Colchicine Binding to Tubulin Monomers: A Mechanistic Study†

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ABSTRACT: The kinetic and thermodynamic parameters for colchicine−tubulin and deacetamidocolchicine−tubulin interaction, under the condition where tubulin is predominantly in its dissociated state (∼80% monomer), have been determined. We observe that the kinetic parameters exihibit marked change when colchicine interacts with the monomeric form of tubulin rather than with the dimeric form of tubulin. The reaction of colchicine with tubulin monomers is characterized by an enhanced association rate which is a consequence of the lowering of activation energy. Colchicine−tubulin interaction, which is only poorly reversible, becomes partially reversible under this condition. Differences were also noticed in the thermodynamic parameters: the reaction of colchicine with tubulin monomers is enthalpy driven with small positive entropy, while with tubulin dimers a large positive entropy change was reported. However, no such changes in the binding parameters were observed for the reaction involving deacetamidocolchicine (a colchicine analog devoid of a side chain at the C-7 position of B-ring) with tubulin monomers. We therefore conclude that a single subunit of tubulin is capable of binding colchicine and that the unusual properties of colchicine−tubulin interactions such as the slow association rate, high activation energy, and the poor reversibility are due to the possible contact(s) of the C-7 substituent (in the B-ring) of colchicine with the other subunit of tubulin.

Colchicine, a major alkaloid in *Colchicum autumnale*, is medically used for the treatment of gout (Beck, 1932) and Familial Mediterranean fever (Zemer et al., 1974). Colchicine exerts its antimitotic property upon binding to a single site on tubulin dimer with high affinity (Weisenberg et al., 1968; Wilson, 1970; Bhattacharyya & Wolff, 1974). Unfortunately, there is no unanimity in the literature regarding the location of the high-affinity binding site in the dimer. Colchicine binding to a site on β-tubulin has been proposed on the basis of indirect experiments dealing with the reactivity of cysteine residues in β-tubulin (Roach et al., 1985) and from the fact that most tubulin mutations that confer colchicine resistance occur in β-tubulin genes (Sheir-Neiss et al., 1978; Cabral et al., 1980). Covalent attachment of a colchicine derivative to tubulin followed by sequence analysis is the most direct method for locating the sequence(s) of tubulin associated with the colchicine binding site. Yet even this method can lead to incongruous results. At least five different affinity and photoaffinity labeling derivatives of colchicine have been shown to form covalent bonds with tubulin. Results presented in these studies are confusing when taken together and support the assignment of the colchicine binding site solely to the α-subunit (Schmitt & Atlas, 1976; Williams et al., 1985), solely to the β-subunit (Grover et al., 1992), to both (Lin et al., 1989; Floyd et al., 1989), or at the interface of the two subunits (Wolff et al., 1991; Uppuluri et al., 1993).

If the results of affinity and photoaffinity experiments are analyzed, one observes that when the affinity group is in the side chain of the B-ring of colchicine (see Figure 1A for structure), the labeled peptide obtained belongs to the α-subunit (Schmitt & Atlas, 1976; Williams et al., 1985). On the other hand, when chemically reactive A-ring modified analogs of colchicine were used, the bulk of the radio label was found on the β-subunit (Grover et al., 1992). These results immediately tell us that the A-ring is part of the β-subunit and the B-ring side chain is close to the α-subunit.

