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Kinetics of Dissociation of the Tubulin-Colchicine Complex

COMPLETE REACTION SCHEME AND COMPARISON TO THERMODYNAMIC MEASUREMENTS*

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The slow dissociation reaction of the tubulin-colchicine complex has been characterized in purified calf brain tubulin and microtubule protein preparations, using [^{3}H]colchicine and fluorometric measurements. It fits to a single exponential phase, within the accuracy of these measurements. The dissociation is a kinetically unfavorable reaction, with activation energy values of 114 ± 10 and 94 ± 10 kJ mol^{-1} (purified tubulin and microtubule protein, respectively). The kinetic scheme previously proposed for the tubulin-colchicine association (Lambeir, A., and Engelborghs, Y. (1981) *J. Biol. Chem.* 256, 3279–3282) is:

$$T + C \underset{}{\overset{K_1}{\rightleftharpoons}} TC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (TC)'$$

where step 1 is a fast reversible binding and step 2 is a slow conformational change, whose backward rate constant (k_{-2}) was neglected for the association study. This kinetic scheme has now been completed to include the measurements of the rate-limiting dissociation step (k_{-2}), and of the purified calf brain tubulin preparation. The overall binding standard free energy change, calculated from the kinetic measurements, is −42.0 ± 0.1 kJ mol^{-1} (fast phase of binding in 10 mM sodium phosphate buffer, 0.1 mM GTP, pH 7.0, at 37 °C). The binding is exothermic and the calculated enthalpy change is −26 ± 13 kJ mol^{-1}, which coincides with the recently determined calorimetric enthalpy value, −21 ± 2 kJ mol^{-1} (Menendez, M., Laynez, J., Medrano, F. J., and Andreu, J. M. (1989) *J. Biol. Chem.* 264, 16367–16371), suggesting that the kinetic scheme and measurements are essentially correct.

Specific binding of colchicine to its main cellular target, tubulin, leads to the inhibition of microtubule assembly and mitotic arrest (Taylor, 1965). Colchicine binds slowly to a single high affinity site of tubulin; the unoccupied sites denature rapidly and the dissociation is very slow, hampering equilibrium binding studies (Wilson and Bryan, 1974). However, equilibrium binding measurements can be made with simple colchicine analogues, which bind more rapidly and reversibly to the colchicine site (Andreu and Timasheff, 1982a; Andreu *et al.*, 1984; Bane *et al.*, 1984; Medrano *et al.*, 1989). The cellular effects of some of these colchicine analogues are reversible (Diez *et al.*, 1987; Mollinedo *et al.*, 1989; Herman *et al.*, 1989).

The kinetics of association of colchicine to tubulin was examined by Garland (1978) and Lambeir and Engelborghs (1981). It consists of two parallel phases (corresponding to two types of binding sites) of which only the fast phase was characterized. A two step mechanism was proposed which consists of a fast reversible binding followed by a slow conformational change:

$$T + C \underset{}{\overset{K_1}{\rightleftharpoons}} TC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (TC)' \qquad (I)$$

where K_1 is the equilibrium association constant of the first step of binding of colchicine (C) to tubulin (T) and k_2 and k_{-2} are the rate constants of the forward and backward second step. This leads to the slow, high affinity formation of the practically stable fluorescent end product (TC)′ (Garland, 1978; Lambeir and Engelborghs, 1981). The rate constant k_{-2} had a very small value under the conditions of the first kinetic study (Garland, 1978), which was neglected in the stopped-flow study of the association kinetics (Lambeir and Engelborghs, 1981). Therefore, the system was rigorously characterized only in terms of K_1 and k_2. For the first step an apparent standard enthalpy change $\Delta H^0_1 = -33 \pm 12$ kJ mol^{-1} was determined, an activation energy $E_{a2} = 100 \pm 5$ kJ mol^{-1} for the second step, and the activation energy of the backward second step (E_{a-2}) was not determined.

For the proposed reaction scheme (I) it holds that the apparent standard enthalpy change of the overall equilibrium is equal to the sum $\Delta H^0_1 + E_{a2} - E_{a-2}$. The colchicine-tubulin interaction was known to be strongly temperature-dependent and van't Hoff estimates of the enthalpy change of approximately 50–70 kJ mol^{-1} were reported in the literature (Bryan, 1972; Bhattacharyaa and Wolff, 1974; Barnes *et al.*, 1983). This would require E_{a-2} values of approximately −17 to 3 kJ mol^{-1}, which does not seem consistent with a kinetically unfavored dissociation reaction. However, the specific binding of different bifunctional colchicine analogues to tubulin is characterized by small van't Hoff enthalpy changes comprised between −28 and 8 kJ mol^{-1} (Bane *et al.*, 1984; Andreu *et al.*, 1984; Medrano *et al.*, 1989). Recently, the enthalpy change of binding of the bicyclic colchicine analogue MTC[1] was determined calorimetrically to be −19 ± 1 kJ mol^{-1}; the value for colchicine, under the limited conditions in which attainment of equilibrium could be ensured, was essentially the same, −21 ± 2 kJ mol^{-1} (Menendez *et al.*, 1989). This indicated that the equilibrium binding of colchicine to tubulin was moder-

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[1] The abbreviations used are: MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

ately exothermic (that is, the temperature dependence commonly observed was due to kinetic effects) and suggested that according to the kinetic mechanism (I) the activation energy of the backward second step should be about 100 kJ mol^{-1} (Menendez *et al.*, 1989).

