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Effect of the B Ring and the C-7 Substituent on the Kinetics of Colchicinoid–Tubulin Associations†

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ABSTRACT: The kinetics of four B-ring derivatives of colchicine binding to tubulin has been examined quantitatively. The bindings of deacetamidocolchicine, deacetylcolchicine, demecolcine, and *N*-methyldemecolcine to tubulin were biphasic processes. The association rate constants were determined as a function of temperature, and the thermodynamic parameters for the transition states of the fast phase were calculated. The kinetic parameters for the formation of the deacetylcolchicine–tubulin, demecolcine–tubulin, and *N*-methyldemecolcine–tubulin complexes were very similar to each other, but different from the parameters for the colchicine–tubulin association. In particular, the global activation enthalpies for the formation of the three aminocolchicinoid–tubulin complexes were 3–5 kcal/mol greater than the global activation enthalpy of colchicine binding to tubulin. These results indicate that electronic rather than steric properties of the B-ring substituent are of greater importance in the activation enthalpy of colchicinoids binding to tubulin. In contrast, the global activation enthalpy for deacetamidocolchicine, which lacks a substituent on the C-7 carbon, binding to tubulin was virtually identical to the global activation enthalpy previously found for the colchicine analog that lacks the B ring, 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone, binding to tubulin (Bane, S., Puett, D., Macdonald, T. L., & Williams, R. C., Jr. (1984) *J. Biol. Chem. 259*, 7391–7398). This result demonstrates that the carbons of the B ring are not involved in the transition state for the formation of colchicinoid–tubulin complexes. The first-order dissociation rate constants of the colchicinoid–tubulin complexes were determined at 37 °C. The dissociation profiles of the colchicinoid–tubulin complexes also consisted of two phases. The equilibrium constants for the two kinetic phases were determined from the rate constants for each ligand at 37 °C. The equilibrium constants for each kinetic phase of the aminocolchicinoid–tubulin complexes were indistinguishable within experimental error. The equilibrium constants for the two kinetic phases of the deacetamidocolchicine–tubulin complex differed by up to a factor of 4 and were in reasonably good agreement with the equilibrium constant previously found for the association by equilibrium methods (Choudhury, G. G., Banerjee, A., Bhattacharyya, B., & Biswas, B. B. (1983) *FEBS Lett. 161*, 55–59). It is concluded that tubulin heterogeneity with respect to colchicinoid binding may be detected by kinetic means but is unlikely to be observed in unfractionated tubulin using equilibrium methods.

Colchicine (Figure 1) belongs to a class of compounds that exert their biological effects by disrupting normal microtubule assembly. Colchicine inhibits microtubule assembly by binding to a single high-affinity site on the tubulin heterodimer. A variety of compounds, including podophyllotoxin, steganacin, combretastatin, and certain benzimidazole carbamates such as nocodazole, bind to the colchicine site on tubulin (Hamel, 1990).

The mechanism by which colchicine and other colchicine site binding ligands interfere with normal microtubule assembly has received a great deal of attention (Hamel, 1990). It is now well established that the colchicine–tubulin complex and not soluble colchicine is the inhibiting species (Skoufias & Wilson, 1992). Low concentrations of the colchicine–tubulin complex perturb normal assembly dynamics by adding to one or both of the microtubule ends (Margolis & Wilson, 1977; Sternlich & Ringel, 1979; Farrell & Wilson, 1984; Bergen & Borisy, 1986). The structural features of the colchicine–tubulin complex that are responsible for its disturbance of the normal assembly–disassembly processes of the microtubule have not been established. It is known that colchicine binding to tubulin induces a conformational change in the protein, as shown by a decrease in the dissociation constant of the α–β dimer (Detrich et al., 1982), induction of

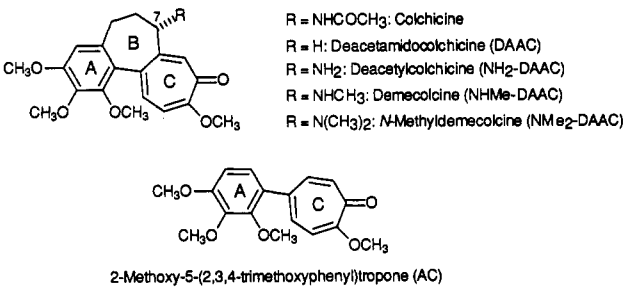

FIGURE 1: Structures of colchicine, B-ring analogs of colchicine, and AC.

GTPase activity (David-Pfeuty et al., 1979), and alteration of the far-CD spectrum of tubulin (Andreu & Timasheff, 1982). It may be that the colchicine-induced conformational change in tubulin is related to the conformational changes that occur when tubulin is incorporated into the microtubule (Skoufias & Wilson, 1992).

It is clear that the kinetics of colchicinoid binding to tubulin is related to its effects on microtubule polymerization. Colchicine binding to tubulin is a slow, highly temperature dependent process, forming a poorly reversible but noncovalent complex (Garland, 1978; Lambeir & Engelborghs, 1981). In contrast, the colchicine analog 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone (AC;[1] Figure 1) binds to tubulin about 500 times faster than colchicine at 37 °C, and the dissociation rate constant of the AC–tubulin complex is 10 000 times

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greater than the dissociation rate constant of the colchicine–tubulin complex (Bane et al., 1984; Engelborghs & Fitzgerald, 1987; Diaz & Andreu, 1991). The apparent potency of colchicine as an inhibitor of in vitro microtubule assembly increases if colchicine is preincubated with tubulin prior to induction of assembly (Margolis & Wilson, 1977), while preincubation of AC (Hastie, unpublished observation) or other drugs that bind rapidly to tubulin (Lin et al., 1989) with tubulin has no effect on their potencies. The actions of colchicine and AC on different cell lines have also been investigated (Diez et al., 1987). Both drugs arrested cell growth and increased the mitotic index. Removal of AC from the culture medium of PtK2 cells resulted in rapid recovery of the microtubule network, while no microtubule reassembly was found 4 h after colchicine removal.