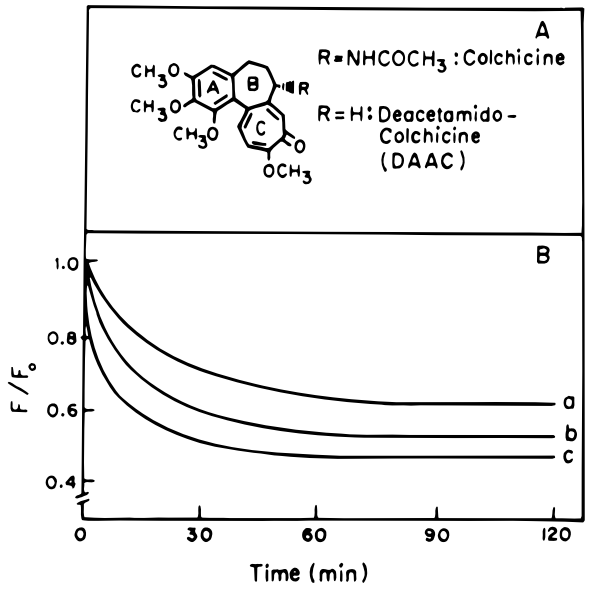

FIGURE 1: (A) Structures of colchicine and deacetamidocolchicine (DAAC). (B) Quenching of intrinsic protein fluorescence upon colchicine binding to tubulin. Tubulin concentrations were 0.2 (a), 1.0 (b), and 3.0 μM (c), and colchicine concentrations were 20 times higher than the respective protein concentrations. F_0 is the intrinsic tryptophan fluorescence of tubulin at zero time of mixing, and F_t is the fluorescence at time t after mixing of colchicine. Excitation and emission wavelengths were 280 and 336 nm, respectively. The kinetic curves have been best fitted to a sum of two exponentials.

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Recently, Wolff and Knipling (1995), by direct photolabeling of lactoperoxidase-induced tubulin monomers with [^{3}H]-colchicine, showed that the bulk of the radioactivity become attached to β-tubulin. They also proposed that the B-ring of colchicine faces α-tubulin, while the A- and the C-rings are in contact with β-tubulin (Wolff & Knipling, 1995). Shearwin and Timasheff (1994) have also put forth a very similar model.

Similar arrangements of colchicine in the binding site of tubulin molecule have also been suggested to explain several properties of colchicine−tubulin interactions (Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996). The reaction of colchicine with tubulin is slow, highly temperature dependent and a poorly reversible process (Wilson, 1970; Bhattacharyya & Wolff, 1974; Cortese et al., 1977; Garland, 1978; Lambeir & Engelborghs, 1981; Bane et al., 1984). These unusual properties are found to be related to its side chain at the C-7 position of the B-ring (Ray et al., 1981; Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996). Thus, an analog without the side chain at C-7 position such as DAAC[1] (deacetamidocolchicine, see Figure 1A) binds tubulin faster, renders the reaction reversible, and lowers the global activation energy to ∼11−13 kcal/mol (Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996) compared to 16−26 kcal/mol for colchicine−tubulin interaction (Lambeir & Engelborghs, 1981; Diaz & Andreu, 1991; Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996). It has been proposed by Pyles and Bane Hastie (1993) as well as by us (Chakrabarti et al., 1996) 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(s) with protein.

If the colchicine binding site is in the β-subunit and the protrusion of the side chain at the C-7 position along with its contact with the α-subunit is responsible for the unusual properties of colchicine−tubulin interaction, then the binding properties of colchicine with tubulin in its dissociated state should be very similar to those of DAAC (the analog devoid of the side chain) with dimeric tubulin. In this communication, we report a recently completed study of colchicine binding with tubulin at very low protein concentrations (viz. 0.2 μM), where 80% of tubulin is in the dissociated state (Panda et al., 1992). Under these conditions, colchicine binds faster—only one of the dissociated subunits of tubulin binds colchicine (data not shown), and the activation energy of the reaction is lowered to 13 kcal/mol, a value similar to DAAC−tubulin (dimer) interaction (Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996). The reaction of colchicine with monomeric tubulin is partially reversible but is poorly reversible with tubulin dimers.

MATERIALS AND METHODS

Materials. Pipes, GTP, EGTA, colchicine, and podophyllotoxin were purchased from Sigma. Deacetamidocolchicine was a gift from Prof. T. J. Fitzgerald (Florida A&M University). All other reagents used were of analytical grade. Radioactive colchicine ([^{3}H] ring C methoxy, 70.0 Ci/mmol) was obtained from New England Nuclear.

Preparation of Tubulin. Goat brain tubulin, free from microtubule-associated proteins, was prepared by two cycles of assembly−disassembly in PEM buffer (0.05 M Pipes, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.9, at 25 °C) in presence of 1 mM GTP followed by two more cycles in 1 M glutamate buffer (Hamel & Lin, 1981) and stored at −70 °C. The concentration of protein was determined by the method of Lowry et al. (1951).

Stock solutions of colchicine and its analogs were prepared either in water or in dimethyl sulfoxide (Me_2SO). The maximum concentration of Me_2SO in the reaction mixture was 5% for DAAC. The concentrations of the ligands were determined from their respective extinction coefficients (Pyles & Bane Hastie, 1993).

