Effect of Colchicine Analogues on the Dissociation of αβ Tubulin into Subunits: The Locus of Colchicine Binding†

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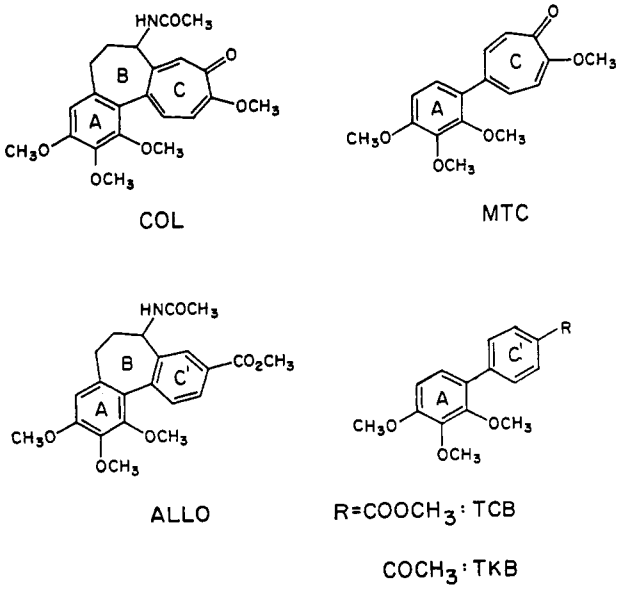
ABSTRACT: A combination of ligand binding and sedimentation equilibrium studies was used to characterize the thermodynamic linkages between αβ tubulin association, nucleotide binding, and the interaction of colchicine analogues with dimeric and dissociated tubulins. The strength of binding of allocolchicine to the tubulin dimer was identical (8×10^5 M^{-1}) whether the exchangeable nucleotide site (E site) was occupied by GTP or GDP. This drug bound to dimeric (αβ) tubulin and to one of the monomeric subunits, and the binding affinity for the dissociated state was linked to occupancy of the exchangeable nucleotide site. When the exchangeable site was occupied by GTP, the drug bound with very similar affinities to the dimeric and dissociated states of the protein. For tubulin-GDP, the binding of the drug to the dissociated state was significantly weaker (6.3×10^4 M^{-1}) than to the dimeric state, suggesting the existence of an E-site-related conformational change in the dissociated state. Podophyllotoxin, which contains the A-ring portion of colchicine, bound with equal affinity to the dimeric and dissociated forms of both tubulin-GTP and tubulin-GDP, indicating that it is the C-ring portion of colchicine that is linked to the E-site-related conformational change. Given that the nonexchangeable nucleotide site does not exchange with free nucleotide following dimer dissociation [Shearwin, K. E., Perez-Ramirez, B., & Timasheff, S. N. (1994) (preceding paper in this issue)], the colchicine binding site and the exchangeable site must be located on the same subunit; this is the β subunit [Geahlen, R. L., & Haley, B. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 4375–4377]. Examination of the free energy linkages between drug binding and tubulin dimer dissociation shows that the α subunit can contribute at most 10% of the free energy of binding of the drug to the dimer. It is proposed that the positioning of colchicine on the β subunit of tubulin is such that ring A is juxtaposed to the α–β subunit interface.

In the preceding paper in this issue (Shearwin et al., 1994), it was shown that αβ tubulin association is linked to the binding of Mg^{2+} ions. The observed effect is stronger on tubulin-GDP[1] than tubulin-GTP. In the absence of magnesium, the stability of both αβ dimers was found to be identical and it was concluded that tubulin-GTP and tubulin-GDP exist in the same conformation in magnesium-free solution.

Colchicine, an antimitotic drug, and its structural analogues (Chart I) are known to influence the self-assembly behavior of tubulin. For example, under conditions in which tubulin assembles into microtubules, the tubulin–colchicine complex polymerizes into anomalous structures, such as spirals and aggregated protofilaments (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b). In contrast, formation of another self-assembly product of tubulin, 42S double rings, is almost unaffected by this drug (Shearwin & Timasheff, 1992). Colchicine binding also induces in tubulin a GTPase activity (David-Pfeuty et al., 1977; Andreu & Timasheff, 1981). Furthermore, studies on the dissociation of αβ tubulin-GTP in the presence of colchicine have led to the conclusion that the binding of this drug stabilizes the dimeric structure (Detrich et al., 1982; Mejillano & Himes, 1989; Panda et al., 1992).

Chart I



Given the wide range of effects of these ligands on the conformation and the polymerization properties of the αβ dimer, it seemed of interest to investigate in greater depth their effects by examining the linkages between their binding, the occupancy of the E site, and the association of the α and β subunits. Furthermore, careful examination of the ther-

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[1] Abbreviations: ALLO, allocolchicine; E site, exchangeable nucleotide binding site; EDTA, ethylenediaminetetraacetic acid; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; N site, nonexchangeable nucleotide binding site; PODO, podophyllotoxin; TCB, 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl; TKB, 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl; TMB, 2,3,4,4′-tetramethoxy-1,1′-biphenyl; NAM, *N*-acetyl mescaline; TME, tropolone methyl ether.

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modynamic linkages between the binding of colchicine-like ligands and tubulin $\alpha\beta$ association should permit identification of the subunit (α or β) on which the colchicine binding site is located.

MATERIALS AND METHODS

The purification of tubulin and preparation of samples were performed as described in the preceding paper (Shearwin et al., 1994). Colchicine was from Aldrich Chemical Co. Allocolchicine (ALLO), TKB, TCB, and MTC were synthesized by Dr. M. J. Gorbunoff, as described elsewhere (Medrano et al., 1991). The tubulin–colchicine complex was prepared by incubation of the protein with an excess of colchicine for 10 min at 20 °C (Andreu & Timasheff, 1982b).

Ultracentrifugation. All sedimentation equilibrium experiments were carried out as before in a buffer consisting of 0.01 M sodium phosphate, 0.1 mM EDTA, and 10 μM nucleotide, pH 7.0, supplemented with the drug of interest. The data were analyzed by the methods described in the preceding paper (Shearwin et al., 1994).

