Kinetics and Mechanism of Colchicine Binding to Tubulin: Evidence for Ligand-Induced Conformational Change†

Donita L. Garland

ABSTRACT: The kinetics of tubulin–colchicine complex formation were reinvestigated using both isotopic labeling and fluorescence techniques. The time course for association is composed of a relatively fast step, which is responsible for most of the reaction, followed by a slower step, which accounted for 5–10% of the bound colchicine. The origin of the slow step is not known. Analysis of the fast step showed that, under pseudo-first-order conditions, rate constants for association, k_{obsd}, increased linearly with both increasing colchicine and tubulin concentration and then deviated from linearity at high concentrations. Plots of $(k_{obsd} - k_{-2})^{-1}$ vs. concentration^{-1} were linear. The simplest mechanism that can explain the data is:

$$P + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (PC)'$$

Kinetic constants were obtained by computer curve fitting of the data. These yield an equilibrium constant for the rapid equilibrium step $K_1 = 6 \times 10^3\ M^{-1}$; $k_2 = (2\text{–}3) \times 10^3\ s^{-1}$ and $k_{-2} = (5\text{–}9) \times 10^{-6}\ s^{-1}$. The rate constant for the dissociation of the tubulin–colchicine complex determined by the isotopic labeling method was $5.3 \times 10^{-6}\ s^{-1}$. Therefore, k_{-2} is the rate-limiting step for colchicine dissociation from the tubulin–colchicine complex. The dependency of k_{obsd} on solvent viscosity was determined using sucrose to vary the viscosity. At low colchicine concentrations the apparent second-order rate constant is decreased with increasing viscosity, whereas at high colchicine concentrations the rate dependency on viscosity is significantly reduced. The fluorescence enhancement observed when colchicine binds to tubulin is a consequence of the colchicine-induced conformational change step. Tubulin prepared by cycles of polymerization–depolymerization was separated into two molecular weight species [Weingarten, M. D., Suter, M. M., Littman, D. R., and Kirschner, M. W. (1974), *Biochemistry 13*, 5529], peak 1 containing the high-molecular-weight species and peak 2 consisting only of tubulin dimers. The apparent second-order rate constants for association of colchicine to tubulin in both peaks were essentially the same, 1.7 and $1.4 \times 10^2\ M^{-1}\ s^{-1}$, respectively. When tubulin was preincubated with colchicine prior to chromatography, bound colchicine was only found in peak 2 as the tubulin dimer.

The binding of colchicine, an antimiotic drug, to tubulin has been studied extensively because colchicine prevents the assembly and causes the disassembly of microtubules both in vivo and in vitro (Inoue, 1952; Taylor, 1965; Olmsted and Borisy, 1973). The results of these studies revealed the following. (1) Colchicine binds to the tubulin heterodimer, but binding to the intact microtubule has not been demonstrated (Weisenberg et al., 1968; Shelanski and Taylor, 1968; Wilson and Meza, 1973). (2) For each tubulin dimer, there exists only one tight colchicine binding site (Bryan, 1972, Owellen et al., 1972; Wilson and Bryan, 1974). However, the location of this binding site on the tubulin dimer is not known. Recent work of Schmitt and Atlas (1977) suggests that the high-affinity site may be on the α subunit. (3) Tubulins isolated from widely diverse sources exhibit very similar colchicine binding properties (Wilson and Friedkin, 1967; Borisy and Taylor, 1967; Shelanski and Taylor, 1967; Weisenberg et al., 1968; Wilson, 1970; Bhattacharyya and Wolff, 1974a). (4) The rate of colchicine binding to brain tubulin is slow, with an apparent rate constant of $\sim 10^2\ M^{-1}\ s^{-1}$ as determined by an isotopic labeling technique (Borisy and Taylor, 1967; Wilson, 1970; Garland and Teller, 1975, Sherline et al., 1975; McClure and Paulson, 1977).

In order to understand the role of colchicine in microtubule disassembly, a reinvestigation of the kinetics of tubulin–colchicine complex formation using both radioactive isotopic labeling and fluorescence techniques was done. The data thus obtained can be explained by a mechanism which requires a colchicine-induced protein conformational change step. In fact, the results indicate that this ligand-induced conformational change step is essential for obtaining the nearly irreversible binding of colchicine which is believed to be the cause for shifting the assembled microtubules to the dissociated form.

Materials and Methods

Colchicine [ring C, methoxy-^{3}H] was obtained from New England Nuclear. Guanosine 5′-triphosphate, colchicine, and Pipes[1] were purchased from Sigma. Porcine brains were provided by USDA Research Center, Beltsville, Md. Hydromix was purchased from Yorktown.

Protein Preparation. Porcine brain tubulin was prepared by a modified method of Shelanski et al. (1973). The cerebrum, cleaned of the meninges and washed, was homogenized (1:1, w/v) in 50 mM Pipes buffer, pH 6.9, containing 1 mM GTP and 1 mM EGTA. Homogenization was done for 1.5 min in a Virtis homogenizer. The homogenate was centrifuged for 75 min at 80 000g at 4 °C and the pellet was discarded. Glycerol was added to the supernatant to give a final concentration of 25%, and the sample was incubated for 35 min at 37 °C. After centrifugation at 80 000g for 1 h at 30 °C, the supernatant was discarded. The pellet was resuspended in buffer and left on ice for 45 min. This was centrifuged at 80 000g for 45 min. The

† From the Laboratory of Biochemistry, Section on Enzymes, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received January 12, 1978.* This work was supported by National Research Service Award 1 F 32 GM 05105-01.