Association Kinetics. The association kinetics of colchicine and DAAC with tubulin were studied under pseudo-first-order conditions using a Hitachi F-3000 spectrofluorometer. The temperature was controlled with a circulating water bath accurate to ±0.5 °C. Quenching of intrinsic tryptophan fluorescence of tubulin by drug (colchicine, DAAC) was used to measure the association rate constant with an excitation wavelength of 280 nm and the emission wavelength at 336 nm (slit widths of 5 nm were used). The quenching data were analyzed according to Pyles and Bane Hastie (1993) using the bi-exponential equation

$$F_t = A \exp(-k_1 t) + B \exp(-k_2 t) + C \qquad (1)$$

where F_t is the fluorescence of the drug−tubulin complex at time t, A and B are the amplitudes for the fast and the slow phases, respectively, k_1 and k_2 are the pseudo-first-order rate constants for the fast and slow phases, respectively, and C is the integration constant. As for all tubulin−colchicinoid complexes studied (Lambeir & Engelborghs, 1981; Chakrabarti et al., 1996), the amplitude of the slow phase, B, was small relative to the fast phase, A, and the slow phase was not analyzed further. The apparent second-order rate constant (k_{on}) was obtained by dividing the observed rate constant for the fast phase (k_1) by the drug concentration. For the calculation of activation energy (E_a), k_{on} was determined at different temperatures (T), ranging from 17 to 37 °C, and plotted against (1/T), according to the Arrhenius equation, $k_{on} = A \exp(-E_a/RT)$, where A is a pre-exponential factor.

Dissociation Kinetics. The dissociation of the tubulin−colchicine complex was monitored by determining the loss of bound [^{3}H]colchicine from the tubulin−[^{3}H]colchicine complex (Ray et al., 1984). The protein-bound [^{3}H]colchicine was assayed by the DEAE cellulose filter disk method (Weisenberg et al., 1968; Williams & Wolff, 1972) using PM buffer (10 mM phosphate and 10 mM $MgCl_2$, pH 6.9).

The dissociation rate constant (k_{off}) of colchicine−tubulin complex at 0.2 μM tubulin was measured by monitoring the time-dependent decrease of [^{3}H]colchicine binding upon addition of a 100-fold excess unlabeled colchicine or podophyllotoxin. k_{off} for tubulin (1 μM) and DAAC interaction was obtained by monitoring the time-dependent increase of intrinsic protein fluorescence as the ligand was released from its binding site on tubulin upon 300-fold dilution of the complex (Bane et al., 1984; Chakrabarti et al., 1996).

Equilibrium Constant (K_a). For the reaction of drugs with tubulin at concentration (0.2 μM), K_a was obtained from the intercept at abscissa (negative) of the double-reciprocal plot

[1] Abbreviations: DAAC, deacetamidocolchicine; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); AC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; C-termini, carboxy-termini.

Table 1: Association Rate Constants and Activation Energies of Colchicine−Tubulin and DAAC−Tubulin Interaction at Low and High Tubulin Concentrations

ligand	[tubulin] (μM)	monomer[a] (%)	k_{on}[b] (M^{-1} s^{-1})	A[c] (M^{-1} s^{-1})	E_a[d] (kcal mol^{-1})
cholchicine	10.0[f]	15			16 ± 4
	3.0[e]	25	72	210	20.9 ± 0.6
	2.0[g]	30			21 ± 2
	0.2[e]	67	270	717	13.1 ± 0.2
DAAC	3.0[e]	25	1340	3102	13.0 ± 1.3
	0.2[e]	67	2440	5014	13.6 ± 0.6

[a] Obtained from dimer ⇌ monomer equilibrium using $K_D = 2.7 \times 10^{-7}$ M, in presence of colchicine (Panda et al., 1992). [b] Determined at 32 °C. [c] A, pre-exponential factor of the Arrhenius equation. [d] E_a, activation energy. [e] This study. [f] From Lambeir and Engelborghs (1981). [g] From Diaz and Andreu (1991).

$1/Q$ vs $1/[D]$, where Q is the quenching of intrinsic fluorescence of tubulin upon binding of drug and [D] is the drug concentration. For the reaction of DAAC with 1 μM tubulin, K_a was obtained from the relation $K_a = k_{on}/k_{off}$ (Bane et al., 1984; Chakrabarti et al., 1996).

Data Analysis. For both association and dissociation kinetics the unknown parameters were varied and the best-fit values giving minimum χ^2 were obtained by the method of iteration on their respective equations, using a BASIC program written for this purpose (Chakrabarti et al., 1996).