The structural features of colchicine responsible for its slow association and dissociation kinetics have not been clearly defined. At least two hypotheses have been put forth to explain the differences in the kinetics of colchicine, AC, and B-ring analogs of colchicine binding to tubulin. One proposal was that colchicine binding to tubulin is accompanied by a conformational change in the ligand as well as the protein (Detrich et al., 1981). According to this hypothesis, the activation energy of AC binding to tubulin is decreased compared to colchicine binding to tubulin as a result of the relatively unhindered rotation of AC about the phenyl–tropone bond (Bane et al., 1984). Subsequently, a substantial body of evidence has been found to indicate that colchicine retains its solution conformation in its complex with tubulin (Hastie, 1991).

A second hypothesis suggested that steric features of colchicinoids in the B-ring region of the molecule are responsible for the high activation energy of colchicine binding to tubulin. The rates of B-ring analogs of colchicine binding to tubulin were found to increase with decreasing size of the C-7 substituent and with complete removal of the B ring (Ray et al., 1981; Bhattacharyya et al., 1986). A later study reported that the activation energies of B-ring analogs of colchicine binding to tubulin decreased as colchicine > deacetylcolchicine > deacetamidocolchicine ≅ AC > demecolcine (Maity et al., 1988;[2] see Figure 1 for structures.)

Although the kinetics of colchicine and AC binding to tubulin has been thoroughly investigated, the kinetics of colchicine B-ring analogs binding to tubulin has received less quantitative treatment. In this work, we have quantitatively determined the kinetic parameters for the global reactions of four B-ring analogs of colchicine binding to tubulin: deacetamidocolchicine (DAAC), deacetylcolchicine (NH_2-DAAC), demecolcine (NHMe-DAAC), and *N*-methyldemecolcine (NMe_2-DAAC). Our findings indicate that electronic rather than steric features of the C-7 substituent are important in the transition state of colchicinoids binding to tubulin. We have also found that the carbons of the B ring make no contribution to the activation energy of the process. We propose that ligands that bind to the colchicine site on tubulin through a relatively low energy pathway ($E_a \leq 13$ kcal/mol) do so because they possess no structural features that correspond to the region of the C-7 substituent of colchicine.

EXPERIMENTAL PROCEDURES

Materials. Pipes, EGTA, $MgSO_4$, GTP (type II-S) were purchased from Sigma. Colchicine was obtained from U.S. Biochemical Corp.; it was used without further purification for synthesis and was recrystallized from ethyl acetate prior to kinetic measurements. Podophyllotoxin and spectrophotometric grade DMSO were purchased from Aldrich. All other reagents were obtained from Fisher and were ACS certified. Silica gel plates, Merck F_{245} with a thickness of 0.25 mm, were employed for thin-layer chromatography.

Tubulin Purification and Protein Determination. Bovine brain tubulin, free of microtubule-associated proteins, was prepared by two cycles of assembly–diassembly followed by phosphocellulose chromatography as described previously (Williams & Lee, 1982) and stored in liquid nitrogen. Prior to use, the frozen pellets were gently thawed, centrifuged at 800*g* for 10 min at 4 °C, and then desalted into PMEG buffer (0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM GTP, pH 6.90 at 23 °C) on 1-mL G-50 Sephadex columns according to the method of Penefsky (Penefsky, 1977). The concentration of tubulin was determined spectrophotometrically by the use of an extinction coefficient at 278 nm of 1.23 $(mg/mL)^{-1}$ in PMEG buffer (Detrich & Williams, 1978). Near-UV absorption spectra were measured on a Hewlett-Packard Model 8451A diode array spectrometer.

Synthesis. *N*-Methyldemecolcine (NMe_2-DAAC), demecolcine (NHMe-DAAC), and *N*-deacetylcolchicine (NH_2-DAAC) were synthesized from colchicine as previously described (van Tamelen et al., 1961; Capraro & Brossi, 1979). Deacetamidocolchicine (DAAC) was synthesized from trimethylcolchicinic acid by the method of Eschenmoser (Schreiber et al., 1961). Separation of the two isomers, DAAC and deacetamidoisocolchicine, was accomplished by successive recrystallizations from acetone–petroleum ether. DAAC and deacetamidoisocolchicine migrated as a single spot under all TLC conditions tested, so the purity of DAAC was assessed by ^1H NMR spectroscopy. No deacetamidoisocolchicine was detected by ^1H NMR spectroscopy in the DAAC used for kinetic analyses. The structure and purity of the other B-ring analogs were determined by TLC analysis and by ^1H NMR spectroscopy.

The concentrations of the ligands employed in this study were determined spectrophotometrically by use of an extinction coefficient in aqueous solution determined at the near-UV absorption maximum. The extinction coefficients used were as follows: colchicine at 352 nm, $\epsilon = 1.69 \times 10^4$ M^{-1} cm^{-1} (Chabin et al., 1990); demecolcine at 354 nm, $\epsilon = 1.58 \times 10^4$ M^{-1} cm^{-1}; NMe_2-DAAC at 356 nm, $\epsilon = 1.45 \times 10^4$ M^{-1} cm^{-1}; NH_2-DAAC at 352 nm, $\epsilon = 1.40 \times 10^4$ M^{-1} cm^{-1}; DAAC at 352 nm, $\epsilon = 1.62 \times 10^4$ M^{-1} cm^{-1} (Pyles et al., 1992); and podophyllotoxin at 290 nm, $\epsilon = 3.70 \times 10^3$ M^{-1} cm^{-1} (Andreu & Timasheff, 1982).

Association Kinetics. The association of the colchicinoids with tubulin was measured under pseudo-first-order conditions using an SLM 8000 spectrophotometer. The temperature was controlled with a circulating water bath accurate to ±0.5 °C. Enhancement of colchicine fluorescence was monitored with excitation and emission wavelengths of 360 and 440 nm, respectively. Quenching of intrinsic protein fluorescence was monitored with an excitation wavelength of 300 nm (new source lamp) for NH_2-DAAC and NMe_2-DAAC binding to tubulin and with an excitation wavelength of 280 nm (old source lamp) for NHMe-DAAC and colchicine binding to tubulin. The emission wavelength was 335 nm. An excitation

[1] Abbreviations: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMEG buffer, 0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM GTP, pH 6.90 at 23 °C; DMSO, dimethyl sulfoxide; TLC, thin-layer chromatography; AC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone; DAAC, deacetamidocolchicine; NH_2-DAAC, deacetylcolchicine; NHMe-DAAC, demecolcine; NMe_2-DAAC, *N*-methyldemecolcine.