In the absence of drug, the association constant, defined as $K_{\alpha\beta} = [\alpha\beta]/[\alpha][\beta]$, could be set numerically to $[\alpha\beta]/([\beta])^2$, since $[\alpha] = [\beta]$. In the presence of a drug however, the association constant, $K_{\alpha\beta}$, which must be defined again as [dimers]/[monomers]^2 is only an apparent quantity, $K_{\alpha\beta}^{app}$. Assuming that only one subunit binds the ligand and assigning this arbitrarily to the β subunit,[2] gives $[\alpha] = [\beta] + [\beta S]$ and

$$K_{\alpha\beta}^{app} = \frac{[\alpha\beta] + [\alpha\beta S]}{[\alpha]([\beta] + [\beta S])} \quad (1)$$

Introducing this into the sedimentation equilibrium equation (see preceding paper) gives

$$c_{T,r} = 2c_{\alpha,r_0} \exp\left[A_\beta M_\beta\left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right] + c_{\alpha\beta,r_0} \exp\left[A_{\alpha\beta} M_{\alpha\beta}\left(\frac{r^2}{2} - \frac{r_0^2}{2}\right)\right] \quad (2)$$

In each experiment, the data obtained from scans of three cells, each of which contained a different initial protein concentration (0.1–0.35 mg/mL), were fitted simultaneously, as described in the preceding paper (Shearwin et al., 1994).

Ligand Concentrations. The concentrations of colchicine and its analogues were determined spectrophotometrically using the following extinction coefficients: colchicine, 15 950 M^{-1} cm^{-1} at 353 nm; ALLO, 11 860 M^{-1} cm^{-1} at 288 nm and 4680 M^{-1} cm^{-1} at 315 nm; TCB, 12 100 M^{-1} cm^{-1} at 284 nm; TKB, 14 400 M^{-1} cm^{-1} at 295 nm; MTC, 17 600 M^{-1} cm^{-1} at 343 nm. For sedimentation equilibrium experiments, free drug concentrations were limited by drug solubility and by the maximum absorbance which could be compensated for by the ultracentrifuge optical system.

Ligand Binding. The binding of drugs to tubulin was followed fluorometrically in a Perkin-Elmer 650-40 spectrofluorometer. Temperature was controlled by a circulating water bath. Varying amounts of drug were added to a fixed concentration of protein, the samples were incubated at the required temperature for 1 h, and the fluorescence intensity was recorded. Narrow path length cells (10 × 2 mm) were used, with the shorter path length oriented in the excitation beam in order to minimize the inner filter effect. The fluorescence intensity of ligand solutions in the absence of protein was noted and the values, practically negligible, were subtracted from further measurements. Values of maximum fluorescence were calculated via the procedure of Shanley et al. (1985). Briefly, the total ligand concentration was used as a first estimate of free ligand concentration and (fluorescence (F)/free ligand) was plotted versus F. The abscissa intercept then gives a first estimate of F_{max}. Free ligand concentrations are then recalculated and the plot is repeated until no change is found in values of free ligand concentration. This procedure does away with the need to titrate a fixed amount of ligand with increasing amounts of protein and hence removes the uncertainty involved in the resulting double-reciprocal ($1/F$ vs $1/$[protein]) plot.

The excitation and emission wavelengths used were 315 and 390 nm for ALLO, 315 and 373 nm for TCB, and 350 and 423 nm for MTC. Slit widths were 5 and 10 mm for excitation and emission, respectively. The kinetics of ALLO binding were followed fluorometrically, the data (approximately 1000 points) being acquired directly by a personal computer. Data were fitted, via a nonlinear least-squares procedure, to the equation for a double exponential

$$F_t = A(1 - e^{-k_1 t}) + B(1 - e^{-k_2 t}) + C \quad (3)$$

where F_t is the fluorescence at time t, A and B are the amplitudes of the two phases, k_1 and k_2 are the rate constants, and C is an integration constant.

RESULTS

Effect of Colchicine and Its Analogues on the $\alpha\beta$ Association. A sedimentation equilibrium study was carried out of the effect of colchicine (COL), its reversibly binding analogue allocolchicine (ALLO), and a number of two-ring analogues (MTC, TCB, and TKB). The bulk of the study concentrated on ALLO, since its binding is reversible and it has favorable fluorescence and binding affinity characteristics for the present analysis. Values of the apparent dimerization constants, $K_{\alpha\beta}^{app}$, calculated from the sedimentation equilibrium data are listed in column 4 of Table I. A full description of these interactions, however, requires that all species be taken into account and that the analysis be carried out in terms of the complete thermodynamic box (Scheme I). In this scheme it is assumed that the drug (S) can bind to the dimer ($\alpha\beta$) and to only one of the dissociated monomers and that both liganded and unliganded protein can participate in the association–dissociation reaction. Such a system, in which self-association is linked to ligand binding, can be examined quantitatively in two ways—(i) the effect of drugs on the $\alpha\beta$ dissociation (horizontal equilibria in Scheme I) and (ii) the effect of dissociation on the binding of the drug (vertical equilibria)—yet it must be kept in mind that the two processes are linked.

Dissociation Study. Let us consider first the approach which distinguishes species on the criterion of molecular weight only. Application of Scheme I to eq 1 gives for the measured dimerization constant

$$K_{\alpha\beta}^{app} = \frac{[\alpha\beta](1 + K_{\alpha\beta S}[S])}{[\alpha][\beta](1 + K_{\beta S}[S])} = \frac{K_{\alpha\beta}(1 + K_{\alpha\beta S}[S])}{(1 + K_{\beta S}[S])} \quad (4)$$

Equation 4 shows that if $K_{\alpha\beta}$, the dimerization constant in the absence of ligand, and $K_{\alpha\beta S}$, the binding constant to the dimeric protein are known, the sedimentation equilibrium results can yield $K_{\beta S}$, the binding constant to the tubulin subunit.

All previous studies of the strength of binding of colchicine analogues to tubulin had been performed only on tubulin

[2] It is immaterial which subunit is assumed as binding the drug, since the two are stoichiometrically identical.

Table I: Binding Constants for Tubulin–Drug Interactions Deduced from Sedimentation Equilibrium