[1] Abbreviations used: GTP, guanosine 5′-triphosphate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl.

supernatant was incubated for 25 min at 37 °C and centrifuged at 80 000*g* for 1 h. This pellet was frozen in liquid nitrogen and stored at −75 °C. To prepare for use the pellet was resuspended in 50 mM Pipes buffer containing 0.8 M sucrose, 0.2 mM GTP, and 1.8 mM $CaCl_2$. The sample remained on ice for 45 min and was centrifuged at 100 000*g* for 30 min. At this stage the sample contained about 80% tubulin, 15% high-molecular-weight proteins, and several minor protein bands as revealed by $NaDodSO_4$–polyacrylamide electrophoresis.

Tubulin obtained from two cycles of polymerization–depolymerization was further purified by chromatography on DEAE Bio-Gel A. The sample was added to a column equilibrated with 50 mM Pipes buffer containing 0.2 mM GTP and 0.05 M NaCl. Protein was eluted from the column with a linear salt gradient of NaCl (0.05–0.4 M). Alternatively, the sample obtained from two cycles of polymerization–depolymerization was fractionated on Bio-Gel A-15m into two size species, one containing the high-molecular-weight proteins and a second containing only tubulin 6S dimers (Weingarten et al., 1974). Protein obtained from the ion-exchange column and that in peak 2 from the gel-filtration column was at least 95% tubulin. No high-molecular-weight proteins were present. The stoichiometry of colchicine binding was measured on the 6S tubulin fraction eluted from the Bio-Gel A-15m column. As determined from Scatchard plots of binding data, 0.7 mol of colchicine bound per mol of tubulin dimer.

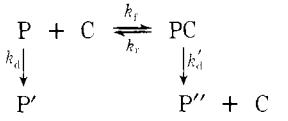
[^3H]Colchicine Binding Assay. An aliquot of [^{3}H]colchicine was dried with a stream of nitrogen. Unlabeled colchicine was then added to obtain the desired specific activity.

Tubulin-bound [^{3}H]colchicine was measured by binding to Whatman DE-23 ion-exchange cellulose (Harris and Teller, 1973). Columns containing 0.5 mL of ion-exchange cellulose were equilibrated with 10 mL of buffer. Samples of 50–100 μL were added and they were immediately washed with 1.5 mL of buffer to remove unbound colchicine. The cellulose was quantitatively transferred to 15 mL of Hydromix scintillant for counting.

Fluorescence Measurements. Colchicine binding to tubulin was also monitored by measuring the enhancement of the colchicine fluorescence which occurs upon its binding to tubulin (Arai and Okuyama, 1973). Fluorescence measurements were made using a Hitachi-Perkin-Elmer MPF-2A spectrofluorometer equipped with a Hewlett-Packard 7004B X-Y recorder and a homemade voltage offset circuit. All measurements were made at 37 °C. Constant temperature was maintained using a thermostated cell holder and a constant-temperature circulating bath. The fluorescence emission of the tubulin–colchicine complex was measured with an excitation wavelength of 380 nm to yield an emission spectrum with a maximum at 440 nm.

Isotopic Tracer Measurements. The rate of dissociation of the tubulin–colchicine complex was monitored by determining the loss of [^{3}H]colchicine from the tubulin–[^{3}H]colchicine complex (Garland and Teller, 1975). In this experiment, tubulin was incubated with 50 μM tritiated colchicine at 37 °C for 2 h. The sample was gel filtered on a Bio-Gel P-10 column to remove unbound [^{3}H]colchicine. Portions of the protein fraction were reincubated with either (1) 50 μM [^{3}H]colchicine of the same specific activity as that used in the initial incubation, (2) 50 μM unlabeled colchicine, or (3) buffer alone. In all three cases, samples were assayed for bound [^{3}H]colchicine at various time intervals for up to 12 h. This experimental design is based on a simplified binding scheme (Scheme I), where P, P′, and P″ are active tubulin and inactive tubulin transformed from P and tubulin–colchicine complex, PC, respectively; C is colchicine; and k_i is the rate constant for the designated step. The rate observed for the loss of bound [^{3}H]colchicine in the presence of added [^{3}H]colchicine (case 1) was equal to k_d', the rate of irreversible loss of colchicine binding activity due to the conversion of PC to P″ + C. In case 2 where unlabeled colchicine was added, the rate measured for the loss of [^{3}H]colchicine is a function of k_r and k_d'.

SCHEME I



The rate of [^{3}H]colchicine dissociation observed for case 3 where no colchicine was added is a function of k_r, k_d' and k_d. The value of k_d, the rate constant for converting P to P′ in the absence of colchicine, was determined as follows: tubulin was incubated at 37 °C, and, at intervals, fractions were removed and to these 50 μM of [^{3}H]colchicine was added. These samples were reincubated for 1 h at 37 °C and assayed for bound [^{3}H]colchicine.