The purposes of this study were to characterize the complete kinetic pathway of the tubulin-colchicine interaction, including the dissociation, and to verify the kinetics with independent thermodynamic measurements under rigorously identical conditions. It is shown that the dissociation of tubulin-colchicine is a kinetically unfavored reaction and that the kinetically derived enthalpy value for the complete binding and dissociation scheme is coincident with the calorimetric enthalpy change.

MATERIALS AND METHODS

Experimental—Calf brain tubulin (W-tubulin) was purified by the Weisenberg procedure and its concentration was measured spectrophotometrically as described (Weisenberg *et al.*, 1968; Lee *et al.*, 1973; Andreu and Timasheff, 1982b). Calf brain microtubule protein was prepared as described (Karr *et al.*, 1982) and its concentration was measured (Bradford, 1976) using purified tubulin as standard. Subtilisin-cleaved tubulin (S-tubulin) was prepared by differential C-terminal cleavage of purified tubulin in Mes buffer (De la Viña *et al.*, 1988) and assembly-disassembly of the S-tubulin polymers. Colchicine and podophyllotoxin were from Aldrich; [^{3}H]colchicine was from Amersham Corp. (ring C methoxy tritiated, 40 Ci/mmol, lot no. 2425-231). The colchicine concentration was determined spectrophotometrically, using an extinction coefficient of 15,950 M^{-1} cm^{-1} at 353 nm in aqueous buffer (Andreu and Timasheff, 1982b), and by scintillation counting in a LKB 1219 spectrometer. GTP, dilithium salt, was from Boehringer Mannheim. The tubulin-colchicine complexes were prepared essentially as described (Andreu and Timasheff, 1982b) by incubation of the concentrated protein with 5×10^{-3} M colchicine 30 min at room temperature. Excess colchicine was eliminated by chromatography at 4 °C in 20 × 1-cm Sephadex G-25 columns equilibrated in PG buffer (10 mM sodium phosphate, 0.1 mM GTP buffer, pH 7.0) (for W-tubulin and S-tubulin) or in MKMEG buffer (50 mM Mes, 70 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, pH 6.4 (Lambeir and Engelborghs, 1981) containing 1 mM GTP) (for microtubule protein). The stoichiometry of the complexes was 0.96 ± 0.03 mol of colchicine/10^5 g of purified tubulin and 0.65 ± 0.05 mol of colchicine/10^5 g of microtubule protein.

The dissociation of tubulin-[^{3}H]colchicine was measured as follows. Aliquots of freshly prepared 2×10^{-6} M protein-ligand complex were incubated at constant temperature during given times in PG buffer containing 10^{-4} M unlabeled colchicine or [^{3}H]colchicine of exactly the same specific activity. The remaining tubulin-colchicine complex and the dissociated [^{3}H]colchicine were separated by Sephadex G-25 as above. The tubulin-associated and dissociated colchicine concentrations were determined by scintillation counting; the tubulin concentration was determined with a Cary 16 spectrophotometer, using an extinction coefficient of 1.16 liter g^{-1} cm^{-1} at 276 nm, after correction for light scattering and for the small contribution of bound colchicine at this wavelength (Andreu and Timasheff, 1982b). Alternately, the dissociation of approximately 2×10^{-6} M tubulin-colchicine complex in buffer containing 10^{-4} M podophyllotoxin (a nonfluorescent colchicine site ligand; Andreu *et al.*, 1984; Engelborghs and Fitzgerald, 1987) or 10^{-4} M colchicine was measured fluorometrically in a Fica MKII spectrofluorometer, with excitation at 365 nm (slit 2.5 nm) and emission at 430 nm (slit 7.5 nm). The sample was held during each experiment in the thermostated cuvette holder connected to a Lauda K2RD bath and was illuminated only at given times, to minimize possible photolysis; the temperature of the sample was measured with a thermocouple.

The association of colchicine to W-tubulin in PG buffer was measured fluorometrically under pseudo-first order conditions, with identical sample temperature control, using a Shimadzu RF540 spectrofluorometer with excitation wavelength 358 nm (slit 2 nm) and emission wavelength 430 nm (slit 10 nm); sample photolysis was not significant in the time scale of these measurements.