nucleotide	drug	concn (μM)	$K_{\alpha\beta}^{app}$ (M^{-1})	$K_{\alpha\beta}^{app}/K_{\alpha\beta}$	$K_{\alpha\beta S}$ (M^{-1})	$K_{\beta S}$ (M^{-1})
GTP			9.6×10^6 (–9.02)[a]			
GDP			1.2×10^7 (–9.15)			
GTP	ALLO	16	3.9×10^6 (–8.53)	0.41	8×10^5 (–7.65)[a]	2.0×10^6 (–8.12)[a]
GTP	ALLO	8.1	7.5×10^6 (–8.91)	0.78	8×10^5	1.0×10^6 (–7.77)
GTP	ALLO	5.6	1.3×10^7 (–9.20)	1.35	8×10^5	5.6×10^5 (–7.45)
GDP	ALLO	27.4	9.6×10^7 (–10.34)	8.00	8×10^5	6.8×10^4 (–6.26)
GDP	ALLO	16	9.4×10^7 (–10.33)	7.80	8×10^5	4.8×10^4 (–6.07)
GDP	ALLO	5.8	3.9×10^7 (–9.83)	3.25	8×10^5	1.3×10^5 (–6.61)
GTP	MTC	14.7	1.1×10^7 (–9.14)	1.15	5.2×10^5 (–7.40)[b]	4.4×10^5 (–7.32)
GDP	MTC	14.2	6.6×10^7 (–10.10)	5.50	5.2×10^5	3.7×10^4 (–5.92)
GTP	TCB	23.3	6.4×10^6 (–8.81)	0.67	1.3×10^5 (–6.63)[h]	2.2×10^5 (–6.91)
GDP	TCB	25.6	1.7×10^7 (–9.36)	1.42	1.3×10^5	8.0×10^4 (–6.35)
GTP	TKB	15	3.3×10^6 (–8.45)	0.34	1.6×10^5 (–6.74)[c]	6.0×10^5 (–7.49)
GDP	TKB	15	1.7×10^6 (–9.36)	1.42	1.6×10^5	9.3×10^4 (–6.44)
GTP	colchicine	[e]	2.1×10^7 (–9.48)	2.19	$\sim 2.7 \times 10^7$ (–9.63)[d]	1.2×10^7 (–9.19)[f]
GDP	colchicine		3.8×10^7 (–9.82)	3.17	$\sim 2.7 \times 10^7$	8.5×10^6 (–8.98)[f]
GTP	PODO	53.7	4.8×10^7 (–9.95)	5.00	5×10^5 (–7.38)[g]	8.5×10^4 (–6.39)
GDP	PODO	56.8	5.1×10^7 (–9.98)	4.25	5×10^5	1.0×10^5 (–6.49)

[a] Numbers in parentheses are the free energy values in kilocalories per mole. [b] Andreu et al. (1984). [c] Medrano et al. (1991). [d] Diaz & Andreu (1991). [e] Prepared as the tubulin–colchicine complex. [f] Calculated from equation 7. [g] Cortese et al. (1977). [h] Medrano et al. (1989).

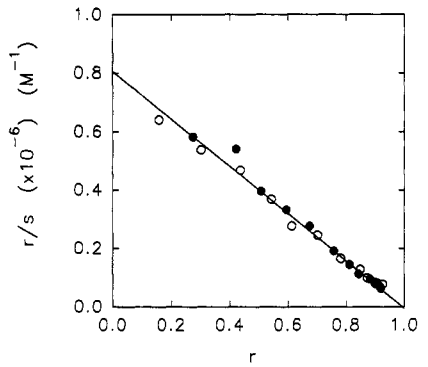
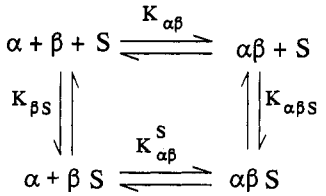


FIGURE 1: Binding study to determine the affinity ($K_{\alpha\beta S}$) of ALLO for dimeric tubulin-GTP (open symbols) and dimeric tubulin-GDP (solid symbols), presented as a Scatchard plot. Binding was measured by fluorescence titration at 25 °C. The protein concentration was 6 μM in each case.

Scheme I



containing GTP at the E site (Medrano et al., 1989, 1991; Hastie, 1989). Since the dissociation studies were done on tubulin in both states of E-site occupancy, experiments were carried out to determine whether the nucleotide that occupies the exchangeable site affects the affinity of tubulin for the drugs. The binding of ALLO to tubulin-GTP and tubulin-GDP was measured, therefore, by following the change in ligand fluorescence intensity upon binding to tubulin under conditions where the protein exists predominantly as the $\alpha\beta$ heterodimer (25 °C, 6 μM tubulin). The results are shown in Figure 1 in the form of a Scatchard plot. Identical binding constants ($K_{\alpha\beta S}$ = 8.0×10^5 M^{-1}) were obtained for tubulin-GTP and tubulin-GDP, demonstrating that the E-site nucleotide does not affect the interaction of ALLO with dimeric ($\alpha\beta$) tubulin. This result is consistent with the conclusion of the preceding paper (Shearwin et al., 1994) that tubulin-GTP and tubulin-GDP exist in the same conformation in the absence of magnesium.

The nature of the dependencies of $K_{\alpha\beta}^{app}$ on the free concentration of the drug [S], predicted by eq 4, was calculated

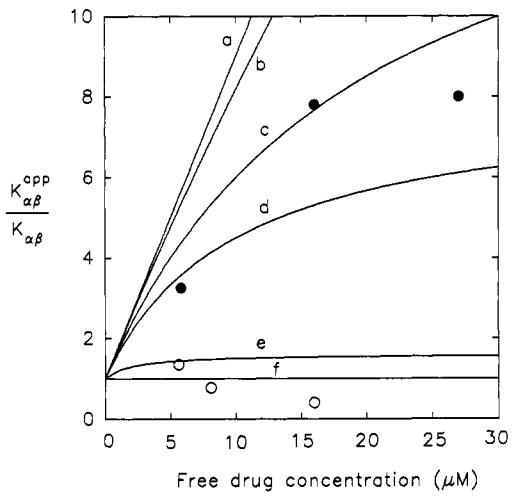


FIGURE 2: Effect of free drug concentration on the association of the α and β subunits of tubulin. The ratio of the apparent association constant in the presence of ligand to the intrinsic association constant, obtained in the absence of ligand ($K_{\alpha\beta}^{app}/K_{\alpha\beta}$), was calculated as a function of free ligand concentration for various values of $K_{\beta S}$, according to eq 4. $K_{\alpha\beta S}$ was fixed at 8×10^5 M^{-1}, the binding constant for the interaction of ALLO with dimeric tubulin. The values of $K_{\beta S}$ used were (a) 0, (b) 1×10^4, (c) 5×10^4, (d) 1×10^5, (e) 5×10^5, and (F) 8×10^5. The symbols represent the results of sedimentation equilibrium experiments performed on tubulin-GTP (○) or tubulin-GDP (●), in the presence of known free concentrations of ALLO. Experiments were carried out in 0.01 M sodium phosphate, 0.1 mM EDTA, and 10 μM nucleotide, pH 7.0 at 10.0 °C.