RESULTS

Association Kinetics. In the present study the association rate constants of DAAC (deacetamidocolchicine) and colchicine with tubulin were determined by the drug-induced quenching of tubulin fluorescence under psuedo-first-order conditions. Figure 1B shows a representative kinetic profile for the binding of colchicine to various concentrations of tubulin (0.2−3.0 μM), at 25 °C. Apparent second-order rate constants (k_{on}) at different tubulin concentrations were determined from bi-exponential (Figure 1B) curves for fluorescence versus time and fitted to experimental data as described in Materials and Methods. There is no qualitative change in the biphasic nature of the quenching of tryptophan fluorescence upon dilution of tubulin. The rate of colchicine binding increases 3−4-fold upon dilution of tubulin. It is to be noted that the dilution of tubulin from 3 to 0.2 μM changes the monomer concentration from 38% to 82% in absence of colchicine and from 25% to 67% in presence of colchicine (calculation based on $K_D = 0.72$ and 0.27 μM for dimer ⇌ monomer equilibrium in absence and presence of colchicine, respectively) (Panda et al., 1992). Activation energies for colchicine−tubulin and DAAC−tubulin interactions, for both monomeric and dimeric forms of tubulin, were determined as described in Materials and Methods. A comparison of the variation of association rates (k_{on}), activation energies (E_a), and the pre-exponential factors (A) of colchicine−tubulin and DAAC−tubulin interactions at 3 and 0.2 μM tubulin concentrations is presented in Table 1. It is clear that the association rate increases and the activation energy decreases for the colchicine−tubulin interaction as the monomer state of tubulin increases in solution (Figure 2A). According to Arrhenius equation, the rate constant is a product of the activation energy term (E_a) and the pre-exponential factor (A), i.e., $k_{on} = A \exp(-E_a/RT)$. It is observed that the pre-exponential factor A increases significantly from 210 to 717 for colchicine−tubulin interaction upon dilution of tubulin concentration from 3 to 0.2 μM. However, under identical conditions, there is no change in the activation energy of DAAC−tubulin interaction (Figure 2B), although slight variations in the association rate and the pre-exponential factor have been noted (Table 1).

Dissociation Kinetics and Reversibility. Colchicine−tubulin interaction is poorly reversible, and removal of the C-termini of tubulin increases the dissociation rate of tubulin−colchicine complex (Mukhopadhyay et al., 1990). In the present study, we have measured the reversibility of colchicine−tubulin complex using [^{3}H]colchicine. Reversibility was tested at 0.2 and 3 μM tubulin concentrations (Figure 3A,B). Tubulin and [^{3}H]colchicine were incubated together at 37 °C, a 100-fold excess of unlabeled colchicine or podophyllotoxin was added to the reaction mixture (indicated by arrow, Figure 3), and the incubation was continued for the indicated period. Aliquots were withdrawn at different time intervals and binding assay were carried out according to Weisenberg et al. (1968) using DE-81 filter disks. Any loss of protein bound radioactivity upon addition of the unlabeled ligand reflected the displacement of the initially bound labeled ligand and hence the reversibility of the reaction. It is evident from Figure 3A that about 50% of the initially bound radioactivity was displaced within 1 h when the tubulin concentration was 0.2 μM. In contrast, the initial radioactivity bound to 3 μM tubulin (Figure 3B) in a parallel experiment remained largely unaffected upon addition of 100-fold excess of unlabeled colchicine or podophyllotoxin. The kinetic profile of the dissociation of colchicine−tubulin complex, as shown in Figure 3A, is clearly biphasic in nature and was best described by a sum of two exponentials (Bane et al., 1984). The dissociation constant obtained using data from Figure 3A (i.e., 0.2 μM tubulin containing dimer:monomer in the ratio 33:67) has been found to be 6.6×10^{-4} s^{-1} (for the fast phase) compared to 0.18×10^{-4} s^{-1} (Diaz & Andreu, 1991) reported for 2 μM tubulin (the dimer:monomer ratio being 70:30). It should be mentioned that this dissociation constant for the colchicine−tubulin interaction, at low tubulin concentration, is very similar to that reported for the DAAC−tubulin interaction (Banerjee et al., 1994). Percentage monomers as a function of tubulin concentration is plotted along with percent dissociation of [^{3}H]colchicine−tubulin complex after 120 min of displacement with unlabeled colchicine, in Figure 3C. It is apparent that both the enhanced reversibility and the fraction of tubulin in the monomeric state present in the sample have similar patterns of reduction with tubulin concentration. Thus, the reversibility changes being related to the appearance of tubulin monomers cannot be ruled out.