Data Treatment. Unless it is specified, the kinetic data were obtained at 37 °C under pseudo-first-order conditions. The observed first-order rate constants were analyzed based on the simplest mechanism possible for explaining the data. The mechanisms considered involved a one- and a two-step binding process (Rhee and Chock, 1976). The one-step reaction is:

$$P + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PC \tag{1}$$

where k_1 and k_{-1} are forward and reverse rate constants, respectively. Under the pseudo-first-order conditions, e.g., $[C]_0 \gg [P]_0$ ($[C]_0$ and $[P]_0$ are the total concentrations of C and P added, respectively), the observed rate constant is predicted to be a linear function of $[C]_0$, as shown by eq 2.

$$k_{obsd} = k_1[C]_0 + k_{-1} \tag{2}$$

A two-step reaction mechanism can involve either a tubulin isomerization step (eq 3) or a colchicine-induced tubulin conformational change step as described in eq 4.

$$P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P' + C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P'C \tag{3}$$

$$P + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (PC)' \tag{4}$$

When $[C]_0 \gg [P]_0$, with the steady-state assumption, the observed pseudo-first-order rate constants for the reactions described by eq 3 and 4 are given in eq 5 and 6, respectively.

$$k_{obsd} = \frac{k_1k_2[C]_0 + k_{-2}(k_1 + k_{-1})}{k_1 + k_{-1} + k_2[C]_0} \tag{5}$$

$$k_{obsd} = \frac{k_1[C]_0(k_2 + k_{-2}) + k_{-1}k_{-2}}{k_1[C]_0 + k_{-1} + k_2} \tag{6}$$

Since only one exponential function within the interested time range was observed whether $[C]_0$ or $[P]_0$ was in excess, it implies that $k_{-1} \gg k_1$ for mechanism 3, and eq 5 can be further simplified.

If the tubulin isomerization step in eq 3 proceeds via a rapid equilibrium mechanism, then eq 5 becomes

$$k_{obsd} = \frac{k_2[C]_0}{1 + K_1^{-1}} + k_{-2} \tag{7}$$

Equation 6 can be simplified to eq 8 when $k_{-1} \gg k_2$,

$$k_{obsd} = \frac{k_2}{1 + (K_1[C]_0)^{-1}} + k_{-2} \tag{8}$$

TABLE I: Predicted Dependence of k_{obsd} on Reactant Concentration.

models	conditions	
	$[C]_0 \gg [P]_0$	$[P]_0 \gg [C]_0$
(1) $P + C \rightleftharpoons PC$	linear	linear
(2) $P \rightleftharpoons P' + C \rightleftharpoons P'C$	nonlinear	linear
(3) $P + C \rightleftharpoons PC \rightleftharpoons (PC)'$	nonlinear	nonlinear
exptl obsd	nonlinear	nonlinear

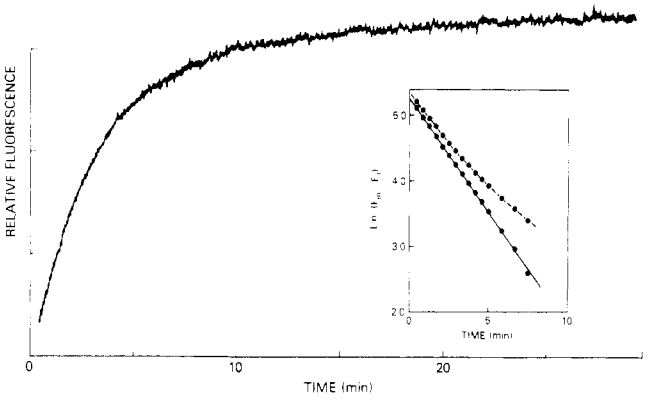


FIGURE 1: Fluorescence change for the formation of the tubulin-colchicine complex. Buffer contains 50 mM Pipes, 0.2 mM GTP, 2 mM $CaCl_2$, 0.8 M sucrose, pH 6.9. Colchicine was 80 μM and tubulin was 1.8 μM. The inset is the plot of ln $(F_m - F_t)$ vs. time, where F_m is the fluorescence at the maximum and F_t is the fluorescence at time t. The dotted line is a plot of the data and the solid line is a plot of the fluorescent data after the slow increase has been subtracted. Excitation wavelength was 380 nm; emission wavelength was 440 nm.

with $K_1 = k_1/k_{-1}$.

When experiments were performed with $[P]_0 \gg [C]_0$, the mechanism described by eq 4 would yield a similar k_{obsd} expression as that described by eq 9 with $[P]_0$ as the variable. With the mechanism described by eq 3, k_{obsd} is predicted to be a linear function of $[P]_0$ when (1) rapid equilibrium is assumed for the isomerization step (eq 9)

$$k_{obsd} = k_2 K_1 [P]_0 + k_{-2} \quad (9)$$

or (2) when the isomerization step is rate limiting. In Table I the predicted dependence of k_{obsd} on either the colchicine or tubulin concentration is indicated for the limiting conditions when $[C]_0 \gg [P]_0$ and $[P]_0 \gg [C]_0$, respectively, for the mechanisms described by eq 1, 3, and 4. All data analysis was performed with a PDP 10 computer using an interactive curve-fitting and graphic program.