for the various possible relations between $K_{\alpha\beta}^{app}$ and $K_{\alpha\beta}$, i.e., between the apparent association constant obtained in the presence of various patterns of drug binding and the intrinsic association constant obtained in its absence. In this calculation, $K_{\alpha\beta}$ was set at 9.6×10^6 M^{-1} [the value obtained for tubulin-GTP; see preceding paper (Shearwin et al., 1994)], $K_{\alpha\beta S}$ at 8×10^5 M^{-1} (the value for ALLO binding to dimeric tubulin), and the ratio $K_{\alpha\beta}^{app}/K_{\alpha\beta}$ was calculated over a range of $K_{\beta S}$ values (strength of binding of drug to monomer). The dependencies for three cases are shown in Figure 2. Curve a corresponds to the limiting case in which the drug does not bind to monomer, i.e., $K_{\beta S} = 0$. Equation 4, then, reduces to

$$\frac{K_{\alpha\beta}^{app}}{K_{\alpha\beta}} = 1 + K_{\alpha\beta S}[S] \qquad (5)$$

and $K_{\alpha\beta}^{app}/K_{\alpha\beta}$ increases linearly with increasing drug concentration. If the tubulin monomer and dimer were to bind

the drug with equal affinity, then

$$\frac{K_{\alpha\beta}^{app}}{K_{\alpha\beta}} = 1 \quad (6)$$

for all concentrations of the drug (curve f). The results expected for the situation where the drug binds to the monomeric protein but with a lower affinity than to the dimeric protein ($K_{\beta S} < K_{\alpha\beta S}$) are shown for various values of $K_{\beta S}$ by curves b–e.

The points on Figure 2 are the experimental results obtained in the presence of known free concentrations of ALLO for tubulin-GTP (open circles) and for tubulin-GDP (solid circles). It is evident that, for tubulin-GDP, dissociation of the protein lowers the affinity. Solution of eq 4 with these values of $K_{\alpha\beta}^{app}/K_{\alpha\beta}$ and $K_{\alpha\beta S}$ fixed at 8×10^5 M^{-1} and $K_{\alpha\beta}$ set to its experimental value [see preceding paper (Shearwin & Timasheff, 1993)] yielded mean values of $K_{\beta S} = 1.0 \times 10^6$ M^{-1} ($\Delta G^\circ = -7.77$ kcal/mol) for tubulin-GTP and 7.3×10^4 M^{-1} ($\Delta G^\circ = -6.31$ kcal/mol) for tubulin-GDP (final column, Table I). These results show that the binding affinities of ALLO to monomeric ($\alpha + \beta$) and dimeric ($\alpha\beta$) tubulins-GTP are approximately equal. In the case of tubulin-GDP, however, the affinity of ALLO for the protein subunit is ca. 14 times lower than its affinity for the dimeric $\alpha\beta$ species. Hence, there is essentially a 1 order of magnitude difference in the binding affinities of this drug for the two forms of monomeric tubulin. Similar results were obtained for the other reversibly binding colchicine analogues that contain both rings A and C (or C′), for which it was assumed, by analogy with ALLO, that $K_{\alpha\beta S}$ is equal for tubulin-GTP and tubulin-GDP. The results are listed in Table I. For each of the compounds examined, the affinity of the drug for the monomeric species was lower for tubulin-GDP than for tubulin-GTP. On the other hand, podophyllotoxin, which contains only the trimethoxyphenyl moiety (ring A) of colchicine, bound with equal affinity to the GTP and GDP forms of tubulin in the dissociated state, but the binding to the dissociated form was weaker than that to the dimer. In the case of colchicine, precise quantitation of the binding affinities via eq 4 is difficult, since the kinetics of colchicine binding are very slow (Garland, 1978) and the free concentration of drug cannot be defined accurately. Therefore, samples were prepared as the stable tubulin–colchicine complex in which the binding sites are saturated. The apparent association constant obtained in sedimentation equilibrium studies then is $K_{\alpha\beta}^S$, i.e., the constant of the fully liganded species (Scheme I). It follows that

$$\frac{K_{\alpha\beta}^S}{K_{\alpha\beta}} = \frac{K_{\alpha\beta S}}{K_{\beta S}} \quad (7)$$

Using this relation, the values of $K_{\alpha\beta}^S$ determined for the colchicine–tubulin-GTP and -GDP, and the binding constant ($K_{\alpha\beta S}$) deduced by Diaz and Andreu (1991), values of $K_{\beta S}$ were obtained for tubulin-GTP and for tubulin-GDP. Therefore, just as the analogues, colchicine binds to the dissociated form of tubulin with a 2–3-fold lower affinity than to the dimeric species, a result consistent with the conclusion of Detrich et al. (1982) that colchicine binding stabilizes the $\alpha\beta$ dimer.

Binding Study. Having obtained estimates of the relative binding affinities of several colchicine analogues for the two association states of tubulin in the two occupancies of the E site, let us turn now to the examination of the second approach, that of the interaction through measurements of binding. This distinguishes species only on the criterion of liganding and is blind to molecular weight changes. For a system described

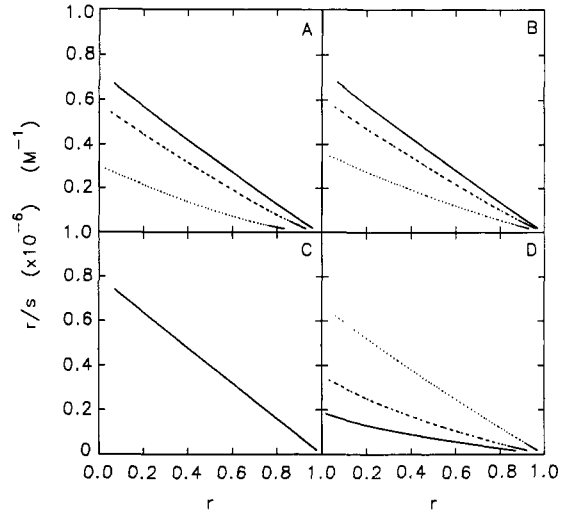


FIGURE 3: Scatchard plots expected for binding studies performed on the system shown in Scheme I. The forms of the Scatchard plots were calculated by substituting values of the various binding constants into eq 8. $K_{\alpha\beta}$ was fixed at 9.6×10^6 M^{-1} (the association constant for tubulin-GTP) in each case. Solid lines represent the result when the total protein concentration (C_T) = 10 μM, dashed lines represent $C_T = 1$ μM, and dotted lines represent $C_T = 0.1$ μM. The magnitudes of the ligand binding constants for each plot are (A) $K_{\alpha\beta S} = 8 \times 10^5$, $K_{\beta S} = 0$; (B) $K_{\alpha\beta S} = 8 \times 10^5$, $K_{\beta S} = 1 \times 10^5$; (C) $K_{\alpha\beta S} = K_{\beta S} = 8 \times 10^5$; and (D) $K_{\alpha\beta S} = 1 \times 10^5$, $K_{\beta S} = 1 \times 10^6$ M^{-1}.