Kinetic Data Analysis—The dissociation of the tubulin-colchicine complex was analyzed according to the scheme (Garland, 1978):

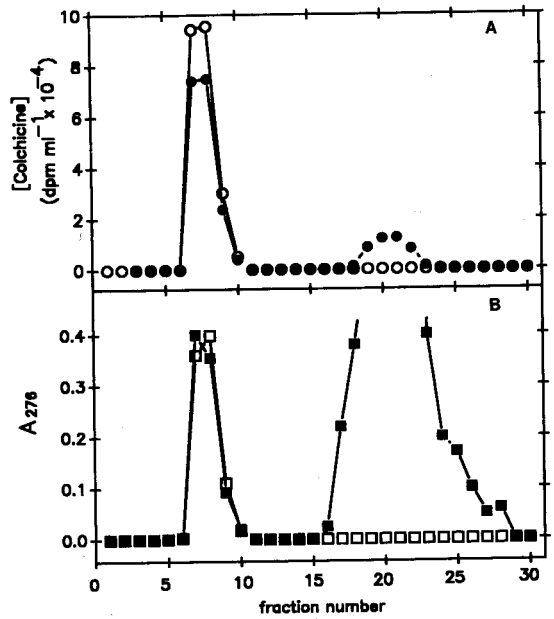

FIG. 1. **Sephadex G-25 chromatography of the purified tubulin-[^{3}H]colchicine complex (1 mg).** *Open symbols*, freshly prepared complex. *Filled symbols*, after 6-h incubation at 30 °C with 100-fold excess unlabeled colchicine. *A*, [^{3}H]colchicine elution profile; the specific activity of [^{3}H]colchicine used in this experiment was 3.344×10^{10} dpm $mmol^{-1}$. *B*, absorbance at 276 nm; note that the second peak is due to the excess colchicine.

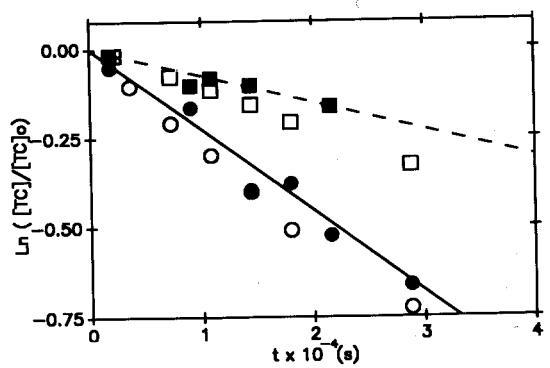

FIG. 2. **Time course of dissociation of 2×10^{-6} M purified tubulin complex in PG buffer at 35 °C.** *Solid circles*, apparent [^{3}H]colchicine dissociation in 50-fold unlabeled colchicine excess. *Solid squares*, apparent [^{3}H]colchicine dissociation in 50-fold excess of labeled colchicine of the same specific activity. *Open circles*, colchicine dissociation measured fluorometrically in 50-fold excess of podophyllotoxin. *Open squares*, fluorometric measurements in 50-fold colchicine excess. The *solid* and *dashed lines* are the least squares linear fits to the ^{3}H-labeled colchicine data.

$$\begin{array}{ccccc} & & k_f & k_d' & \\ T + C & \rightleftharpoons & (TC)' & \longrightarrow & T'' + C \\ k_d \downarrow & & k_r & & \\ T' & & & & \end{array} \qquad \text{(II)}$$

which contains no assumptions about the binding mechanism (Scheme I) by which the measured end product (TC)′ is generated, and where k_r is the intrinsic dissociation rate constant, k_f is the apparent bimolecular association rate constant, k_d the denaturation rate constant of the unbound colchicine site, k_d' the denaturation rate constant of the bound colchicine site, and T′ and T″ the denatured colchicine site. The total rate of loss of colchicine binding sites is $k_d'[(TC)'] + k_d[T]$. However, for the experimentally determined values of k_d (Menendez *et al.*, 1989) and k_f (see "Results" below), and in large excess of free ligand, it holds that

$k_d[T] \ll k_d[T][C]$; that is, the colchicine sites T, generated by dissociation, will reassociate to (TC)′ instead of denaturing to T′. Hence, the contribution of the rate of denaturation of the unliganded site, $k_d[T]$, can be neglected for the purpose of the present analysis. Measurement of the decay rate of the tubulin-colchicine complex in large excess of the same colchicine employed to make it affords in good approximation the denaturation rate constant k'_d. Measurement of the decay rate of the complex the presence of a large enough excess of competitor (unlabeled colchicine or podophyllotoxin) affords the uncorrected dissociation rate constant $k_r + k'_d$. The difference $(k_r + k'_d) - k'_d$ gives the dissociation rate constant k_r.

Alternately, the dissociation of tubulin-colchicine can be measured in the absence of free ligand, in which case the following expression applies to the dissociation scheme (II):

$$d[(TC)']/dt = k_f[T][C] - k_r[(TC)'] - k'_d[(TC)'] \quad (1)$$

The observed initial rate is $k_r + k'_d$; at long times a steady state is approached in which $k_f[T][C] = k_r[(TC)']$, excess free ligand is generated and the rate tends to k'_d.