Results

Rates of Colchicine Binding. Both radiolabeled colchicine and fluorescence techniques were utilized to monitor the rate of tubulin-colchicine complex formation. Figure 1 depicts a time course observed for the fluorescence enhancement at 440 nm due to colchicine binding under the condition that $[C]_0 \gg [P]_0$. The reaction was composed of two parts: an initial relatively rapid reaction which is responsible for 90 to 95% of the total signal change, followed by a slow fluorescence change which accounts for 5 to 10% of the total reaction amplitude. The existence of two reaction times was also indicated by the nonlinear first-order plot (dashed line) shown in the inset. When the slow fluorescence change was subtracted from the total reaction amplitude, a linear first-order plot was obtained (see inset) from which k_{obsd} was determined. A similar time course was observed when the reaction was monitored by the formation of tubulin-[^{3}H]colchicine under conditions where $[C]_0 \gg [P]_0$. The slow reaction described above was not observed by either the fluorescence technique or the radiolabeled colchicine method when $[P]_0 \gg [C]_0$ (Figure 2). In these experiments, 2 mM $CaCl_2$ was present to prevent polymerization. The value for k_{obsd} was calculated from the slope of the first-order plot depicted in the inset of Figure 2.

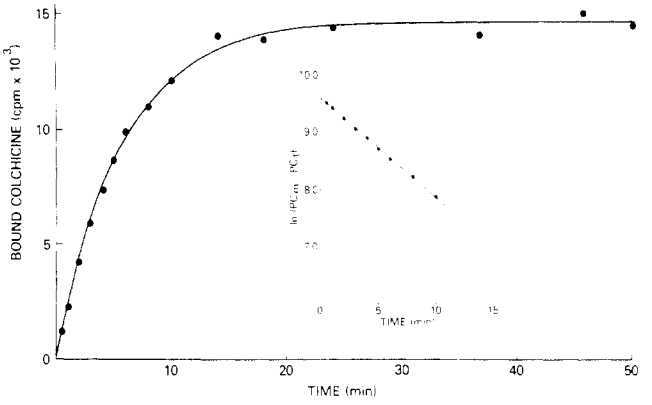


FIGURE 2: Binding of [^{3}H]colchicine to tubulin. Buffer conditions are the same as in Figure 1. Colchicine was 1 μM and tubulin was 20 μM. The inset is a plot of ln $([PC]_m - [PC]_t)$ vs. time, where $[PC]_m$ is the concentration of tubulin-colchicine complex at the maximum and $[PC]_t$ is the concentration at time t.

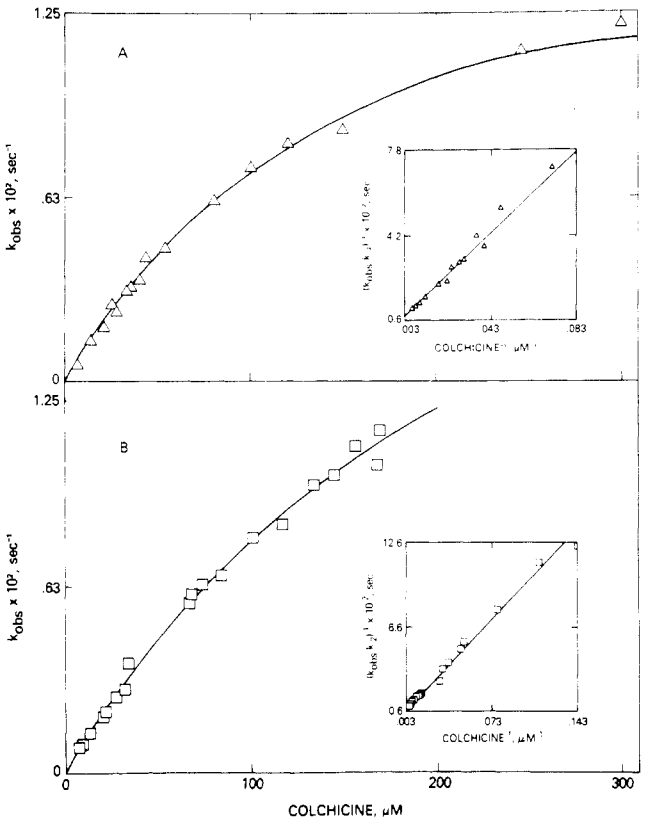


FIGURE 3: The rate of tubulin-colchicine complex formation as a function of colchicine concentration. Binding was determined by (A) the isotopic method and (B) monitoring fluorescence change. Experimental conditions were the same as Figure 1. Measurements were made at 37 ° C. The inset is the data plotted as $(k_{obsd} - k_{-2})^{-1}$ vs. [colchicine]$^{-1}$. The solid line is a theoretical fit of the data.