by Scheme I, the binding isotherm, expressed as extent of liganding, r, is defined as follows (the derivation is given in the Appendix):

$$r = \{K_{\beta S}[\beta][S] + K_{\alpha\beta S}K_{\alpha\beta}[\beta]^2[S](1 + K_{\beta S}[S])\}/ \{K_{\alpha\beta}[\beta]^2(1 + K_{\beta S}[S]) + K_{\alpha\beta S}K_{\alpha\beta}[\beta]^2[S](1 + K_{\beta S}[S]) + K_{\beta S}[\beta][S] + [\beta]\} \quad (8)$$

where $[\beta]$ is the concentration of the β subunit, [S] is the concentration of the ligand, and the various equilibrium constants are defined by Scheme I (see also Appendix). This relation permits construction of Scatchard (1949) plots for various combinations of $K_{\beta S}$, $K_{\alpha\beta S}$, $K_{\alpha\beta}$, C_T, and [S]. It should be noted that the right-hand side of eq 8 contains a term in total protein concentration (C_T) (see eq A4b). The Scatchard plots, therefore, can be expected to be dependent on protein concentration. Figure 3 shows theoretical plots, based on eq 8, expected for various combinations of ligand binding constants and protein concentrations. In each case the dimerization constant in the absence of drug ($K_{\alpha\beta}$) was again fixed at 9.6×10^6 M^{-1}, the value obtained experimentally for tubulin-GTP at pH 7.0. In Figures 3A–C, $K_{\alpha\beta S}$ was set at 8×10^5 M^{-1}, the binding constant of ALLO to tubulin-GTP. Four basic cases were treated. In case I the monomer does not bind the ligand ($K_{\beta S} = 0$, Figure 3A). The very slightly curvilinear Scatchard plots exhibit a marked protein concentration dependence; they are very slightly curvilinear and the slope increases with increasing protein concentration. In case II, the tubulin monomer binds the ligand with a lower affinity than the dimer ($K_{\beta S} < K_{\alpha\beta S}$, Figure 3B). The slightly curvilinear Scatchard plots have a protein concentration dependence similar to that of case I. In case III, the monomer and dimer bind the ligand with equal affinity (Figure 3C). Concentration dependence is abolished and all points fall on the same straight line. The slope in this case is equal to $K_{\beta S}$ ($=K_{\alpha\beta S}$). In case IV, the monomer binds the ligand more strongly than does the dimer ($K_{\alpha\beta S} < K_{\beta S}$). The slightly curvilinear plots (Figure 3D) are again concentration-dependent, but the order is reversed from cases I and II, i.e., as the protein concentration is increased, the apparent slope

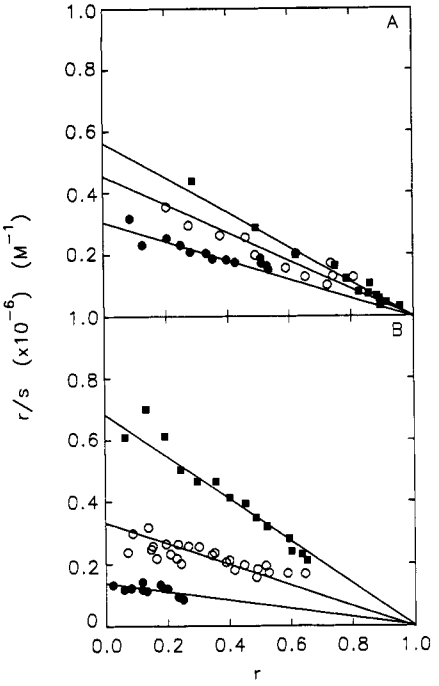


FIGURE 4: Scatchard plots of the binding of ALLO to (A) tubulin-GTP and (B) tubulin-GDP at 10 °C. Data were obtained by measuring the increase in fluorescence emission intensity of the ligand upon binding to tubulin, following a 1-h incubation. Protein concentrations were (A) 0.054 μM (●), 0.54 μM (○), and 4.83 μM (■) and (B) 0.050 μM (●), 0.503 μM (○), and 4.89 μM (□). The lines represent the apparent slope only.

of the Scatchard plot decreases. Comparison of cases I and II (Figure 3A,B) shows that this technique is relatively insensitive to changes in $K_{\beta S}$ when $K_{\beta S}$ is small ($<10^5$). Furthermore, for the three cases which exhibit curvilinearity, the deviations from linearity are so small that, given experimental uncertainty, they would be undetectable.

Figure 4 presents the results of binding measurements of ALLO to tubulin, carried out by fluorescence titration at 10 °C. Protein concentrations were in the range 0.05–4.9 μM. It was assumed that the quantum yields of the tubulin–drug interaction are identical for the four protein species involved (dimeric and dissociated tubulin-GTP and tubulin-GDP). The results for both tubulin-GTP (Figure 4A) and tubulin-GDP (Figure 4B) display an obvious dependence upon tubulin concentration. The apparent slopes of the Scatchard plots increase with an increase in tubulin concentration. Hence, by the prediction of Figure 3B, $K_{\beta S} < K_{\alpha\beta S}$. Values of $K_{\beta S}$ and $K_{\alpha\beta S}$ were obtained by analyzing the data in Figure 4 in terms of eq 8. All the data for a given nucleotide state of tubulin were fitted globally to eq 8 by nonlinear least squares, using the values of $K_{\alpha\beta}$ given earlier. The results are listed in Table II. Their comparison with those deduced from sedimentation equilibrium shows substantial agreement between the thermodynamic parameters of tubulin–ALLO interaction obtained from the examination by independent means of the two aspects of the thermodynamic box (Scheme I), namely, protein self-association and ligand binding.

Other Results. None of the other systems examined by ultracentrifugation could be subjected to binding studies: colchicine because of its very slow binding kinetics (Garland, 1978; Diaz et al., 1991) and TKB and MTC because they do not exhibit sufficient fluorescence intensity to permit accurate quantitation of their binding at low protein concentrations. For TCB, because of its relatively weak binding, fluorescence titration data could not be obtained with sufficient precision to determine meaningful values of $K_{\beta S}$ from the fitting procedure.

In the above studies, dissociation of $\alpha\beta$ tubulin could be detected only if the experiments were performed at low temperature (10 °C). Since the binding of ALLO is slow (Medrano et al., 1989; Hastie, 1989), an incubation period of 1 h was required to ensure attainment of equilibrium. If this incubation was carried out at 25 °C, no concentration dependence of binding was observed, and the binding constant was always on the order of 8×10^5 M^{-1} for tubulin-GTP and tubulin-GDP both. Correspondingly, essentially no dissociation into monomers could be detected by sedimentation equilibrium at 25 °C.