[2] However, the association rate constants at 37 °C listed in their Table I do not agree with rate constants obtained by extrapolation from their Arrhenius plot.

wavelength of 300 nm was chosen to avoid photochemical reactions of tubulin, which apparently lead to the formation of aggregates (as assessed by the appearance of scattering in the kinetic measurements). It is known that high-energy light (≤285 nm) can alter spectral properties of proteins by affecting their aggregation state (Claesson, 1956). Moreover, it has been shown that irradiation of tubulin at 280 nm at a dose rate of 2×20^{-4} W/cm^2 leads to damage to tubulin dimers (Zaremba et al., 1984). The damage includes production of higher molecular weight cross-linked species. The apparent photoaggregation of tubulin was observed upon irradiation at 280 nm (excitation slits = 16 nm) when a new source lamp was placed in the instrument. The extent of aggregation appears to be a function of lamp intensity at 280 nm. No detectable tubulin aggregates were formed when the solution was excited at 300 nm, regardless of the age of the lamp. The wavelength dependence of the apparent photoaggregation is in accordance with the data of Zaremba et al. (1984). To ensure that similar rates could be obtained from either excitation wavelength (280 nm or 300 nm), colchicine binding to tubulin was monitored with excitation wavelengths of 300 and 280 nm at 30.5 °C. The apparent second-order rate constants for colchicine binding to tubulin were equivalent within experimental error using either excitation wavelength.

A 10 × 10 mm fluorescence cell was employed for the kinetic measurements, and the procedure used was as follows. A solution of colchicinoid in PMEG buffer was stored in a brown bottle to avoid photochemical decomposition. The concentration of the ligand was adjusted to give a final ligand concentration of about 110 μM. It is important to maintain a similar ligand concentration for all kinetic measurements since it was previously shown that the rate of colchicine binding to tubulin is concentration dependent in a nonlinear fashion (Lambeir & Engelborghs, 1981). After equilibration of the colchicinoid to the desired temperature, the shutters were closed and the tubulin solution was added to give the final concentration of 2.0 μM. The volume of the tubulin added to the cell was ≤5% of the total sample volume. The solution was rapidly mixed without removing the cell from the compartment, and the shutters were opened. The maximum time that it took to add the tubulin, mix the solution, and open the shutters was ≤15 s. The data were collected on an IBM PC interfaced to the instrument and were analyzed using the program KINFIT (On-Line Instrument Systems, Inc., Jefferson, GA). The fit of the data to the kinetic equations was evaluated by the distribution of the residuals about zero (see Figure 2) and by the magnitude of the Durbin–Watson factor. The rate constants are an average of 3–11 data sets at each temperature.

The effect of colchicinoid concentration on the rate of the association reaction was investigated for NHMe-DAAC binding to tubulin. The association rate was measured using a fixed concentration of tubulin (2 μM) and increasing concentrations of NHMe-DAAC (50–320 μM) at 25 °C. It was not possible to measure the kinetics at higher ligand concentrations due to a severe inner filter effect. The association rate was found to be a linear function of NHMe-DAAC concentration in this range. On the basis of this analysis and previous determinations for AC binding to tubulin (Bane et al., 1984), it is assumed that the kinetics of the colchicinoids binding to tubulin is determined at concentrations within the linear range of the data.

DAAC binding to tubulin was monitored on a Durrum stopped-flow photometer integrated to a Digital VT105 computer system with MINC 23 BASIC software. The concentration of DAAC after mixing was 204 μM. The

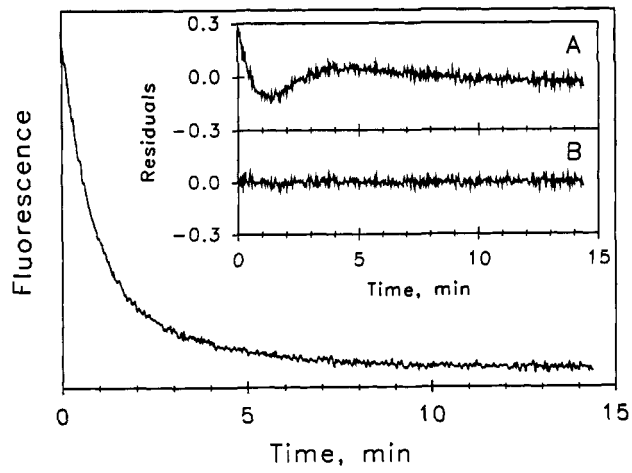

FIGURE 2: Quenching of intrinsic protein fluorescence upon NHMe-DAAC binding to tubulin. Insets: Plots of the residuals between the experimental and the theoretical curves for (A) a single exponential and (B) a sum of two exponentials.

concentration of the ligand was doubled with respect to the other ligands employed in the kinetic measurements to account for the two conformers of DAAC which exist in a 1:1 ratio (Yeh et al., 1988). Since only one conformer binds tubulin (Berg et al., 1991), the concentration of DAAC in the active conformation to tubulin was 102 μM. This factor was considered in the analysis of the data. The excitation wavelength was 296 nm, and emission beyond 320 nm was observed using a Dionex 16331 filter. The temperature was controlled by a circulating water bath and was accurate to ±0.5 °C. The maximum time required to collect sufficient kinetic data was 360 s, and the time constant was 10 ms. The data were transferred to an IBM PC-AT and analyzed by the same method as described above. The rate constants are an average of 5–7 data sets at each temperature.