Figure 3A,B demonstrates the dependence of the computed k_{obsd} on colchicine concentration when $[C]_0 \gg [P]_0$. The data derived from either the isotopic method (Figure 3A) or the fluorescence technique (Figure 3B) show that k_{obsd} increases

TABLE II: Kinetic Constants for a Two-Step Binding Scheme.[a]

	K_1 (M^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	K_a (calcd)[b]	K_a (titrat)[c]
fluorescence	3.8×10^3	2.8×10^{-2}	9.3×10^{-6}	1.1×10^7	
[^{3}H]colchicine, $[C]_0 \gg [P]_0$	6×10^3	1.9×10^{-2}	8.3×10^{-6}	1.4×10^7	2×10^6
[^{3}H]colchicine, $[P]_0 \gg [C]_0$	5.6×10^3	2.4×10^{-2}	5.2×10^{-6}	2.3×10^7	
			5.3×10^{-6} [d]		

[a] Measurements were made under the buffer conditions described in Figure 1. The constants were computed according to eq 8. [b] K_a was calculated from the computed kinetic constants, $K_a = (K_1 k_2)/k_{-2}$. [c] K_a was determined from a Scatchard plot under the same experimental conditions (Garland and Teller, 1975). [d] k_r was experimentally measured (see Materials and Methods.)

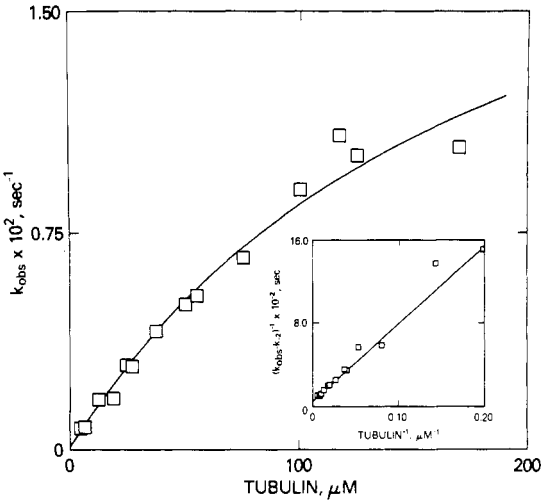


FIGURE 4: The rates of tubulin-[^{3}H]colchicine complex formation as a function of tubulin concentration. For experimental conditions, see Figure 1. The inset is a plot of $(k_{obsd} - k_{-2})^{-1}$ vs. tubulin μM^{-1} for the same data. The solid line is a theoretical fit of the data.

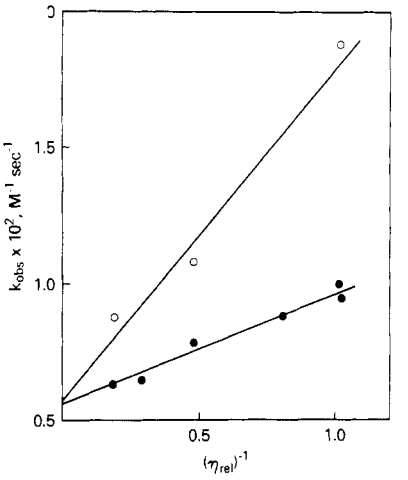


FIGURE 5: Dependence of the observed rate constants on the solvent viscosity. Rates were measured at (○) 15 and (●) 83 μM colchicine by monitoring fluorescence. Solvent viscosity was changed using sucrose from 0 to 1.5 M.

linearly with increasing colchicine concentration and then deviates from linearity at high colchicine levels.

The results of computer curve fitting show that the data best fit a rate expression described by eq 8, as demonstrated by the solid line in Figure 3A,B. The rate and equilibrium constants thus computed are tabulated in Table II. Furthermore, as predicted by eq 8, a linear plot was obtained when $(k_{obsd} - k_{-2})^{-1}$ was plotted against $[C]_0^{-1}$ (see inset of Figure 3A,B). When $[P]_0 \gg [C]_0$, k_{obsd} is proportional to $[P]_0$ at low tubulin concentration and again it deviates from linearity at high tubulin concentration (Figure 4). The data were best described by eq 8 with $[P]_0$ substituted for $[C]_0$, as shown by the solid line in Figure 4. The rate and equilibrium constants evaluated are in good agreement with those obtained under the conditions when $[C]_0 \gg [P]_0$ (Table II).

Rate of Colchicine Dissociation. Table I shows that k_{-2}, as determined by colchicine-binding studies, is very small, e.g., $(5\text{–}9) \times 10^{-6}$ s^{-1}. Therefore, k_{-2} is likely the rate-limiting step for colchicine dissociation from the tubulin–colchicine complex. To confirm this thesis, the rate constant for the dissociation of the tubulin–colchicine complex, k_r (Scheme I), was determined with the isotopic experiment described under Materials and Methods. The experiments were designed such that k_r determined was corrected for the irreversible loss of colchicine-binding activity of tubulin in the presence and absence of colchicine. The same values were obtained whether no colchicine, 50 μM unlabeled colchicine, or 1 mM unlabeled colchicine was added prior to the second incubation (tubulin was first incubated with 50 μM [^{3}H]colchicine). In the presence of 0.8 M sucrose, k_d' and k_r computed are 4.2×10^{-6} and $(5.3 \pm 1.6) \times 10^{-6}$ s^{-1}, respectively. The rate for the irreversible loss of colchicine-binding activity in the absence of colchicine, k_d, is 4×10^{-5} s^{-1}, which is considerably faster than that observed for tubulin in the tubulin–colchicine complex.