The simple scheme (II) relates to the proposed complete kinetic scheme (I) as follows. The measured dissociation rate constant k_r corresponds to the rate-limiting dissociation constant k_{-2}; therefore, an Arrhenius plot of k_r yields E_{a-2}. The apparent bimolecular association rate constant k_f is:

$$k_f = K_1k_2/(1 + K_1[C]) + k_{-2}/[C] \quad (2)$$

Due to the facts that at low colchicine concentrations $K_1[C] \ll 1$ and that k_{-2} has a very small value, Equation 2 reduces in good approximation (Lambeir and Engelborghs, 1981) to:

$$k_f = K_1k_2 \quad (3)$$

The product K_1k_2 can be measured with good accuracy. The Arrhenius equation for this apparent association rate constant is:

$$\ln k_f = \ln K_1 + \ln k_2 = \text{constant} - (\Delta H^0_1 + E_{a2})/RT \quad (4)$$

and therefore the slope of a plot of $\ln k_f$ *versus* $1/T$ measures the sum of the standard enthalpy change of the first step (ΔH^0_1) plus the activation energy of the second step (E_{a2}). The overall standard free energy and enthalpy changes of binding can be calculated from the parameters of the kinetic scheme (I) as:

$$\Delta G^0_{app} = -RT \ln(K_1k_2/k_{-2}) \quad (5)$$

$$\Delta H^0_{app} = H^0_1 + E_{a2} - E_{a-2} \quad (6)$$

RESULTS

To characterize the slow dissociation reaction of the tubulin-colchicine complex a sensitive measurement method and correction for the denaturation of the binding site were required. Fig. 1 shows a chromatographic measurement of the dissociation of the purified tubulin-[^{3}H]colchicine complex in excess unlabeled colchicine. The colchicine bound to tubulin (first peak) decreased from 0.97 ± 0.03 to 0.78 ± 0.02 mol of colchicine/mol tubulin during 6 h at 30 °C; the free [^{3}H] colchicine (second peak) increased from 0.00 ± 0.01 to 0.19 ± 0.02 mol of colchicine dissociated per mol of tubulin. Both measurements agree and for small degrees of dissociation the free colchicine peak provides a more sensitive measurement of dissociation than the difference in bound colchicine. Actually, dissociation of 2% of the tubulin-colchicine complex could be accurately measured by this procedure. Nevertheless the measurements were extended to 20–70% dissociation, depending on the temperature.

Fig. 2 shows the first order time course of dissociation of the purified tubulin-[^{3}H]colchicine complex in PG buffer at 35 °C, in presence of unlabeled colchicine excess (*solid circles*) and in [^{3}H]colchicine excess (*solid squares*); the first is the measurement of the apparent dissociation rate constant of the tubulin-colchicine complex ($2.32 \pm 0.04 \times 10^{-5}$ s^{-1}) and the second is the measurement of its denaturation rate constant ($8.0 \pm 0.4 \times 10^{-6}$ s^{-1}); this rate was measured only from the decrease in bound colchicine and was generally of lower

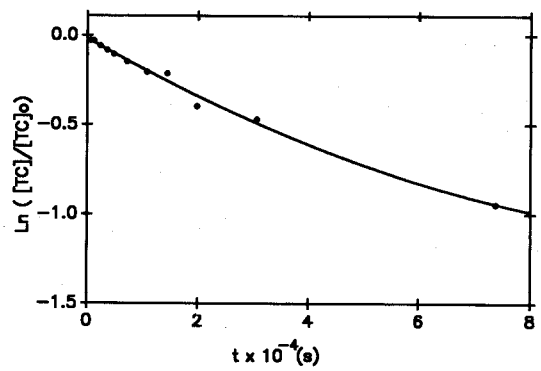

FIG. 3. **Dissociation of 10^{-5} M purified tubulin-[^{3}H]colchicine complex in PG buffer at 35 °C, in the absence of other ligands.** The line is drawn simply to show the trend of the data.

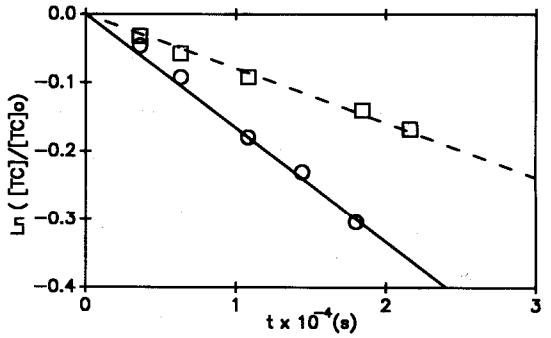

FIG. 4. **Dissociation of 0.2 mg ml^{-1} microtubule protein-colchicine complex in MKMEG buffer at 35 °C, monitored fluorometrically in 10^{-4} M podophyllotoxin (*circles*) or colchicine (*squares*).**

accuracy than the first rate). The difference between the two rate constants is the corrected dissociation rate constant of the tubulin-colchicine complex (see "Kinetic Data Analysis"), which was $15.2 \pm 0.6 \times 10^{-6}$ s^{-1} under the conditions of the experiment. Fig. 2 also shows the results of a parallel experiment in which the dissociation of the tubulin-colchicine complex was measured fluorometrically in excess podophyllotoxin (*empty circles*) and in excess colchicine (*empty squares*) (see "Materials and Methods"), under otherwise identical conditions. Although the fluorometric method gave slightly larger rates than the chromatographic procedure, the net rate constant of dissociation of tubulin-colchicine was $15.6 \pm 0.8 \times 10^{-6}$ s^{-1}, which is identical to the radioactive measurement. The dissociation of tubulin-colchicine was a single first order process, within the accuracy of the measurements, under all the conditions examined in this study.