Due to the known photochemical sensitivity of colchicine (Roigt & Leblanc, 1973), an additional experiment was performed to determine the amount of decomposed colchicinoid upon exposure to light in the spectrofluorometer. Colchicinoid in PMEG buffer (~110 μM) was irradiated for 2.5 h (excitation wavelength = 300 nm). DAAC was not included in this experiment since it binds quickly to tubulin even at low temperatures, and thus the time the ligand was exposed to light was minimal. An absorption spectrum of colchicinoid was taken before and after irradiation to determine the change in the intensity at the absorption maximum. The intensity decreased by 1.2%, 2.1%, 3.0%, and 3.7% for colchicine, NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC, respectively. In addition, the irradiated solutions were extracted with dichloromethane, dried over sodium sulfate, filtered, evaporated, and then analyzed by thin-layer chromatography (90:10 dichloromethane–methanol). Minimal photochemical decomposition was observed for the colchicinoids by thin-layer chromatographic analysis. Colchicinoid decomposition was therefore neglected in the calculation of the apparent second-order rate constants.

Dissociation Kinetics. The dissociation of colchicinoid–tubulin complexes was measured by monitoring the time-dependent decrease in colchicinoid fluorescence as the ligand was released from the binding site on tubulin. Reassociation of the complex was prevented by the inclusion of excess podophyllotoxin in the solutions (Banerjee & Luduena, 1987; Engelborghs & Fitzgerald, 1987; Diaz & Andreu, 1991). The dissociation of the complexes was monitored using an SLM 8000 spectrophotometer at 36.5 °C with a 2 × 10 mm fluorescence cell oriented such that the light beam passed

Table I: Association Rate Constants and Activation Energies of Colchicine and B-Ring Analogs of Colchicine Binding to Tubulin

ligand	[ligand], μM	[tubulin], μM	k_f, M^{-1} s^{-1} [a]	k_s, M^{-1} s^{-1} [a]	A_f/A_s [b]	Ea_f, kcal/mol [c]	Ea_s, kcal/mol [c]
colchicine[d]	105	2.0	138(2)[e]	29(1)	2.1	19.3(0.1)	26.0(0.5)
NMe_2-DAAC	109	2.0	303(22)	63(13)	3.8	23.9(0.3)	25.3(4.0)
NHMe-DAAC	119	2.0	178(4)	42(3)	3.1	21.9(0.5)	23.5(2.2)
NH_2-DAAC	109	2.0	488(45)	109(13)	3.1	23.9(0.4)	27.6(2.8)
DAAC	204	2.0	6140(730)	516(60)	1.7	11.7(0.7)	9.2(1.1)
AC[f]	100	4.5	52400(4240)	10640(1600)	2.3	13.2(0.6)	9.3(1.0)

[a] k_f and k_s are the apparent second-order rate constants of the fast and slow phases, respectively, at 37 °C. [b] Ratio of the amplitudes of the fast (A_f) and slow (A_s) phases. [c] Ea_f and Ea_s are the activation energies of the fast and slow phases, respectively. [d] Quenching of tubulin fluorescence monitored. Equivalent results were obtained when enhancement of colchicine fluorescence was monitored. [e] Values in parentheses are the standard deviations of the terms. [f] Obtained from data previously published by Bane et al. (1984).

through the shorter path. The excitation and emission wavelengths were 360 and 440 nm, respectively. Aliquots of colchicinoid–tubulin complexes in PMEG buffer were incubated at 37 °C until equilibrium was reached. The complexes were prepared prior to each run to avoid exposing the protein to warm temperatures for long periods of time. An aliquot of the complex was added to a preequilibrated solution of podophyllotoxin in 10% DMSO–PMEG, and the solution was rapidly mixed. Typically, the ratios of podophyllotoxin: colchicinoid:tubulin after mixing were as follows: NH_2-DAAC and NHMe-DAAC, 200:2:1; DAAC, 200:4:1; and NMe_2-DAAC, 80:2:1. The protein concentration was 2.0 μM except in the case of the NMe_2-DAAC–tubulin complex, in which the protein concentration was increased to 5.0 μM to augment the fluorescent signal. The time that it took to add the complex, mix the solution, and open the shutters was ≤20 s. As was true for the association reactions, the dissociation of colchicinoid–tubulin complexes was best described by a biexponential equation. The data were analyzed in a manner similar to that used for the data obtained for the association reactions described above. The dissociation rate constants for the fast and slow phases are averages of 8–14 data sets.

The dissociation of the DAAC–tubulin complex was also measured using a low concentration of DMSO (1.8%). Due to the poor solubility of podophyllotoxin at this low DMSO concentration, the concentration of the DAAC–tubulin complex was decreased by a factor of 2. The dissociation rate constants of the DAAC–tubulin complex were identical within experimental error at the two DMSO concentrations and slightly greater than those previously reported by Banerjee et al. (1987). In addition, Engelborghs and Fitzgerald (1987) have shown that the dissociation rate constants of the AC–tubulin complex are unaffected by increasing the DMSO concentration from 5% to 10%. Therefore, the effect of DMSO on the dissociation rate constants appears to be negligible.

RESULTS

Association Kinetics. The kinetics of colchicine and AC binding to tubulin has been extensively investigated by monitoring the enhancement of ligand fluorescence as a function of time (Cortese et al., 1977; Garland, 1978; Lambeir & Engelborghs, 1981; Bane et al., 1984; Engelborghs & Fitzgerald, 1987; Diaz & Andreu, 1991). The DAAC–tubulin complex is strongly fluorescent (Ray et al., 1981), and the increase of DAAC fluorescence upon tubulin binding as a function of time may be used to monitor the binding of DAAC to tubulin (Maity et al., 1988). In contrast, colchicinoids possessing an amine at the C-7 carbon are considerably less fluorescent in their complexes with tubulin (Bhattacharyya et al., 1986; Pyles & Hastie, 1992). In these cases, a better signal-to-noise ratio was obtained when quenching of intrinsic tubulin fluorescence was used as the spectroscopic probe. For consistency, the kinetics of DAAC binding to tubulin as well as the kinetics of the aminocolchicinoids binding to tubulin was monitored by quenching of tubulin fluorescence. The kinetics of colchicine binding to tubulin was measured using the same experimental conditions to ensure proper comparison of the kinetic parameters. In addition, the colchicine–tubulin association was monitored by both enhancement of ligand fluorescence and quenching of protein fluorescence as a function of temperature. There was no significant difference in the kinetic parameters obtained for colchicine binding to tubulin using the two different signals (data not shown).