In order to see if the time course of ligand binding was affected by the state of $\alpha\beta$ tubulin association or the nature of the nucleotide that occupies the exchangeable site, the kinetics of binding of the slowly interacting drug ALLO were followed at 10 °C by monitoring the increase in fluorescence emission intensity. In agreement with previous results (Medrano et al., 1989; Hastie, 1989), the kinetic curves were best fit as the sum of two exponentials, where the magnitude of the slow phase was small compared to that of the fast phase. Only the results for the fast phase are presented. The apparent first-order rate constant was obtained at several drug concentrations for tubulin-GTP (Figure 5A) and tubulin-GDP (Figure 5B) at 10 °C. The slopes of the plots, which give the apparent second-order rate constant, were identical, within experimental error, for tubulin-GTP (125 ± 4 M^{-1} s^{-1}) and tubulin-GDP (134 ± 5 M^{-1} s^{-1}). Decreasing the tubulin concentration from 8.7 μM (~90% dimer) to 0.87 μM (~70% dimer) did not affect the rate constant for either tubulin-GTP or tubulin-GDP. Therefore, neither E-site occupancy nor dissociation of $\alpha\beta$ tubulin affects the rate of drug binding.

DISCUSSION

The Colchicine Binding Site Is on the β Subunit of Tubulin. The interaction of the antimitotic drug colchicine, and its analogues, with tubulin was studied by examining the thermodynamic linkages between binding of the drug and tubulin $\alpha\beta$ association (Scheme I). In this model, only one of the subunits was considered capable of binding the drug, since binding stoichiometries greater than 1 have never been reported for the interaction of colchicine or its analogues with tubulin. A similar scheme had been considered by Detrich et al. (1982) for colchicine. The detailed studies were carried out with the colchicine analogue ALLO, since its binding is reversible and it mimics COL in all its tubulin-directed properties. The present study has shown that the monomeric form of tubulin is indeed capable of interacting with colchicine-like drugs. Therefore, the 2–3-fold stabilization of the $\alpha\beta$ tubulin-GTP dimer by colchicine reported in other studies (Detrich et al., 1982; Mejillano & Himes, 1989; Panda et al., 1992) appears to be linked to stronger binding of the drug to the dimeric form of the protein.

A particularly striking result is the finding that the strength of the interaction of dissociated tubulin with the drug ligands was dependent on the nature of the nucleotide that occupies the nucleotide binding site. The dissociated form of tubulin-GTP consistently exhibited a higher affinity for the colchicine analogues than did the dissociated form of tubulin-GDP. There are two possible means by which the free energy of ligand binding to a tubulin subunit could be influenced by (be linked to) the nature of the nucleotide liganding state of tubulin. The first explanation is that the exchangeable nucleotide site and the colchicine binding site are located on the same subunit and that the conformational differences between the subunit in the GTP and GDP states are reflected in the affinity of that subunit for the drug. The second possibility is that, upon

Table II: Comparison of Binding Constants Obtained by Analytical Ultracentrifugation and Ligand Binding

	sedimentation equilibrium		ligand binding	
nucleotide	$K_{\alpha\beta S}$[a] (M^{-1})	$K_{\beta S}$ (M^{-1})	$K_{\alpha\beta S}$ (M^{-1})	$K_{\beta S}$ (M^{-1})
GTP	8.0×10^5 (–7.65)[b]	1.0×10^6 (–7.77)	6.4×10^5 (–7.52)	2.2×10^5 (–6.92)
GDP	8.0×10^5 (–7.65)	6.3×10^4 (–6.31)	6.1×10^5 (–7.50)	5.0×10^4 (–6.09)

[a] Obtained from Figure 1. [b] Numbers in parentheses are the corresponding free energy values in kilocalories per mole.

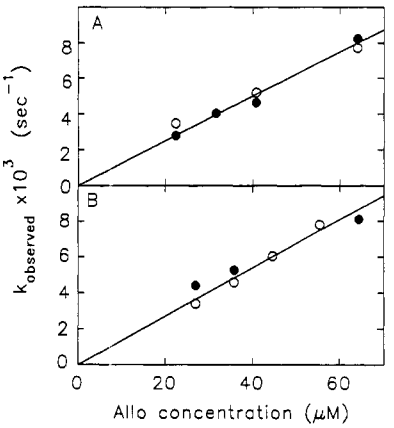
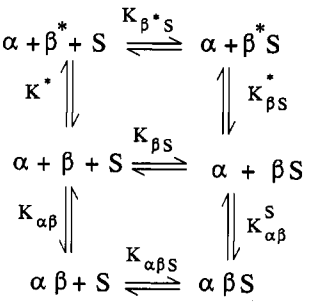


FIGURE 5: Observed first-order rate constants obtained for the fast phase of the binding of ALLO to tubulin, as a function of total drug concentration. The slopes give the apparent second-order rate constants. (A) Tubulin-GTP; (B) tubulin-GDP. Open symbols represent the results obtained with 8.7 μM tubulin, while the closed symbols show the results obtained with a 10-fold lower protein concentration. Experiments were performed at 10 °C.

dissociation of the dimer into its subunits, the nonexchangeable site becomes exchangeable with free nucleotide, which in turn would alter the conformation of that subunit and as a consequence its affinity for the analogue. Results presented in the preceding paper (Shearwin et al., 1994) show that the N site remains nonexchangeable upon dimer dissociation. This eliminates the second possibility, leaving as the only explanation that colchicine-like ligands bind to tubulin on the subunit that contains the exchangeable nucleotide site. That site is known to be located on the β subunit (Geahlen & Haley, 1977; Hesse et al., 1985, 1987). This means that the colchicine binding site must be located on the β subunit.

Does the α Subunit Contribute to the Binding? Having established that colchicine and its analogues interact primarily with the β subunit, one can ask where on the subunit do the ligands bind and what contribution, if any, does the α subunit make to the liganding to dimeric tubulin? The similarities of the affinities of the drugs for the dimeric ($\alpha\beta$) and dissociated (β) forms of GTP tubulin strongly point to the majority of the free energy of the interaction being contributed by the β subunit. A number of studies on this question however, have led to apparently contradictory conclusions. Genetic evidence in which tubulin mutants confer colchicine resistance (Sheir-Neiss et al., 1978; Cabral et al., 1980) suggest that the β subunit is the site of colchicine binding, as does indirect evidence based on the reactivity of cysteine residues in β-tubulin (Roach et al., 1985) and the observation that different isotypes of the β subunit in the $\alpha\beta$ dimer lead to different kinetics of colchicine binding (Banerjee & Luduena, 1992). Direct photoaffinity labeling of tubulin with colchicine resulted in the bulk of the labeling occurring on the β subunit, with some labeling of the α subunit at longer times, suggesting that the site is located near the $\alpha\beta$ interface (Wolff et al., 1991), although the portion of the colchicine molecule involved was not clear. On the other hand, photolabeling studies involving colchicine derivatives with long spacer arms on ring B showed labeling of α-tubulin (Williams et al., 1985), while shorter