Fig. 3 shows the dissociation of purified tubulin complex in the absence of added ligand at 35 °C. This first order plot is curved because the finite concentration of free colchicine generated by the dissociation binds significantly to the empty sites. The initial slope measures the apparent dissociation rate constant (2.35×10^{-5} s^{-1}, coincident with the value from Fig. 2), and the dissociation rate constant at long times tends to the value of the denaturation rate constant of the complex ($\sim 9.8 \times 10^{-6}$ s^{-1}, which is compatible with the value calculated from Fig. 2) (see "Kinetic Data Analysis").

Fig. 4 shows the dissociation of the microtubule protein-colchicine complex (*i.e.* tubulin-colchicine plus microtubule-associated proteins) determined fluorometrically in MKMEG buffer at 35 °C. The value of the dissociation rate constant was $9.4 \pm 0.3 \times 10^{-6}$ s^{-1}, which is 1.6 times smaller that the value for purified tubulin above; this difference could be

attributed to the presence of the associated proteins, the different preparation procedure or the different buffer, and it was not further investigated because of its small amplitude. The values of the dissociation rate constants determined at different temperatures and under the different conditions examined are shown in Table I.

Fig. 5 shows the Arrhenius plots for the dissociation of the purified tubulin-colchicine complex (*circles*) and the microtubule protein-colchicine complex (*squares*). The activation energies determined are similar, 114 ± 12 and 94 ± 10 kJ mol^{-1}, respectively. The activation energies of denaturation of the liganded colchicine site were 150 ± 22 and 169 ± 10 kJ mol^{-1} for purified tubulin and microtubule protein, respectively.

Since the association kinetics of colchicine to purified tubulin had not been characterized under our conditions, the binding time course was monitored fluorometrically under pseudo-first order conditions (see Fig. 6*A*). The association is biphasic (Fig. 6*B*), as for the other tubulin preparations (see the Introduction). The apparent bimolecular association rate constants of the fast and slow phase at 35 °C were 168 ± 8 and 34 ± 3 M^{-1} s^{-1}, respectively (Fig. 6*C*). They were determined at different temperatures and rigorously constant colchicine concentrations. Fig. 7 shows the plots of ln k_f *versus* $1/T$. The apparent activation energies of colchicine association to purified tubulin were 88 ± 6 and 89 ± 13 kJ mol^{-1} for

TABLE I

Kinetics of dissociation of the tubulin-colchicine complex

Protein	T	Method[a]	$k'_d \times 10^6$	$k_r \times 10^6$
	°C		s^{-1}	
Purified tubulin[b]	25	R	3.3 ± 0.3	3.6 ± 0.5
Purified tubulin[b]	30	R	3.7 ± 0.2	6.6 ± 0.4
Purified tubulin[b]	35	R	8.0 ± 0.4	15.2 ± 0.6
Purified tubulin[b]	35	F	11.4 ± 0.2	15.6 ± 0.7
Purified tubulin[b]	37	F	20.5 ± 0.7	18.0 ± 0.9
Purified tubulin[b]	40	R	31.0 ± 2.0	33.0 ± 2.2
Purified tubulin[b]	40	F	35.8 ± 0.8	36.7 ± 1.2
Subtilisin-cleaved purified tubulin[b]	40	F	30.0 ± 2.0	42.2 ± 2.2
Microtubule protein[c]	30	F	2.3 ± 0.1	5.1 ± 0.3
Microtubule protein[c]	35	F	7.5 ± 0.5	9.2 ± 0.9
Microtubule protein[c]	37	F	12.0 ± 0.5	14.0 ± 1.1
Microtubule protein[c]	40	F	19.1 ± 0.9	16.4 ± 1.3

[a] R, release of radioactive labeled colchicine; F, decay of fluorescence of the tubulin colchicine complex.
[b] PG buffer, pH 7.0.
[c] MKMEG buffer, pH 6.4.

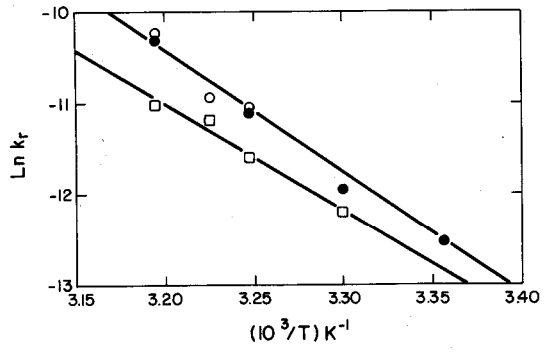

FIG. 5. **Arrhenius plots of the dissociation of the tubulin-colcicine complexes.** *Solid circles*, dissociation rate constants of the purified tubulin-[^{3}H]colchicine complex. *Open circles*, dissociation rate constants of the purified tubulin-colchicine complex determined fluorometrically. *Open squares*, fluorometric dissociation rate constants of the microtubule protein-colchicine complex. The lines are the least squares linear fits to the tubulin and microtubule protein, respectively.