Similar results were obtained when colchicine binding sites were initially saturated with unlabeled colchicine followed by incubation with [^{3}H]colchicine. These measurements were done in the presence of 1 M sucrose. In these experiments, [^{3}H]colchicine was exchanged into the protein fraction at a rate comparable to k_r. In addition, k_r measured for the crude supernatants was the same as that determined for tubulin obtained from two cycles of polymerization–depolymerization. Identical k_r values were also obtained in the absence of sucrose and when vinblastine was added to stabilize the tubulin against the loss of colchicine-binding activity. When the purified 6S tubulin dimer (see later) was studied, a k_r of 5.8×10^{-6} s^{-1} was obtained.

Viscosity and the Apparent Second-Order Rate Constants. When a reaction proceeds via a mechanism described by either eq 1 or 3 with k_2 as the rate-limiting step, the second-order rate constant will be strongly dependent on the viscosity of the solvent. To determine the effect of viscosity on the second-order rate constant, the reaction was studied in the presence of various concentrations of sucrose (0–1.5 M). Sucrose was used instead of glycerol to vary the viscosity, because at the concentrations of tubulin used glycerol induces polymerization of tubulin into microtubules whereas sucrose does not (as judged by turbidity measurements at 350 nm). Figure 5 depicts the dependence of the apparent second-order rate constants on the relative viscosity of the solvent when 15 (opened circles) and 83 μM (closed circles) colchicine reacted with 1.8 μM tubulin (binding sites). The reaction was monitored by the fluorescence enhancement due to the formation of tubulin-

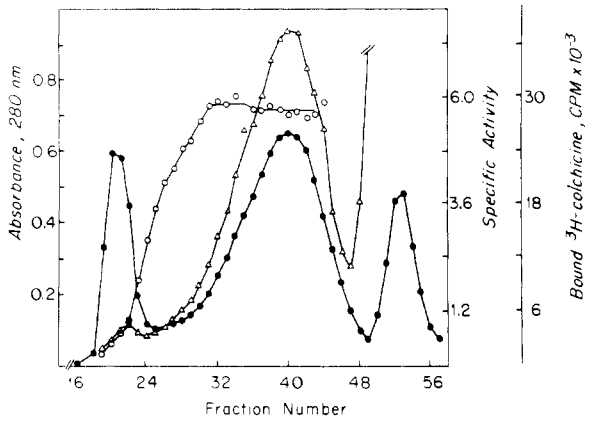


FIGURE 6: Elution profile of tubulin on a Bio-Gel A-15m column after binding [^{3}H]colchicine. The protein was incubated with [^{3}H]colchicine for 2 h at 37 °C and placed onto the column: (●) absorbance 280 nm; (△) colchicine binding activity; (○) specific activity, cpm/OD. The peak of absorbance after fraction 50 represents free GTP.

colchicine complex. At 15 μM colchicine, which is in the range of concentration where the rate of colchicine binding is essentially proportional to colchicine concentration (see Figure 3), the apparent second-order rate constant is inversely proportional to the viscosity of the solvent. At a high concentration of colchicine (83 μM), which is in the range where the rate of complex formation is no longer directly proportional to colchicine concentration (see Figure 3), the dependence of the second-order rate constant on viscosity is significantly reduced.

Colchicine Binding to Peaks 1 and 2 from Bio-Gel A Chromatography. It has previously been shown that tubulin purified by cycles of polymerization–depolymerization was resolved into two molecular weight species by gel filtration (Weingarten et al., 1974). The first peak was composed of structures containing tubulin and high-molecular-weight proteins and the second peak was composed entirely of 6S tubulin dimers. Since the tubulin preparation used here was obtained by this same purification procedure, colchicine-binding properties of both molecular weight species were further investigated.

Approximately 40% of the total protein, as determined by the absorbance at 280 nm, and 20% of the total colchicine-binding activity were found in the first peak, while the second peak, which was identified as the 6S dimer, accounted for 80% of the total colchicine-binding activity. This observation is in accord with work reported by Kirschner et al. (1974). By means of the fluorescence technique, the rates of colchicine binding to tubulin in peaks 1 and 2 were measured with 20 μM colchicine (in excess) in 50 mM Pipes buffer, pH 6.9, containing 2 mM $CaCl_2$ and 0.2 mM GTP. The k_{obsd} evaluated are 3.4 and 2.8 $\times 10^{-3}$ s^{-1} for peaks 1 and 2, respectively. When tubulin was incubated with a saturating concentration of [^{3}H]colchicine prior to fractionation on Bio-Gel A-15m, only 20% of the protein and essentially no colchicine-binding activity was eluted in peak 1. Almost all of the colchicine-binding activity eluted from the column as 6S dimers in peak 2 (Figure 6).