Kinetic measurements of all ligands binding to tubulin were performed under pseudo-first-order conditions at a single concentration of ligand and of tubulin. Figure 2 shows a typical kinetic profile for the binding of NHMe-DAAC to tubulin at 37 °C. The insets show the distribution of the residuals between the theoretical fit and experimental data when the data were analyzed as a single exponential (inset A) and as a sum of two exponentials (inset B). The association reaction was best described as two parallel pseudo-first-order reactions by the following biexponential equation:

$$F_t = Ae^{-\alpha t} + Be^{-\beta t} + C$$

F_t is the fluorescence of the ligand–tubulin complex at time t, A and α are the amplitude and pseudo-first-order rate constant, respectively, for the fast phase, B and β are the corresponding parameters for the slow phase, and C is an integration constant. The binding of colchicine, DAAC, NMe_2-DAAC, and NH_2-DAAC to tubulin was also best described by the above biexponential equation (data not shown). The apparent second-order rate constants for the fast and slow phases of the colchicinoids binding to tubulin at 37 °C are presented in Table I.

The apparent second-order rate constants for the associations of colchicine, NMe_2-DAAC, NHMe-DAAC, NH_2-DAAC, and DAAC with tubulin were determined as a function of temperature. The temperature dependence of the apparent second-order rate constants for the fast and slow phases is shown in Figure 3. The activation energies for the associations were calculated by linear least-squares analysis of the data and are presented in Table I.

Knowledge of the apparent second-order rate constants at 37 °C and the activation energies for the formation of the colchicinoid–tubulin complexes allowed for calculation of the global thermodynamic parameters of the transition states. These parameters for the fast phase are summarized in Table II.

Dissociation Kinetics. The dissociations of the NMe_2-DAAC–tubulin, NHMe-DAAC–tubulin, NH_2-DAAC–tubulin, and DAAC–tubulin complexes at 37 °C were determined by displacement experiments. Only one temperature was evaluated due to the slow rate of dissociation. This method has been previously used to determine the dissociation kinetics of colchicine–tubulin, DAAC–tubulin, and AC–tubulin com-

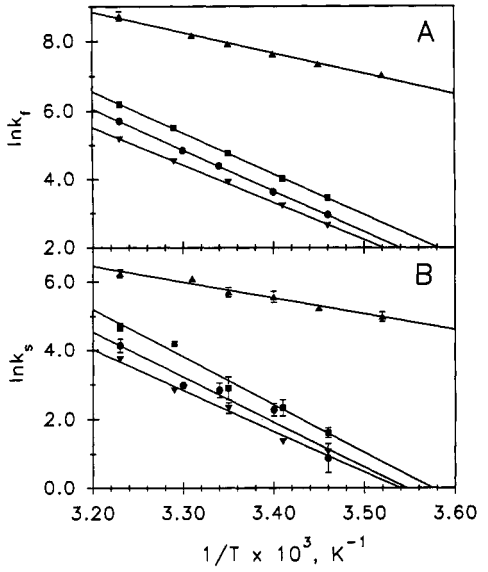

FIGURE 3: Temperature dependence of the apparent second-order rate constants of the fast phase (A) and the slow phase (B) of colchicine B-ring analogs binding to tubulin. The lines were calculated by linear regression analysis and are coded for both phases as follows: DAAC (▲), NH_2-DAAC (■), NHMe-DAAC (▼), and NMe_2-DAAC (●). The standard deviation of the mean of the rates for the fast and slow phases ranged from 2.9% to 10.6% and from 11.4% to 42%, respectively. Some of the error bars are obscured by the symbols of the data points.

Table II: Thermodynamic Parameters for the Transition States of Colchicine and B-Ring Analogs of Colchicine Binding to Tubulin at 37 °C (Fast Phase)

ligand	$\Delta G^{\ddagger}$, kcal/mol[a]	$\Delta H^{\ddagger}$, kcal/mol[b]	$\Delta S^{\ddagger}$, cal/mol·K[c]
colchicine	15.1(0.02)[d]	18.6(0.1)	11.3(0.4)
NMe_2-DAAC	14.7(0.05)	23.3(0.3)	27.7(1.1)
NHMe-DAAC	15.0(0.02)	21.3(0.5)	20.3(1.7)
NH_2-DAAC	14.4(0.06)	23.1(0.4)	28.1(1.5)
DAAC	12.8(0.08)	11.1(0.7)	−5.5(2.8)
AC[e]	11.5(0.08)	12.4(0.7)	2.9(2.8)

[a] At 37 °C, calculated by $\Delta G^{\ddagger} = RT \ln (kT/hk_f)$; Maskill (1985). [b] Calculated from plot of $\ln (k_f/T) = -\Delta H^{\ddagger}/RT + C$. [c] At 37 °C, calculated by $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$. [d] Values in parentheses are the standard deviations of the terms. [e] Obtained from data previously published by Bane et al. (1984).

plexes (Banerjee et al., 1987; Engelborghs & Fitzgerald, 1987; Diaz & Andreu, 1991). The dissociation rates of the colchicinoid–tubulin complexes were measured by monitoring the decrease in colchicinoid fluorescence as a function of time. Reassociation of the colchicinoid was prevented by including an excess of podophyllotoxin in the cell. A typical kinetic profile of the dissociation of the DAAC–tubulin complex is shown in Figure 4. From the insets in this figure, it is clear from the plots of the residuals that the data are better represented by a biexponential equation (inset B) rather than a single exponential equation (inset A). The dissociation of the complexes of tubulin with NMe_2-DAAC, NHMe-DAAC, and NH_2-DAAC were also found to be best described by a sum of two exponentials (data not shown). Due to the weak fluorescence of the NH_2-DAAC–tubulin, the NHMe-DAAC–tubulin, and particularly the NMe_2-DAAC–tubulin complexes, the errors in the dissociation rate constants are rather large (Table III). The first-order dissociation rate constants for the fast and slow phases of the colchicinoid–tubulin complexes are listed in Table III.