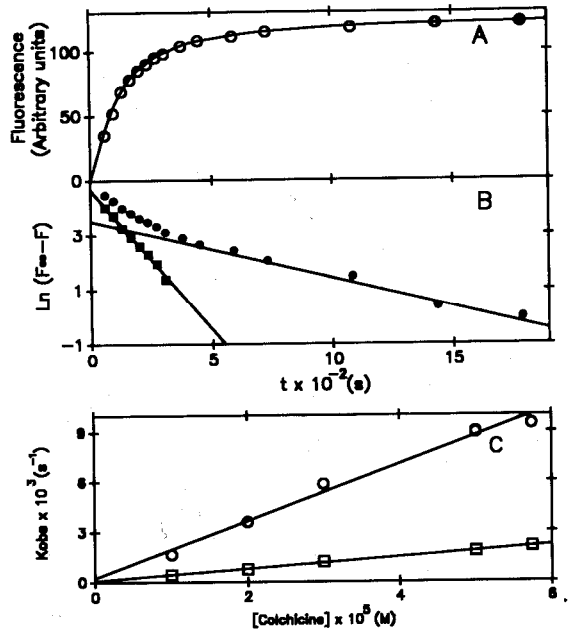

FIG. 6. **Kinetics of association of colchicine to purified calf brain tubulin in PG buffer at 35 °C.** *A*, fluorometric time course of binding of 5.74 × 10^{-5} M colchicine to 2.5 × 10^{-6} M tubulin. *B*, first order plot of the data; the lines show the decomposition into two single-exponential phases (*squares*, calculated fast phase). *C*, observed pseudo-first order association rate constants *versus* ligand concentration (tubulin concentrations varied between 10^{-6} and 2.5 × 10^{-6} M); *circles*, fast phase; *squares*, slow phase.

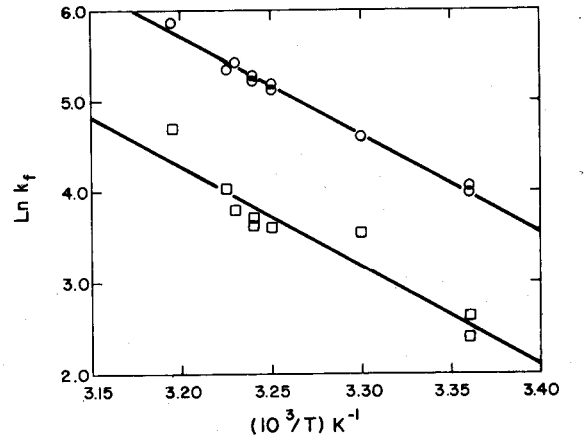

FIG. 7. **Logarithmic plots of the apparent bimolecular rates of association of colchicine to purified tubulin *versus* reciprocal absolute temperature.** *Circles*, fast phase; *squares*, slow phase. The lines shown are the least squares linear fits. The slopes measure the *apparent* activation energies of association (see Scheme I and "Materials and Methods").

the fast and slow phase, respectively. According to the reaction scheme (I), the apparent activation energy of the association reaction corresponds to the sum of the enthalpy change of the first step plus the activation energy of the second step (see "Kinetic Data Analysis").

DISCUSSION

The dissociation of the stoichiometric tubulin-colchicine complex has been characterized in detail, using purified calf brain tubulin and microtubule protein. Correction of the [^{3}H] colchicine and fluorometric measurements for the denaturation rate allowed this very slow reaction to be studied. The differences found with previous measurements at 37 °C (Sher-

TABLE II

Kinetics of the tubulin-colchicine interaction at 37°C

Parameter	Phosphocellulose-purified tubulin	Microtubule protein	W-tubulin
K_1 (M^{-1})	90[a]	60[a]	
ΔH^0_1 (kJ mol^{-1})	−33 ± 12[a]	−33 ± 8[a]	
ΔS^0_1 (J mol^{-1} K^{-1})	−63 ± 40[a]	−71 ± 33[a]	
k_2 (s^{-1})	1.6[a]	1.7[a]	
E_{a2} (kJ mol^{-1})	100 ± 5[a]	100 ± 5[a]	
K_1k_2 (M^{-1} s^{-1})	150[a]	100[a]	210 ± 14[b] (34 ± 3)[b,c]
$\Delta H^0_1 + E_{a2}$ (kJ mol^{-1})	67 ± 12[a]	67 ± 8[a]	88 ± 6[b] (89 ± 13)[b,c]
$k_{-2} \times 10^6$ (s^{-1})	5.3[d]	14.0 ± 1.1[b]	18.0 ± 0.9[b]
E_{a-2} (kJ mol^{-1})		94 ± 10[b]	114 ± 12[b]

[a] Data are taken from the study of Lambeir and Engelborghs (1981) (pig brain microtubule protein and tubulin). The thermodynamic parameters of the first equilibrium were derived from the detailed analysis and temperature dependence of the kinetic data.