Thermodynamic Parameters. Interactions of several colchicinoids such as deacetamidocolchicine (DAAC), deacetylcolchicine (H_2N-DAAC), colcemid (CH_3·NH−DAAC), and methyl colcemid (CH_3·CH_3·N−DAAC) with tubulin have been studied in detail (Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996). Interactions of these analogs with tubulin are entropy driven and are accompanied by an unfavorable positive enthalpy change (Chakrabarti et al., 1996). Studies on the binding thermodynamics of colchicine−tubulin interactions have provided conflicting results. Early equilibrium studies on colchicine−tubulin interaction reported high positive entropy value for the binding reaction (Bryan, 1972; Bhattacharyya & Wolff, 1974). Later, these data were

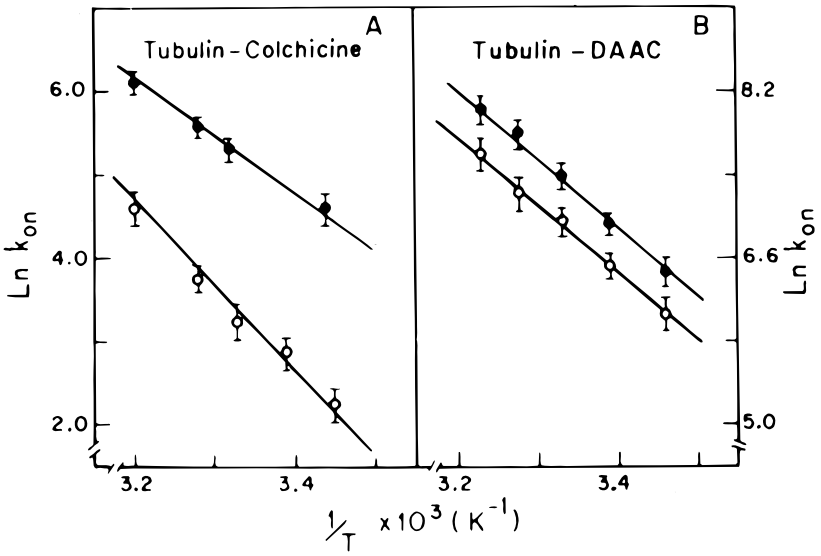

FIGURE 2: Effect of temperature on the apparent second-order rate constant of colchicine and DAAC binding to tubulin. Binding of colchicine (panel A) and DAAC (panel B) with tubulin at concentrations 0.2 (●) and 3.0 μM (○). Excitation and emission wavelengths were same as in Figure 1B.