The dissociation rate constants were not corrected for the irreversible denaturation rate of the bound site. In the case of the colchicine–tubulin complex, the dissociation rate constant for irreversible denaturation of the bound site is about

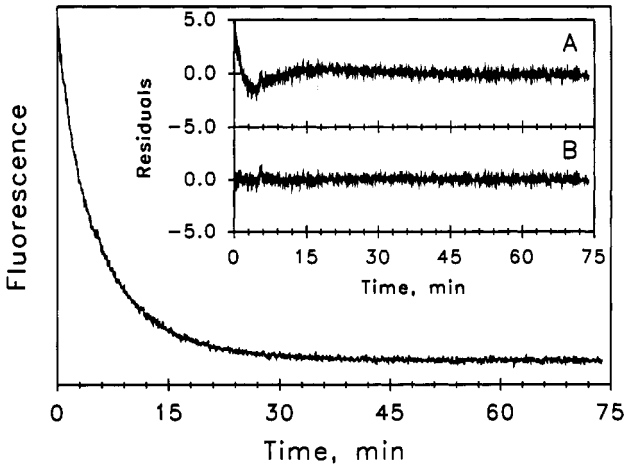

FIGURE 4: Dissociation of DAAC from tubulin at 37 °C. Insets: Plots of the residuals between the experimental and the theoretical curves for (A) a single exponential and (B) a sum of two exponentials.

2×10^{-5} s^{-1} at 37 °C (Diaz & Andreu, 1991). Application of this correction to the dissociation rate constants for both phases does not significantly change the results.

Equilibrium Constants. Knowledge of the association and dissociation rate constants for the fast and slow phases at 37 °C allowed for calculation of the equilibrium constants by the following relations:

$$Ka_f = k_f/k_{-f}$$

$$Ka_s = k_s/k_{-s}$$

Ka_f and Ka_s are the calculated equilibrium constants for the fast and slow phases, respectively, of the complexes. The terms k_f and k_{-f} are the apparent second-order association rate constant and first-order dissociation rate constant, respectively, for the fast phase. The terms k_s and k_{-s} are the corresponding parameters for the slow phase. The calculated equilibrium constants are listed in Table III.

DISCUSSION

Association Kinetics. The bindings of colchicine and AC to tubulin are biphasic processes (Cortese et al., 1977; Garland, 1978; Lambeir & Engelborghs, 1981; Bane et al., 1984; Engelborghs & Fitzgerald, 1987; Kang et al., 1990; Diaz & Andreu, 1991). Each phase has been shown to consist of two steps. The kinetic mechanism for colchicine and AC binding to tubulin is therefore best described by the following equations (Lambeir & Engelborghs, 1981; Engelborghs & Fitzgerald, 1987):

$$T + C \overset{K}{\rightleftharpoons} TC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} TC^* \quad \text{fast phase}$$

$$T' + C \overset{K'}{\rightleftharpoons} T'C \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} T'C^* \quad \text{slow phase}$$

The first step of each phase consists of a rapid association of tubulin (T and T′) and the colchicinoid (C) to form a low-affinity complex (TC and TC′). In the second step of each phase, a slow, unimolecular reaction results in the formation of the stable, fluorescent colchicinoid–tubulin complex (TC* and T′C*).

Separation of the association constants (K and K') from the unimolecular rate constants (k_2 and k_{-2}) may be accomplished by evaluating the apparent second-order rate constants as a function of concentration. For NHMe-DAAC, the association

Table III: Dissociation Rate Constants and Equilibrium Constants for the Colchicinoid–Tubulin Complexes at 37 °C

ligand	$k_f \times 10^3$, s^{-1} [a]	$k_{-s} \times 10^3$ s^{-1} [a]	$Ka_f \times 10^{-4}$, M^{-1} [b]	$Ka_s \times 10^{-4}$, M^{-1} [b]	$Ka_{eq} \times 10^{-4}$, M^{-1} [c]
colchicine	0.0053[d]–0.018[e]	*f*	150–1500[d,e,g]	*f*	200–300[d,h]
NMe_2-DAAC	2.9(0.6)[i]	0.8(1.0)	10.5(2.8)	7.8(11.4)	
NHMe-DAAC	3.3(0.8)	1.0(0.6)	5.4(1.4)	4.3(3.1)	7[j]
NH_2-DAAC	6.3(0.6)	2.4(1.1)	7.7(1.5)	4.5(2.6)	
DAAC	6.5(1.2)	2.2(0.3)	95(17)	24(6)	160[k]
AC[l]	58(10)	25(10)	27(6)	21(6)	48(3)[m]

[a] k_{-f} and k_{-s} are the first-order dissociation rate constants of the fast and slow phases, respectively, at 37 °C. [b] Ka_f and Ka_s are the calculated equilibrium constants of the fast and slow phases, respectively, at 37 °C. [c] Association constants determined from equilibrium methods. [d] From Garland (1978). [e] From Andreu & Diaz (1991). [f] Colchicine dissociation reaction is monophasic. [g] From Menendez et al. (1989). [h] From Bhattacharyya & Wolff (1974). [i] Standard deviations in parentheses. [j] Equilibrium constant for the binding to the colchicine site on tubulin; from Ray et al. (1984). [k] From Coudhury et al. (1983). [l] Kinetic data from Engelborghs & Fitzgerald (1987) at 25 °C. [m] From Andreu et al. (1984); 25 °C.

rate was a linear function of concentration up to 320 μM. Severe inner filter effects precluded obtaining association rates at higher NHMe-DAAC concentrations using our experimental apparatus. In this work, then, colchicinoid–tubulin associations were monitored at a single ligand concentration. Therefore, the data presented here represent the global process for both the fast and slow phases:

$$T + C \underset{k_{-f}}{\overset{k_f}{\rightleftharpoons}} TC^* \quad \text{fast phase}$$

$$T' + C \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} T'C^* \quad \text{slow phase}$$

The terms k_f and k_s represent the apparent second-order association rate constants for the fast and slow phases, respectively. These parameters are also equivalent to the products of the association constant and first-order rate constant (Kk_2 and $K'k'_2$) of each phase of the complete mechanism. The terms k_{-f} and k_{-s} are the first-order dissociation rate constants for the fast and slow phases, respectively.