Scheme II



spacers labeled both subunits (Floyd et al., 1989). All these observations can be reconciled if the ring C binding subsite is located completely on the β subunit, while the ring A binding subsite is located close to, or even partially on, the α subunit. To probe this situation, let us consider the present results in terms of two possible mechanisms of drug binding and examine the free energy linkages for these situations: (i) the ligands bind to the β subunit only or (ii) the binding is at the $\alpha\beta$ interface, with some contribution made by the α subunit. Taking the first mechanism, weakening of the binding of the drugs when tubulin-GDP dissociates into subunits (Table I) implies a linked conformational change in the β subunit, as shown in Scheme II. The decrease of the measured binding constant to the β subunit requires then the altered conformation (β^*) to have a reduced affinity for the ligand. The experimental binding constant to dissociated tubulin, $K_{\beta S}$, would then be an apparent value, such that

$$K_{\beta S}^{app} = \frac{[\beta S] + [\beta^* S]}{[S]([\beta^*] + [\beta])} = \frac{K_{\beta S}(1 + K_{\beta S}^*)}{1 + K^*} = \frac{K_{\beta S} + K^* K_{\beta^* S}}{1 + K^*} \quad (9)$$

For tubulin-GTP, the drugs bind with equal affinity to dimeric and dissociated tubulin ($K_{\alpha\beta S} \approx K_{\beta S}$), and so K^* (and $K_{\beta S}^*$) must equal zero, i.e., tubulin-GTP does not exist in the β^* conformation. It follows that $K_{\beta S}^{app} = K_{\beta S}$. In contrast, ligand binding to the dissociated form of tubulin-GDP is weaker than to the dimeric protein. Therefore, when the E site is occupied by GDP, dissociated tubulin must exist in an equilibrium between the β and β^* conformations. Provided that ligand binding to the β^* form is weaker than to the β conformation, the result will be a decrease in the apparent binding of drug to the dissociated form of tubulin-GDP. Should the β^* state not bind drug at all ($K_{\beta S}^* = 0$), it follows from eq 9 that $K_{\beta S}^{app}$ is simply $K_{\beta S}/(1 + K^*)$. In this case, using the values of $K_{\alpha\beta S}$ and $K_{\beta S}$ obtained from sedimentation equilibrium in the presence of ALLO (Table II), $K^* = 12.7$. Should the β^* conformation of tubulin also bind the ligand ($K_{\beta S}^* > 0$), the value of K^* will increase. Thus, for tubulin-GDP, at least 90% of tubulin monomer should exist in the β^* form. On the basis of Scheme II, then, the reduced affinity of dissociated tubulin-GDP for colchicine analogues can be accounted for by an E-site-induced conformational change in the dissociated β subunit.

The second possible mechanism is that drug binding is at the $\alpha\beta$ interface, with some interaction occurring with the α subunit. For $\alpha\beta$ tubulin-GTP, the binding constants for the

drugs are very close to those for the binding to the dissociated form. This indicates that the major part of the interaction is with the β subunit. On the basis of the data of Table I, the maximum contribution of the α subunit of tubulin-GTP to the free energy of binding of the ligand would be on the order of –0.5 kcal/mol. The weaker binding of the drugs to dissociated tubulin-GDP can be accounted for only by a conformational change in tubulin, similar to that shown in Scheme II, since the binding to the dissociated forms of tubulin reflects solely the contribution of the β subunit. Therefore, the α subunit can contribute at most 10% of the free energy of binding of the ligand, if any.

Ring C Binding Is Linked to the E-Site-Induced Conformational Transition. How do the present results bear on the known binding characteristics of colchicine and its structural analogues? This process has been described in terms of a model (Andreu et al., 1991) that consists of two chemically different and independent subsites on tubulin: a hydrophobic site that binds ring A and a stacking subsite that binds ring C. There is no cooperativity between the two subsites and ring B makes no contribution other than the elimination of the freedom of rotation about the A–C bond. The assessment of the contributions of the bindings of rings A and C to the various association states of tubulin requires that all non-reaction-specific entropic terms be accounted for. This is done by the reduction of all standard entropy changes to unitary entropy ($\Delta S_u = \Delta S° - \Delta S°_{cratic}$) and the calculation of unitary standard free energy changes, $\Delta G°_u$. Since binding results in the loss of the statistical entropy of solution of the ligand, ΔS_u is more positive than $\Delta S°$ by 8 entropy units and $\Delta G°_u$ is more negative by 2.3 kcal/mol at 10 °C. The relative contributions of rings A and C to binding can then be calculated from

$$\Delta G_u° = \Delta G_u°(\text{ring A}) + \Delta G_u°(\text{ring C}) \qquad (10)$$

where ring C is modified in the analogues.

A proper assignment of the free energy contributions of rings A and C to $\alpha\beta$ dissociation requires first the identification of the colchicine ring (A or C or both) whose interaction with tubulin the dissociation is influencing. The binding of podophyllotoxin (PODO), which contains a trimethoxyphenyl (A) ring, may give some insight into this question. Its interaction with the dissociated form of tubulin was found to be independent of the nucleotide at the E site, while the binding to the dissociated form was weaker than that to $\alpha\beta$ tubulin. This result, however, does not give direct information on the A ring binding linkages, since the other half of the PODO molecule also contributes to the binding at a site other than the A or C subsites. There are at least three possible explanations for the observed effect of PODO on dissociation. The A ring moiety of the drug is known to interact with the A ring binding locus of tubulin (Cortese et al., 1977; Andreu & Timasheff, 1982b). The other half of the PODO molecule may (i) bind to a region of β tubulin other than the A or C subsites, (ii) remain unbound in dissociated tubulin, or (iii) bind to a site on the α subunit, such that dissociated tubulin would exist as a mixture of β tubulin with podophyllotoxin bound by the A ring and α tubulin with the drug bound by its other moiety. This latter possibility should give rise to a binding stoichiometry to dissociated tubulin of greater than 1. Free energy calculations should allow us to determine which of these possibilities is the most likely. The unitary free energy change of PODO binding to the tubulin dimer at 10 °C is –9.7 kcal/mol (Cortese et al., 1977). The corresponding value for binding to the monomer is –8.7 kcal/mol. Since the free energy for interaction of ring A alone, $\Delta G°_u$ (ring A), is only –5.8 kcal/mol (Timasheff et al., 1991), the other half of the PODO molecule must make some energetic contribution (~–2.9 kcal/mol) to the binding of this ligand on the β subunit. This eliminates mechanisms (ii) and (iii). Hence, mechanism (i) is the most likely one: both halves of PODO bind to the β subunit. The lack of dependence of $K_{\beta S}$ for PODO on the E-site nucleotide occupancy of tubulin, while there is a marked dependence for the colchicine analogues that contain ring C, suggests that it is the ring C interaction which is linked to the E-site-induced conformational change (Scheme II). This is also consistent with the observation that PODO does not induce the GTPase activity characteristic of COL and its ring A and C containing analogues (David-Pfeuty et al., 1977). A rigorous definition of the linkages between dissociation and ring subsite binding would require a study of the effects of the single-ring analogues NAM (ring A) and TME (ring C) on $\alpha\beta$ dissociation. Unfortunately, the binding of these compounds is much too weak to have an experimentally detectable effect on $\alpha\beta$ dissociation individually (Andreu & Timasheff, 1982a).