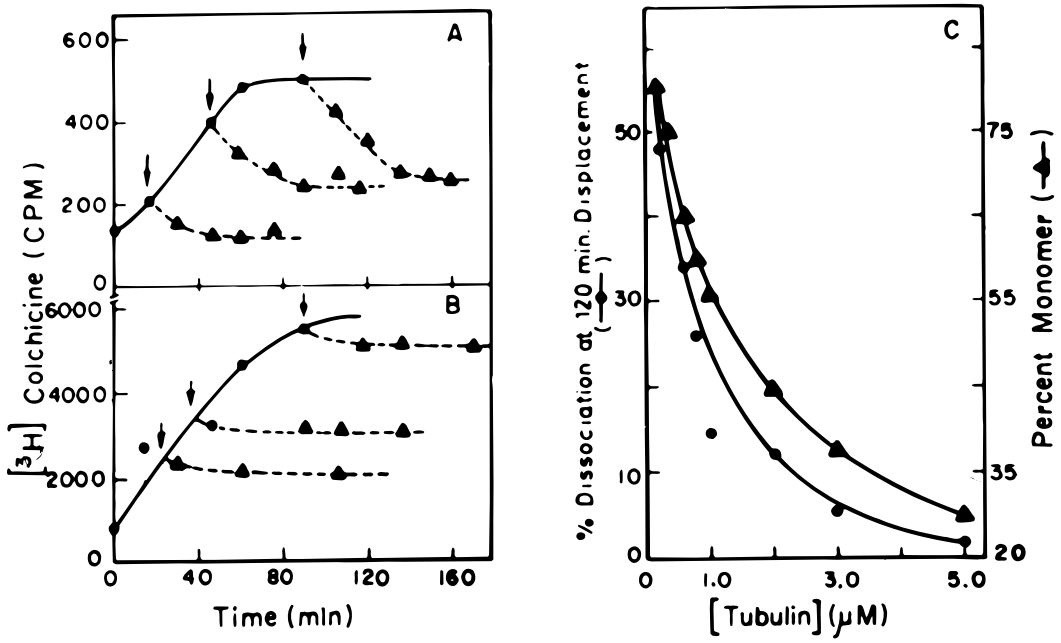

FIGURE 3: Reversibility of colchicine−tubulin interaction. Tubulin and [^{3}H]colchicine were incubated together at 37 °C for different times. At indicated periods (shown by arrow) appropriate amounts of unlabeled colchicine were added to the respective reaction mixtures to make the final concentration 100 times the initial concentration of colchicine. The reaction mixtures were incubated further at 37 °C, and aliquots were withdrawn at different intervals and assayed for the bound radioactivity. (A) Binding of 0.2 μM tubulin and 0.2 μM [^{3}H]colchicine (●) and the effect of addition of 20 μM unlabeled colchicine at 15 (▲), 45 (▲), or 90 min (▲). (B) Binding of 3 μM tubulin and 3 μM [^{3}H]colchicine (●) and the effect of addition of 300 μM unlabeled colchicine at 15 (▲), 45 (▲), or 90 min (▲). (C) Percent dissociation of [^{3}H]colchicine−tubulin complex at 120 min of displacement with unlabeled colchicine and the percent tubulin monomers present in solution. Tubulin at different concentrations (0.2−5 μM) were incubated with [^{3}H]colchicine for 90 min at 37 °C. A 100-fold excess of unlabeled colchicine or podophyllotoxin was then added to the reaction mixtures, the incubation was continued for 120 min, and the amount of binding was determined by the filter disk assay method (Weisenberg et al., 1968; Williams & Wolff, 1972). The extent of dissociation of [^{3}H]colchicine−tubulin complex at 120 min of displacement with unlabeled colchicine is plotted against tubulin concentrations (●). (The decrease in bound radioactivity has been used to determine the percent dissociation and the bound radioactivity before the addition of unlabeled drug has been taken as 100%.) The percent monomer in solution (obtained from dimer ⇌ monomer equilibrium using $K_D = 7.2 \times 10^{-7}$ M) (Panda et al., 1992) for these tubulin concentration are also shown (▲).

questioned for two reasons: first, these were obtained under the conditions where true equilibrium had not been reached; second, proper corrections were not made for the decay of colchicine binding site. After proper corrections for the decay of colchicine binding site, the colchicine−tubulin interaction was found to be accompanied by negative enthalpy change $\Delta H = -27(\pm 18)$ kJ mol^{-1} for pure phosphocellulose tubulin and microtubule-associated proteins (Lambeir & Engelborghs, 1981); $\Delta H = -26(\pm 13)$ kJ mol^{-1} for W-tubulin, (Diaz & Andreu, 1991). A negative enthalpy change for the colchicine−tubulin interaction was also obtained from calorimetric study, i.e., $\Delta H = -21(\pm 2)$ kJ mol^{-1} (Menendez et al., 1989). Contradictory results have also been reported for the interaction of tubulin with

allocolchicine, a colchicine analog with an altered C-ring, where the kinetically estimated overall enthalpy change (19 ± 16 kJ mol^{-1}) (Dumortier et al., 1996) is significantly different from direct calorimetric determination (−10.8 ± 2 kJ mol^{-1}; Menendez et al., 1989) and van't Hoff analysis (Medrano et al., 1989). Interaction of tubulin with AC and DAAC has a negative enthalpy of binding and a small positive ΔS (Andreu et al., 1984; Bane et al., 1984; Engelborghs & Fitzgerald, 1986; Menendez et al., 1989; Chakrabarti et al., 1996).

On the basis of the binding study of several aminocolchicinoids with tubulin it was hypothesized that the B-ring side chain of aminocolchicinoids is on the exterior of the colchicine binding site but makes contact(s) with tubulin (Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996). If this model is true, then the thermodynamic parameters of a colchicine analog without the side chain (such as DAAC) with tubulin should be very much similar, irrespective of the concentration of tubulin. In the present study, we determined the thermodynamic parameters of DAAC tubulin interactions in both high and low tubulin concentrations. We also determined the thermodynamic parameters for colchicine−tubulin interactions at 0.2 μM tubulin concentration. van't Hoff plots of these two drugs are shown in Figure 4. At low tubulin concentration (0.2 μM), both ΔH and ΔS for the interaction with DAAC are favorable. The values of ΔH and ΔS are −11.7 kJ mol^{-1} and +73.1 J K^{-1} mol^{-1}, respectively, for DAAC. Binding of colchicine with 0.2 μM tubulin has shown favorable enthalpy and entropy changes. The values of ΔH and ΔS are −21.4 kJ mol^{-1} and +32.7 J K^{-1} mol^{-1}, respectively. Similar thermodynamic values for the colchicine−tubulin interaction were reported from several laboratories (Lambeir & Engelborghs, 1981; Menendez et al., 1989; Diaz & Andreu, 1991). This is in contradiction to early equilibrium studies (Bryan, 1972; Bhattacharyya & Wolff, 1974). For the purpose of comparison, values of thermodynamic parameters of these two drugs (colchicine and DAAC) with high and low tubulin concentrations are summarized in Table 2. We observe that the changes in protein concentrations do not affect the thermodynamic parameters of DAAC−tubulin interaction, and at both high and low protein concentrations the reaction is enthalpy driven and has positive entropy.