Finally, an attempt was made to characterize the slow reaction observed when $[C]_0 \gg [P]_0$, which accounts for 5–10% of the total signal change. The results of these experiments show that the slow reaction was always observed with no significant change in amplitude, even when the reaction conditions were varied as follows: (1) The tubulin used was from either peaks 1 or 2 resolved by the Bio-Gel A-15m chromatography; (2) the tubulin was prepared by two cycles of polymerization–depolymerization in the presence or absence of glycerol; (3) the tubulin was obtained after the DEAE chromatographic step; (4) the reaction mixture was supplemented with either sucrose, dithiothreitol, or up to 0.25 M NaCl; and (5) the tubulin was subjected to Millipore filtration just prior to assaying to remove any aggregates. However, it is possible that this slow reaction is derived from tubulin aggregates which form during the reaction or from heterogeneity of the tubulin used.

Discussion

The kinetic data in Figures 3 and 4 show that the pseudo-first-order rate constant, k_{obsd}, is not a linear function of colchicine or tubulin concentration; therefore, formation of the tubulin–colchicine complex cannot occur by a simple bimolecular reaction mechanism as described in eq 1. To explain these kinetic data, a reaction mechanism involving a minimum of two steps is required. Equations 3 and 4 describe the two likely two-step mechanisms, one involves isomerization of tubulin prior to colchicine binding and the other involves a colchicine-induced change in the conformation of tubulin.

An initial burst of binding was not observed for the tubulin–colchicine complex formation, even when $[P]_0 \gg [C]_0$. Accordingly, at least 95% of the tubulin would be in the P form if eq 3 were the correct mechanism, and, therefore, $k_{-1} \gg k_1$. It follows that, at low $[C]_0$, $k_2[C]_0 \gg k_{-1}$ and the k_{obsd} would be a linear function of $[P]_0$ as described by eq 9. This prediction is inconsistent with the data shown in Figure 4. Therefore, a mechanism in which tubulin isomerization occurs prior to colchicine addition is contraindicated.

For the colchicine-induced protein conformational change mechanism, k_{obsd} should be a nonlinear function of the colchicine concentration $[C]_0$, when $[C]_0 \gg [P]_0$ (eq 8) and of the tubulin concentration when $[P]_0 \gg [C]_0$ (Table I). The data in Figures 3 and 4 are therefore in accord with the mechanism described in eq 4. Equation 8 was used to compute the values of k_2, k_{-2}, and K_1 (Table II). These values show good agreement between the two methods used. If k_1 is diffusion controlled, e.g., $\sim 5 \times 10^7$ M^{-1} s^{-1} (Eigen and Hammes, 1963), k_{-1} can be calculated from K_1 to be $\sim 1 \times 10^4$ s^{-1}, which is equivalent to a half-time of 70 μs for the dissociation of the PC complex. Since the rate constants obtained using the fluorescent technique are essentially the same as those obtained by the isotopic method which required a lengthy washing procedure to remove free colchicine, both techniques must be monitoring the stable (PC)′ complex. Therefore, the observed fluorescence enhancement observed when colchicine binds to tubulin is probably due to the conformational change.

The data in Table II show that the values of K_1, k_2, and k_{-2} obtained when $[C]_0 \gg [P]_0$ are in good agreement with those obtained when $[P]_0 \gg [C]_0$. This is strong evidence in support of the colchicine-induced tubulin conformational change mechanism and is inconsistent with the previous proposal that colchicine binding proceeds via a simple bimolecular reaction mechanism (Sherline et al., 1975). The evidence for the previously proposed mechanism is that the observed rate for colchicine binding is linear with respect to the concentration of both colchicine and tubulin when they were varied up to 4 μM. Within this concentration range, the same linear function was observed in this report; however, at a high concentration range, k_{obsd} has clearly deviated from linearity (Figures 3 and 4). In addition, the viscosity effect on the reaction rate is much more pronounced when the colchicine concentration is maintained within the linear range (15 μM) compared to that observed with a higher colchicine concentration (85 μM) when the rate is no longer directly proportional to $[C]_0$ (Figure 5). This is

consistent with the ligand-induced fit mechanism in which the rate-limiting step shifts from the initial binding step at a low colchicine level to the conformational change step at a high colchicine concentration. Furthermore, the ligand-induced fit mechanism also reconciles the slow colchicine binding rate observed, which is several orders of magnitude slower than the expected rate for the direct ligand binding to protein (Eigen and Hammes, 1963).

The k_r determined from the direct isotopic method with 6S dimer (5.8×10^{-6} s^{-1}) agrees very well with the computed value of k_{-2} (Table II). This is essentially the same as the value of k_r determined with tubulin in crude supernatants or for the protein obtained from cycles of polymerization–depolymerization. These results indicate that k_{-2} is the rate-limiting step for colchicine dissociation from its tubulin complex, and the tubulin in the complex is the 6S dimer. The k_r values reported here are in good agreement with the value of 8.1×10^{-6} s^{-1} reported by McClure and Paulson (1977) for rat brain tubulin and somewhat higher than the value of 2.5×10^{-6} s^{-1} reported by Sherline et al. (1975). The equilibrium constant calculated from the kinetic parameters is in the range of 1.1 to 2.3×10^7 M^{-1} (Table I) which is considerably higher than the value of 2×10^6 M^{-1} obtained from direct-binding studies under the same experimental conditions (Garland, 1975). To date, values reported for this equilibrium constant vary from 0.7 to 3×10^6 M^{-1} (Borisy and Taylor, 1967; Bryan, 1972; Owellen et al., 1972; Wilson and Meza, 1973; Bhattacharyya and Wolff, 1974b; Sherline et al., 1975). The discrepancy between the equilibrium constant calculated from the kinetic parameters and that obtained from direct binding studies has been reported previously (Garland and Teller, 1975; Sherline et al., 1975). Sherline et al. (1975) explained this discrepancy on the basis of the decay of colchicine-binding activity during the lengthy incubation time required to reach equilibrium. Under the experimental conditions reported here, the half-time for decay of colchicine binding activity of tubulin in the complex is about 90 h. However, the decay of free tubulin is much faster, $t_{1/2}$ is 9 h. In fact, this slow decay of tubulin in the complex should not significantly alter the results; however, the decay of free tubulin would have a much greater effect for obtaining a low association constant. It appears the loss of binding activity during attainment of equilibrium is a reasonable explanation for the discrepancy between the equilibrium constants derived from thermodynamic and kinetic data. Thus, the equilibrium constant calculated from kinetic parameters is probably a more accurate measure of the binding constant.

