$. At high concentrations of nocodazole, Equation 7 can be rearranged to give

$$\frac{1}{k_{obs}} = \frac{1}{k_3} + \frac{1}{k_5} [N].$$

Hence, the intercept of a plot of $1/k_{obs}$ versus $1/[N]$ would yield $1/k_s$. Such an analysis yielded $k_3 = (0.25 \pm 0.07)$ s$^{-1}$ and $K_a = 1 \times 10^{-5}$ M. Knowing $k_a$, $k_b$, and $K_a$, it is possible to estimate the overall apparent equilibrium constant, $K_{d(app)}$, determined by equilibrium binding methods since

$$K_{d(app)} = K_a \times k_a/k_s.$$  

The estimated $K_{d(app)}$ assumes a value of $4 \times 10^{-7}$ M, which is different by more than an order of magnitude from the experimentally determined value of $2.5 \times 10^{-6}$ M (Fig. 4). One is then forced to conclude that the fast and slow phase of the kinetic data cannot be ascribed to the bimolecular binding and unimolecular isomerization step, respectively.

An alternate interpretation of the kinetic data is to ascribe the fast and slow phase to two independent reactions, each with a simple two-step mechanism, shown in Equation 6. Accordingly, the fast and slow phases were analyzed by Equation 7 independently to obtain two sets of values for $K_a$, $k_b$, and $k_c$. Having obtained these values, one can check for internal consistency by comparing the values of $K_{d(app)}$ obtained from equilibrium binding and kinetic data. Taking the values of the kinetic parameters summarized in Table II, the values of $K_{d(app)}$ calculated $(0.8 \pm 0.4) \times 10^{-5}$ M are in fair agreement with that determined by equilibrium techniques $(0.3 \pm 0.1) \times 10^{-5}$ M. Hence, it may be concluded that a simple consistent interpretation of the equilibrium binding and kinetic data is that there are at least two populations of tubulin, each of which react with nocodazole in a simple two-step mechanism of bimolecular binding followed by a unimolecular isomerization step. Furthermore, the calculated values for $K_{d(app)}$ for the two phases are identical; thus, it indicates that equilibrium techniques will not be able to detect the presence of apparent heterogeneity in the system. This prediction is in total agreement with the equilibrium binding data that the binding of nocodazole to tubulin is characterized by a single hyperbolic binding isotherm.

Closer examination of the kinetic data reveals some interesting features of the interaction between nocodazole and tubulin. In either the fast or slow phase, within experimental uncertainties the initial rapid ligand binding step is characterized by identical dissociation constants. The values of these constants are higher at 30°C than those at 25°C by a factor of 3-4. These results indicate that the initial binding of nocodazole to tubulin is favored by the lower temperature. Within experimental uncertainty, the equilibrium constant for the isomerization step, $k_3/k_5$, is also identical. However, temperature affects these steps in an opposite manner, i.e. isomerization is favored by higher temperature by a factor of 3-4, whereas ligand binding is favored to the same extent by lower temperature. Thus, the opposing effect of temperature on these two reaction steps offsets each other leading to the net result of no observable change in the value of $K_{d(app)}$. This conclusion is in good agreement with the results obtained by equilibrium ligand-binding study that there is little observable change in the apparent binding constants at these two temperatures.

Although there is no detectable change in the equilibrium parameters governing the interaction between nocodazole and tubulin, the fast and slow phase are characterized by a difference in $k_s$, the forward rate of isomerization. The fast phase is characterized by a $k_s$ that is about 3 or 4 times that of the slow phase. Apparently the effect of temperature is localized in $k_s$ without any significant influence on $k_a$.

Having identified the similarity and differences between the fast and slow phases, it is of interest to identify the physical properties of these two phases. Are they in equilibrium or do they represent noninteracting components which carry out similar reactions? An examination of the ratio of amplitudes for these two phases may provide some useful information. Evidently the ratio does not change with varying ligand concentration or temperature. Furthermore, presence of tubulin oligomers induced by addition of 2 mM Mg$^{2+}$ does not alter the results. Hence, it may be concluded that the ratio between the amplitudes of the fast and slow phases is constant under these experimental conditions. It implies that there are two similar and parallel reactions. The reactants in the fast and slow phases are not linked by rapid equilibrium that is highly sensitive to nocodazole concentration, temperature, or Mg$^{2+}$ ion. A likely explanation to these results is the heterogeneity of tubulin. Results from two-dimensional gel electrophoresis have demonstrated clearly the high degree of heterogeneity in tubulin (Field et al., 1984; Field and Lee, 1985). This conclusion is well supported by evidence from protein sequencing (Kraubs et al., 1981; Ponsting et al., 1981) and DNA sequencing (Hall et al., 1983; Valenzuela et al., 1981; Sullivan et al., 1983). It is conceivable that there are subtle differences in these subspecies enabling them to undergo isomerization at different rates. Such an interpretation is further supported by a recent report (Engelborghs and Fitzgerald, 1985). The challenge is to identify these subspecies, to characterize their physical and chemical properties, and to establish a biological relevance to the presence of heterogeneity in tubulin.

REFERENCES

Nocodazole-Tubulin Interaction


Brodie, A. E., Potter, J., and Reed, D. J. (1979) Life Sci. 24, 1547-1554


Runge, M. S., Detrich, H. W., III, and Williams, R. C., Jr. (1979) Biochemistry 18, 1688-1698