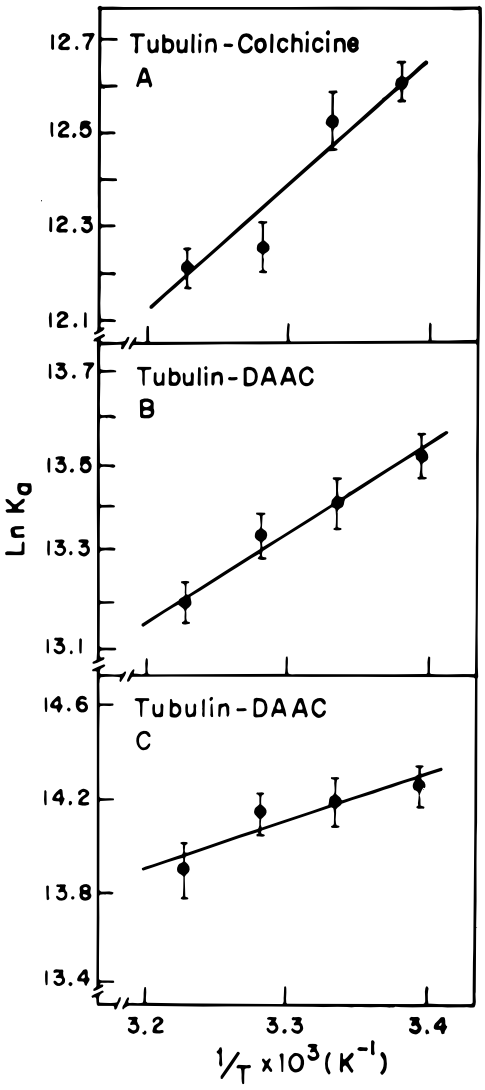

FIGURE 4: Effect of temperature on the equilibrium constants (K_a) of tubulin−colchicine and tubulin−DAAC interactions. van't Hoff plots for colchicine (panel A) and DAAC (panels B and C) with 0.2 μM (panels A and B) and 1 μM tubulin (panel C).

Table 2: Thermodynamic Parameters of Tubulin-Colchicine and Tubulin-DAAC complexes

ligand	[tubulin] (μM)	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J K^{-1} mol^{-1})
cholchine	0.2[a]	−31.5 ± 0.4	−21.4 ± 10.9	+32.7 ± 7.1
	1.0[b]	−38.6	+43.2	+260.4
	2.0[c]	−42	−26.4	+68.4
DAAC	0.2[a]	−36.1 ± 0.8	−11.7 ± 2.5	+73.1 ± 2.1
	1.0[a]	−36.1 ± 2.1	−15.1 ± 2.1	+67.6 ± 6.7

[a] This study. [b] Thermodynamic data from Bhattacharyya and Wolff (1974). [c] Thermodynamic data from Diaz and Andreu (1991).