The apparent second-order rate constant of demecolcine binding to tubulin at 37 °C was found to be about 1.5 times greater than that found for colchicine binding to tubulin. These results are in contrast to a previous determination (Ray et al., 1984), in which the rate constants for the two ligands differed by about a factor of 5. In the earlier analysis, the association rate constant was determined from initial reaction rates under second-order conditions, and the concentration of the demecolcine–tubulin complex was assessed by a filter disk assay. In this study, the association rates for the global process are determined from the full kinetic profile. In addition, the kinetic values found here for the colchicine–tubulin association are in good agreement with previous determinations (Cortese et al., 1977; Garland, 1978; Lambeir & Engelborghs, 1981; Diaz & Andreu, 1991). Thus, we believe our analysis of demecolcine–tubulin kinetics to be more accurate.

The kinetic parameters for the association of a variety of B-ring analogs of colchicine with tubulin are compiled in Tables I and II. Whenever possible, data obtained under the same experimental conditions are compared. In addition, since the amplitude of the fast phase of these ligands binding to tubulin was greater than the amplitude of the slow phase (Table I), this discussion will primarily address the fast phase.

It is seen in Table I that the apparent second-order rate constants at 37 °C for colchicinoids possessing a substituent at the C-7 carbon binding to tubulin differ by up to a factor of 4. In terms of the energetics of the transition state, however, these differences are less than 1 kcal/mol in the free energy of activation (Table II). Thus, there is very little difference in the change in free energy between the ground state and the transition state, regardless of the nature of the C-7 substituent. In contrast, when the C-7 carbon is unsubstituted (DAAC), the rate constant of the association with tubulin dramatically increases, and the free energy for the formation of the transition state decreases by about 2 kcal/mol. The free energy for the formation of the transition state for DAAC binding to tubulin is about 1 kcal/mol greater than that calculated for AC binding to tubulin. In terms of the free energy of activation at 37 °C for colchicinoid–tubulin complexes, then, the B ring itself contributes about 1 kcal/mol and the substituent at C-7 adds an additional 1–2 kcal/mol to the free energy of the transition state.

The activation energy and enthalpy of activation, which are similar parameters, provide more insight into the nature of the transition states of the colchicinoids binding to tubulin (Tables I and II). When these parameters are examined, the similarities and differences in the transition states are more apparent. The enthalpy of activation values for the formation of all three aminocolchicinoid–tubulin complexes are within 2 kcal/mol of each other and are significantly greater than the enthalpy of activation for colchicine binding to tubulin. These data show that the enthalpy for the formation of the transition state is *not* a function of the size of the substituent in this series. The similarities in the free energies of activation must therefore arise from the transition-state entropies. It is not possible to precisely assign the factors that contribute to the different transition-state entropies, which may include contributions from the ligand, protein, and solvent. It is concluded that the nature of the C-7 substituent does contribute to the energetics of colchicinoid–tubulin transition states, but the electronic rather than steric properties of the C-7 substituent appear to be of greater importance.

The activation energy and transition-state enthalpy of DAAC binding to tubulin are significantly less than that of colchicine and the C-7 substituted analogs binding to tubulin. The enthalpy of activation for DAAC binding to tubulin is, in fact, within experimental error of the corresponding parameter for the association of AC with tubulin. This finding indicates that there is no interaction between tubulin and the carbons of the B ring in the transition state. Analysis of the equilibrium enthalpy changes that occur when colchicine, AC, and allocolchicine bind to tubulin led to a similar conclusion for the role of the B ring in the ground state (Menendez et al., 1989). It must be noted here, however, that there is clear evidence for an interaction of the C-7 substituent and tubulin in the transition state which is not believed to occur in the ground state.

A summary of the kinetic and thermodynamic enthalpies of the B-ring analogs of colchicine binding to tubulin is shown in diagram form in Figure 5. On the basis of available data, we propose that the C-7 substituent of the B ring is on the exterior of the colchicine binding site in the equilibrium complex, but makes contact with the protein in the transition state during complex formation, thereby increasing the

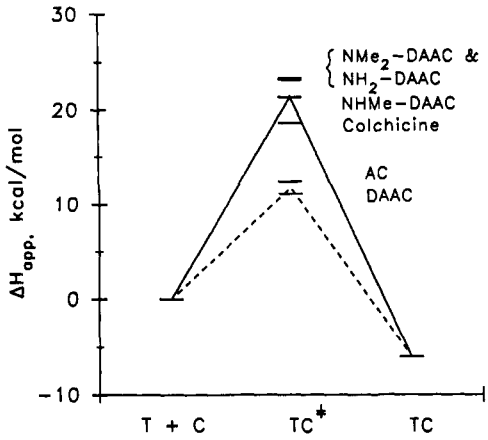

FIGURE 5: Kinetic pathway for the binding of colchicine, AC, and colchicine B-ring analogs binding to tubulin (global reaction for the fast phase). The transition-state enthalpies were determined experimentally (Table II). The equilibrium enthalpy changes for the aminocolchicinoids binding to tubulin are unknown but are estimated to be similar to those of colchicine and AC binding to tubulin (Menendez et al., 1989). The solid line represents the average path for colchicine and C-7 substituted analogs. The average path for AC and DAAC is shown as a dashed line.

enthalpy of activation. According to this model, the conformational change that occurs when colchicine binds to tubulin is assigned an enthalpy of activation of ≤13 kcal/mol. We would then predict that ligands which bind to the colchicine site on tubulin that do not possess substituents in the same region of space as the C-7 substituent of colchicine should bind to tubulin with an activation enthapy of about 13 kcal/mol. Naturally, quantitative analyses of additional ligands binding to tubulin need to be performed to test this hypothesis. It is noted, however, that the activation energy of podophyllotoxin binding to tubulin (13.6 ± 1.9 kcal/mol; Engelborghs & Fitzgerald, 1987) is approximately the same as the global activation energy of AC binding to tubulin (Table I).