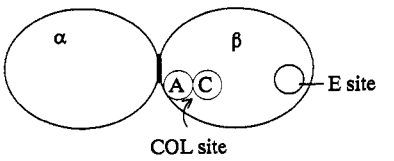


FIGURE 6: Schematic structure of the tubulin $\alpha\beta$ dimer showing the relative positions of the dimer interface, the colchicine binding site, and the exchangeable nucleotide site. It must be noted that this is not a structural model of the protein but only a picture to clarify the discussion.

Relation between COL and Nucleotide Binding Loci. What information can be drawn from this study on the relative positions of the colchicine and E-site nucleotide binding sites on the β subunit of tubulin? Comparison of the present results on the linkage between $\alpha\beta$ subunit dissociation and colchicine binding with the various chemical studies strongly points to the COL binding site being located close to the $\alpha\beta$ interface, with the ring A binding subsite being in greater proximity to the α subunit, if not slightly overlapping it. Fluorescence energy transfer studies have shown, on the other hand, that the high-affinity metal binding site associated with the exchangeable nucleotide site and the colchicine binding site are located at least 2.4 nm apart (Ward & Timasheff, 1988). Given that the tubulin dimer has dimensions of 5 × 8 nm (Amos, 1982), the exchangeable nucleotide site must be located near the outer region (amino-terminal domain) of the β subunit. The corresponding schematic structure is shown on Figure 6. Proteolysis studies with photoaffinity-labeled tubulin have shown that residues 155–174 may be involved in the contact with the ribose portion of the nucleotide (Hesse et al., 1987), while residues in the region 63–77 bind the purine moiety (Kim et al., 1987). Both of these loci are located in the amino-terminal portion of β tubulin, which is considered to be distal from the intersubunit contact area (Kirchner & Mandelkow, 1985). This is consistent with the conclusion drawn from the physicochemical studies.

The linkages described in the present study all refer to experiments carried out in the absence of magnesium. As described in the preceding paper (Shearwin et al., 1994), the association of the α and β subunits into the heterodimer is linked to the binding of two magnesium ions. Furthermore, the conformational state of the heterodimer is also linked to magnesium ion binding. In the absence of magnesium, the curved, or ring-forming, conformation (T*) of tubulin is favored, while in the presence of magnesium, for tubulin-GTP the equilibrium is driven toward the straight or microtubule-forming conformation (T). The binding of colchicine and its analogues described in the present study

refer, therefore, to the curved (T*) conformation. Examination of drug binding in the presence of magnesium may reveal further linkages. For example, the drugs may bind with different free energies to the T and T* conformations. In this connection it is interesting to note the differential effects of colchicine on tubulin self-association. The drug has a significant effect on the type of polymer formed under microtubule-assembling conditions, that is, polymerization of the T conformation (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b), which gives rise to aggregated protofilaments and anomalous structures. Self-assembly of the T* conformation into 42S double rings, on the other hand, is almost unaffected by the binding of colchicine (Shearwin & Timasheff, 1992).

APPENDIX

Derivation of the Binding Equation. Consideration of Scheme I leads to the following equilibria:[2]

$$\alpha + \beta \overset{K_{\alpha\beta}}{\rightleftharpoons} \alpha\beta \quad K_{\alpha\beta} = \frac{[\alpha\beta]}{[\alpha][\beta]}$$

$$\beta + S \overset{K_{\beta S}}{\rightleftharpoons} \beta S \quad K_{\beta S} = \frac{[\beta S]}{[\beta][S]} \qquad \text{(A1)}$$

$$\alpha\beta + S \overset{K_{\alpha\beta S}}{\rightleftharpoons} \alpha\beta S \quad K_{\alpha\beta S} = \frac{[\alpha\beta S]}{[\alpha\beta][S]}$$

The binding function, r, i.e., the extent of liganding, is defined as

$$r = \frac{[\beta S] + [\alpha\beta S]}{C_T} \qquad \text{(A2)}$$

where C_T is the total protein concentration expressed as moles of dimer, $C_T = [\alpha\beta] + [\alpha\beta S] + {}^1/_2[\alpha] + {}^1/_2[\beta] + {}^1/_2[\beta S]$. However, since $[\alpha] = [\beta] + [\beta S]$, algebraically $C_T = [\alpha\beta] + [\alpha\beta S] + [\beta] + [\beta S]$, which gives

$$r = \frac{[\beta S] + [\alpha\beta S]}{[\alpha\beta] + [\alpha\beta S] + [\beta] + [\beta S]} \qquad \text{(A3)}$$

Substitution of the appropriate equilibrium constants (eq 8) and rearrangement of terms leads to

$$r = \{K_{\beta S}[\beta][S] + K_{\alpha\beta S}K_{\alpha\beta}[\beta]^2[S](1 + K_{\beta S}[S])\}/ \{K_{\alpha\beta}[\beta]^2(1 + K_{\beta S}[S]) + K_{\alpha\beta S}K_{\alpha\beta}[\beta]^2[S](1 + K_{\beta S}[S]) + K_{\beta S}[\beta][S] + [\beta]\} \qquad \text{(A4a)}$$

where

$$[\beta] = \{-(1 + K_{\beta S}[S]) + [(1 + K_{\beta S}[S])^2 + 4(K_{\alpha\beta} + K_{\beta S}K_{\alpha\beta}[S] + K_{\alpha\beta S}K_{\alpha\beta}[S] + K_{\alpha\beta S}K_{\alpha\beta}K_{\beta S}[S]^2)C_T]^{1/2}\}/ \{2(K_{\alpha\beta} + K_{\beta S}K_{\alpha\beta}[S] + K_{\alpha\beta S}K_{\alpha\beta}[S] + K_{\alpha\beta S}K_{\alpha\beta}K_{\beta S}[S]^2)\} \qquad \text{(A4b)}$$

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