[b] This study (calf brain microtubule protein and tubulin).

[c] Values in parenthesis are the apparent bimolecular association rate constant and the activation energy of the slow phase.

[d] From Garland (1978).

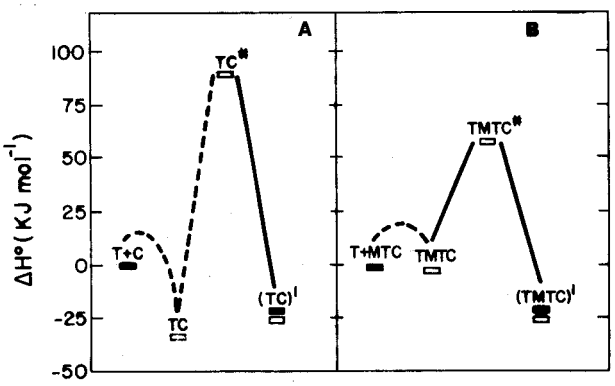

FIG. 8. ***A*, kinetic pathway for the fast phase of colchicine binding to purified calf brain tubulin.** The enthalpy values were determined experimentally (Table II), except the apparent enthalpy change of the first step (see *dashed line*), which was taken equal to that of phosphocellulose-purified pig brain tubulin (Lambeir and Engelborghs, 1981). *B*, shown for comparison, the kinetic pathway for the fast phase of binding of MTC to phosphocellulose-purified pig brain tubulin, taken from Lambeir and Engelborghs (1981). In both panels the reference state, which is the unassociated protein and ligand solution, and the thermodynamically determined global enthalpy change for purified calf brain tubulin (fast and slow phases, Menendez *et al.*, 1989) are marked by the *solid rectangles*. Note the coincidence of kinetic and thermodynamic measurements.

line *et al.*, 1975; McClure and Paulson, 1977; Garland, 1978) can be ascribed to the different protein sources and buffers. In contrast to the association reaction, the dissociation consisted of a single exponential phase, within the precision of the measurements. Therefore, the two association phases, corresponding either to two tubulin conformations (Lambeir and Engelborghs, 1981) or to two tubulin isotypes (Engelborghs and Fitzgerald, 1987; Banerjee and Ludueña, 1987), lead to either the same product or to products with coincident dissociation rates. That is, for our purposes, the same single phase dissociation measured applies to the fast and slow association phases. The finding of a single colchicine dissociation phase is compatible with the fact that the two phases described for the dissociation of the bicyclic colchicine analogue MTC differ by approximately a factor of 2 (Engelborghs and Fitzgerald, 1987). Fast and slow phases of colchicine dissociation had been detected previously at high chaotropic anion concentrations (Ide and Engelborghs, 1981) or with detergents (Andreu *et al.*, 1986); however, under these conditions k_{-2} may speed up and be no longer the rate-limiting constant. The C-terminal cleavage of purified tubulin by subtilisin (see "Materials and Methods") weakly modified the monophasic dissociation and denaturation rates of the colchicine complex at 40 °C (Table II); preliminary results indicated that the colchicine association time course remains biphasic in the subtilisin-cleaved tubulin.

The dissociation of the tubulin-colchicine complex is characterized by similarly large activation energies in purified tubulin and microtubule protein, consistent with a kinetically unfavorable reaction. The kinetic parameters of colchicine binding and dissociation are summarized in Table II. The kinetic reaction pathway (Scheme I) previously proposed for colchicine binding (Lambeir and Engelborghs, 1981) is now fully characterized, at least for the fast phase. Let us now compare the kinetic mechanism and parameters to the thermodynamic parameters of colchicine binding. This is shown in Table III. The binding equilibrium constant could not be measured rigorously as discussed previously (Andreu and Timasheff, 1982a; Menendez *et al.*, 1989); however, the kinetic binding and dissociation rate constants provide calculated values for the overall equilibrium constant and hence the standard free energy change, which for the fast phase of colchicine binding to purified calf brain tubulin varies between 41.2 and 42.0 kJ mol^{-1} in the range of temperatures studied. The kinetically estimated overall reaction enthalpy change for microtubule protein (fast phase) was found to be similar to the calorimetrically measured reaction enthalpy of binding of colchicine to purified tubulin (with contributions of fast and slow phase) (Table III). In order to be able to compare the kinetic and thermodynamic measurements under rigorously identical conditions, the necessary kinetic measurements were extended to the purified tubulin system. The kinetically calculated overall enthalpy change is exothermic, −26 ± 13 kJ mol^{-1}, which coincides within error with the calorimetric measurement. The simplest interpretation is that