DISCUSSION

The mechanism of colchicine binding to tubulin has remained a subject of intensive study since the original isolation of tubulin by tracing this binding with radioactive colchicine (Borisy & Taylor, 1967; Wilson & Freidkin, 1967). In the last three decades, several aspects of colchicine−tubulin interaction and related phenomena were discovered: substoichiometric poisoning of tubulin polymerization by colchicine (Margolis & Wilson, 1977; Sternlicht & Ringel, 1979; Margolis et al., 1980; Koufias & Wilson, 1992), two-step sequence of binding as proposed by Garland (1978) and others (Lambeir & Engelborghs, 1981), and recently the isolation of covalently labeled [^{3}H]colchicine bound to β-tubulin (Wolff & Knipling, 1995). According to the two-step binding mechanism, the second step of interaction involves conformational adjustments in both tubulin and the drug. Promotion of colchicine fluorescence (Bhattacharyya & Wolff, 1974), changes in the CD spectra of colchicine (Detrich et al., 1981), enhancement of GTPase activity of tubulin (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981; Hamel, 1990), and the unfolding of β-tubulin (Sackett & Varma, 1993) are the consequences of the second step of colchicine−tubulin interaction. Other aspects which received significant attention in the last few decades are several unusual properties of colchicine−tubulin interaction such as slow association rate, high activation energy, poor reversibility, and high entropy in interaction (Bhattacharyya & Wolff, 1974; Garland, 1978; Banerjee & Bhattacharyya, 1979; Lambeir & Engelborghs, 1981; Ray et al., 1981; Diaz & Andreu, 1991; Pyles & Bane Hastie,

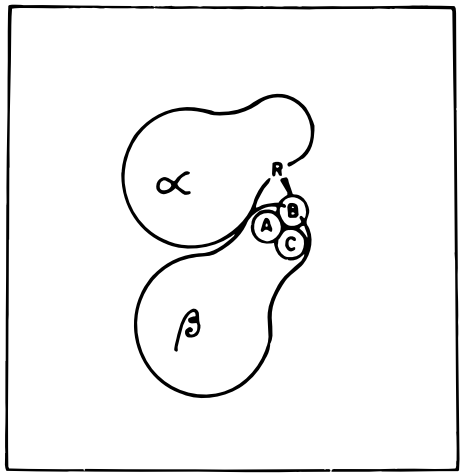

FIGURE 5: Schematic structure of the tubulin dimer showing the colchicine binding site. It must be noted that this model is used only to clarify the discussion and is not a structural model of the protein.

1993; Chakrabarti et al., 1996). It has now been established that these unusual properties of colchicine−tubulin interaction are due to its side chain at the C-7 position of the B-ring (Ray et al., 1981; Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996). This is supported by the fact that analogs devoid of the side chain, such as DAAC, possess no such unusual binding properties with tubulin (Ray et al., 1981; Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996).

It has already been hypothesized that this side chain of colchicine at the C-7 position stays outside the binding site and makes contact(s) with the protein (Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996). A pertinent question at this juncture is whether this side chain makes contact(s) with another part of the same subunit where A- and C-rings reside. If so, then even in the dissociated state of tubulin, the properties of colchicine−tubulin interaction should be similar to that observed with tubulin dimers. In the dissociated state of tubulin, the association rate of tubulin−colchicine interaction increases 3−4-fold and the activation energy is lowered to 13 kcal/mol and becomes similar to that of DAAC (Pyles & Bane Hastie, 1993; Chakrabarti et al., 1996). Futhermore, reversibility of colchicine−tubulin interaction increased dramatically and the off-rate is of the same order of magnitude as that for DAAC (Pyles & Bane Hastie, 1993; Banerjee et al., 1994; Chakrabarti et al., 1996). Finally, colchicine−tubulin interaction, which normally shows a high positive entropy change, becomes enthalpy driven with small positive entropy (like the DAAC−tubulin interaction) when tubulin is in the dissociated state. Thus, we conclude from the results presented here that the side chain of colchicine does not make contact(s) with the same subunit where the A- and C-rings reside.

Results presented here support the model proposed by Shearwin and Timasheff (1994) and later supported by Wolff and Knipling (1995). We believe that the side chain of colchicine makes contact with the α-subunit (possibly with its C-termini). A schematic structure is presented in Figure 5 based on the results obtained. This conjecture is supported by previous findings from this laboratory that the cleavage of the α-subunit by subtilisin increases the off-rate of colchicine−tubulin interaction (Mukhopadhyay et al., 1990). This model also explains why the α-subunit is labeled when the affinity label is in the side chain of colchicine analogs (Williams et al., 1985). We believe that the α-subunit imposes constraints and thus provides impediments to approach toward the binding site by colchicine during the association reaction. This makes the exit and the entry of the drug to the binding site difficult. The lower values of the entropy of the reaction in the monomer state can also be explained by the fact that both the acetamido side chain of colchicine and the subsite of α-subunit (to which this side chain were attached in the dimer) are free, possibly exposed to solvent, and the rearrangements of water structure (which accounts for a large positive entropy value) around them are not involved in the binding reaction (Ross & Subramaniam, 1981; Chakrabarti et al., 1996).

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