Dissociation Kinetics. The dissociation rate constants of the colchicinoid–tubulin complexes were determined at 37 °C. Only one temperature was evaluated due to the slow dissociation rate observed at 37 °C. The dissociations of the colchicinoid–tubulin complexes studied were found to be biphasic in nature. The dissociation of the colchicinoid–tubulin complexes was monitored by the decrease in colchicinoid fluorescence upon release of the ligands from tubulin. Tubulin-bound colchicinoid fluorescence is very weak in the case of the aminocolchicinoids (Pyles & Hastie, 1992), and thus the errors in dissociation rate constants of the slow phase were rather large for these ligands. The error was smaller when a stronger signal was monitored (DAAC; see Table III).

The first-order dissociation rate constants for the fast phase of aminocolchicinoid–tubulin and DAAC–tubulin complexes at 37 °C were about 100-fold greater than the colchicine–tubulin dissociation rate constant (Garland, 1978; Banerjee et al., 1987; Diaz & Andreu, 1991) and about 100-fold less than the AC–tubulin dissociation rate constant (Banerjee et al., 1987; Engelborghs & Fitzgerland, 1987) (Table III). In contrast to the formation of the complexes, the dissociation of the DAAC–tubulin complex is considerably slower than the dissociation of the AC–tubulin complex. Conclusions about the transition states for the dissociation of the colchicinoid–tubulin complex cannot be drawn since the rate constants were determined at a single temperature. These parameters are of value, however, in evaluating the equilibrium constants of the two kinetic phases.

It is interesting to note that the dissociation of the colchicine–tubulin complex is best described by a single exponential equation (Diaz & Andreu, 1991), while a biexponential equation better represents the dissociation of the other colchicinoid–tubulin complexes. We have found that the data for the dissociation of the demecolcine–tubulin complex were better fit by a single exponential equation when relatively short time spans were analyzed (10 vs 75 min). It is possible that the dissociation of the colchicine–tubulin complex is also biphasic, but the long time periods required to detect the second phase coupled with the difficulties in analyzing this system have precluded detection of the second phase. It has been suggested that the dissociation rates for the two phases may be too similar to detect separately (Diaz & Andreu, 1991). It is also noted that the dissociation of the colchicine–tubulin complex induced by salt or detergents is also described by a biphasic process (Ide & Engelborghs, 1981; Banerjee et al., 1987; Andreu et al., 1986).

Equilibrium Constants. The equilibrium constants for the association of NMe_2-DAAC, NHMe-DAAC, NH_2-DAAC, and DAAC with tubulin were calculated from the apparent second-order rate constants and the first-order dissociation rate constants at 37 °C (Table III). The calculated equilibrium constants for the fast and slow phases for each aminocolchicinoid–tubulin complex are indistinguishable from one another within experimental error. The calculated equilibrium constants for the two phases of the DAAC–tubulin complex are distinguishable and differ by a factor of 2–4. In addition, the calculated equilibrium constant for the fast phase is similar to the equilibrium constant experimentally determined from fluorescence titration under equilibrium conditions (Choudhury et al., 1983).

There is a single report of the association properties of demecolcine and tubulin measured by equilibrium methods (Ray et al., 1984). In this study, demecolcine was found to bind to two sites on the tubulin dimer, one of which was blocked by colchicine. The equilibrium constants determined here by kinetic means are in reasonable agreement with the equilibrium constant found for demecolcine associated with the colchicine binding site (see Table III). On the basis of the data presented here, we cannot speculate as to whether the biphasic nature of the demecolcine–tubulin kinetics is related to the two different binding sites found by equilibrium methods.

The two kinetic phases of colchicine binding to tubulin can be attributed to colchicine binding to different tubulin isotypes (Banerjee & Luduena, 1991, 1992). The slow phase of the association appears to be the result of colchicine binding to tubulin containing the β_{III} isotype, while the fast phase may be assigned to colchicine binding to the β_{II} and β_{IV} isotypes. The affinity constants of colchicine binding to the three purified β-tubulin isotypes at 37 °C have recently been measured (Banerjee & Luduena, 1992). The association constants for colchicine binding to $\alpha\beta_{II}$- and $\alpha\beta_{III}$-tubulin were virtually identical, while the association constant for colchicine binding to $\alpha\beta_{IV}$-tubulin was a factor of 10 greater than the value determined for the other two isotypes.

Most equilibrium measurements of colchicinoids binding to unfractionated tubulin have found a single type of binding site on tubulin for colchicinoids (see Hastie (1991)). The recent equilibrium studies on isolated tubulin isotypes coupled with these kinetic determinations of the association constants for the two kinetic phases provide a rationale for the apparent discrepancies between the kinetic and equilibrium findings. We have found the calculated equilibrium constant for the two kinetic phases of the aminocolchicinoids binding to tubulin at 37 °C to be indistinguishable within experimental error. In the case of DAAC binding to tubulin, the apparent association constants were significantly different, but the

difference between the apparent association constants of the two phases is too small for the two types of binding to be detected by equilibrium methods. Therefore, to study the associations of colchicinoids with the different tubulin isotypes, either the isotype population must be separated or kinetic methods must be used.

CONCLUSIONS

Quantitative kinetic analyses of four B-ring analogs of colchicine binding to tubulin have been performed. The kinetic parameters for the global reaction obtained in this study have been compared with those previously found for colchicine and AC binding to tubulin. The high activation energy of colchicine binding to tubulin is attributed to the presence of a substituent at the C-7 carbon but not the B ring itself. The activation energy for the formation of C-7 substituted colchicinoid–tubulin complexes appears to be a function of the electronic nature of the substituent rather than the size of the substituent. We propose that ligands that bind to the colchicine site on tubulin will display a relatively low activation energy ($\leq$13 kcal/mol) unless a component of the ligand overlaps with a region on colchicine corresponding to the C-7 substituent.

Kinetic analyses of the association and dissociation of the colchicinoid–tubulin complexes at 37 °C revealed that both processes were biphasic for the colchicinoids in this study. Equilibrium constants calculated from the kinetic data were generally indistinguishable within experimental error. In one case, the equilibrium constants were demonstratively different, but the differences were too small to have been detected by equilibrium methods. Equilibrium determinations of colchicinoid binding to tubulin isotypes should therefore be performed on separated tubulin isotypes or by kinetic methods.

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