TABLE III

Thermodynamic parameters of colchicine binding to tubulin

Parameter	Phosphocellulose-purified tubulin	Microtubule protein	W-tubulin
ΔG^0_{app} (kJ mol^{-1}), 37°C (from kinetics)[a]	−43.6[b]	−40.4	−42.0 ± 0.1 (−37.3 ± 0.2)
ΔH^0_{app} (kJ mol^{-1}) (from kinetics)[b]		−27 ± 13	−26 ± 13 (−25 ± 18)
ΔH^0_{app} (kJ mol^{-1}) (calorimetric measurement)[c]			−21 ± 2
ΔS^0_{app} (J mol^{-1} K^{-1})		43 ± 27	68 ± 7 (53 ± 7)

[a] Calculated from the parameters in Table II; values in parentheses are those calculated for the slow phase.

[b] From Garland (1978).

[c] From Menendez *et al.* (1989) (global value for the two phases).

TABLE IV

Thermodynamic parameters of MTC binding to tubulin

Parameter	T	Phosphocellulose-purified tubulin[a]	Phosphocellulose-purified tubulin[b]	W-tubulin[c]
	°C			
ΔG^0_{app} (kJ mol^{-1})	25		−31.0 ± 0.5	
(from kinetics)			(−30.3 ± 0.5)	
(Equilibrium measurements)	25			−32.1 ± 0.2
	37	−33		−32.6 ± 0.2
ΔH^0_{app} (kJ mol^{-1})			−24 ± 9	
(from kinetics)			(−11 ± 17)	
(Equilibrium measurement)		−28.4		
(Calorimetric measurement)				−19.0 ± 1.0
ΔS^0_{app} (J mol^{-1} K^{-1})		15	24 ± 15	43 ± 3
			(64 ± 100)	

[a] From Bane *et al.* (1984) (global values for the two phases).
[b] From the study of Engelborghs and Fitzgerald (1987); the values in parentheses are those calculated for the slow phase.
[c] From Andreu *et al.* (1984) and Menendez *et al.* (1989) (global values for the two phases).

the kinetic mechanism (Scheme I) and measurements of colchicine binding and dissociation (Table II) are fully correct. The complete kinetic pathway of the fast phase of colchicine binding is illustrated by Fig. 8*A*.

In the case of the rapid bicyclic colchicine analogue MTC both association and dissociation reactions have been measured (Engelborghs and Fitzgerald, 1986, 1987) and the kinetically derived parameters are compared to thermodynamic measurements (Andreu *et al.*, 1984; Bane *et al.*, 1984; Menendez *et al.*, 1989) in Table IV. Saving the differences in the protein preparations and in the experimental errors, the data are in good agreement, which suggests that the kinetic scheme (I) and measurements are equally correct for MTC. A comparison of the thermodynamic binding parameters of colchicine and MTC binding under rigorously identical solution conditions can be now carried out (compare last columns in Tables III and IV). The binding of colchicine is stronger than the binding of MTC by −9.4 ± 0.2 kJ mol^{-1} (or −8.5 ± 0.5 kJ mol^{-1} for any arbitrary mixture of 60–90% fast phase with slow phase); however, this difference free energy change does not arise from a difference enthalpy change, which is practically nonsignificant (−2 ± 2 kJ mol^{-1}), but from a significant difference entropy change of 25 ± 8 J mol^{-1} K^{-1} (or 22 ± 8 J mol^{-1} K^{-1} for the two phase mixtures above) favorable to colchicine. This essentially entropic difference between the bindings of colchicine and MTC is most simply attributed to the partial immobilization of rings A and C upon binding of MTC, whereas in colchicine the biaryl rotation is already restricted by the presence of ring B (Menendez *et al.*, 1989). Natural colchicine is the (7*S*)(−)-atropoisomer, which adopts predominantly the biaryl a*S* configuration, while unnatural (7*R*)(+)-colchicine prefers the a*R* conformer and is essentially inactive (Yeh *et al.*, 1988; Brossi *et al.*, 1990).

The kinetic pathway of MTC binding is illustrated in Fig. 8*B*. The overall binding is similarly exothermic (within experimental error) for both ligands. However, the first reaction step is exothermic for colchicine and nearly athermic for MTC, and the activation energy of the second step of colchicine binding is roughly twice of the value for MTC; the activation energies of the backward second step are less different. The larger energy of activation of the conformational change step of colchicine binding may be attributed to the presence of ring B, which is absent in MTC and may constitute a transient kinetic impediment to binding. The apparent thermodynamic parameters of the initial binding of colchicine (Table II) were remarkably similar to those of binding of the single ring ligand tropolone methyl ether (Andreu and Timasheff, 1982a). Hence, it was proposed that the tropolone ring of colchicine would bind first (Andreu and Timasheff, 1982b). Clearly this does not hold for MTC binding, as pointed out by Engelborghs and Fitzgerald (1987). However, it is conceivable that in this ligand lacking ring B the initial binding might proceed alternately through rings A or C (Andren *et al.*, 1991).

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