The equilibrium constant for the equilibrium between PC and (PC)′ (eq 4) was calculated from k_2 and k_{-2} to be 3.2×10^3, which yields a $\Delta G°$ value of −5 kcal/mol for this conformational change step at 37 °C. This suggests that the colchicine-induced conformational change step is responsible for the nearly irreversible colchicine-binding phenomenon.

The apparent second-order rate constants for the association of colchicine to peaks 1 and 2 (20 μM colchicine) are essentially the same as that calculated for the cycle-purified tubulin (Figure 5). In addition, the same k_r was observed for the dissociation of colchicine from the cycle-purified tubulin and peak 2 tubulin. These data suggest that colchicine is likely bound only to the dimer (peak 2) and thereby shifts the equilibrium between peaks 1 and 2 to the colchicine-induced dimer form. This thesis is supported by the fact that when colchicine was incubated with tubulin prior to fractionation on Bio-Gel A-15m all the tubulin-bound colchicine was eluted in peak 2. The protein remaining in peak 1 consists primarily of the high-molecular-weight proteins and some tubulin which may be in the form of aggregates. The tubulin species present in peak 1 prior to colchicine addition is believed to exist as an intermediate structure involved in the assembly of microtubules (Borisy et al., 1975; Kirschner et al., 1974). Hence, when colchicine binds to the tubulin dimer, it shifts the equilibrium from the intermediate structure to the dimer. These data do not discount an alternative mechanism in which colchicine binds first to tubulin in the intermediate structure to form a primary complex whose conversion to (PC)′ causes the shift to the free dimer.

These results are in essence similar to those previously reported by Borisy et al. (1975) where colchicine causes the dissolution of the intermediate structures. The results are somewhat different from those of Penningroth and Kirschner (1977) who report that colchicine binds to ring structures found in peak 1 (Weingarten et al., 1974) and to 6S dimers at the same rate but that there is no conversion of the structures to dimers. Rings were not observed by electron microscopic analysis of peak 1 reported here.

Additional studies are necessary to determine if colchicine can bind to tubulin in microtubules or if the colchicine site is blocked. Only limited disassembly occurs at less than millimolar colchicine concentrations, whereas significant disassembly is found at much higher concentrations (Margolis and Wilson, 1977; Gaskin et al., 1975). Margolis and Wilson (1978) have proposed a mechanism for colchicine modulation of assembly and disassembly. An additional mechanism for disassembly may be possible only at high colchicine concentrations. This mechanism is suggested by the binding scheme reported here. Colchicine could interact with tubulin in the microtubule causing disassembly to the dimer as a result of the conformational step, or, alternatively, the shift to the dimer is followed by formation of (PC)′. Colchicine binding to the intact microtubule has not been observed (Wilson and Meza, 1973). However, at very high colchicine concentrations it may be possible to demonstrate the existence of the intermediate form, PC.

In summary, the kinetic data reported here can best be explained by a colchicine-induced tubulin conformational change mechanism. This ligand-induced protein isomerization step is the cause of the nearly irreversible colchicine binding to tubulin for which it shifts the aggregated tubulins to the dimer form, (PC)′.

Acknowledgments

The author thanks Dr. P. B. Chock for many valuable discussions and assistance with the computer modeling and the manuscript, Dr. E. R. Stadtman for reading the manuscript, and Kathy Shriver for doing the electron microscopy.

References

Arai, T., and Okuyama, T. (1973), *Seikagaku 45*, 19.

Bhattacharyya, B., and Wolff, J. (1974a), *Biochemistry 13*, 2364.

Bhattacharyya, B., and Wolff, J. (1974b), *Proc. Natl. Acad. Sci. U.S.A. 71*, 2627.

Borisy, G. G., and Taylor, E. W. (1967), *J. Cell. Biol. 34*, 525.

Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B., and Johnson, K. A. (1975), *Ann. N.Y. Acad. Sci. 253*, 107.

Bryan, J. (1972), *Biochemistry 11*, 2611.

Eigen, M., and Hammes, G. G. (1963), *Adv. Enzymol. 25*, 1.

Garland, D. L. (1975), Ph.D. Thesis, University of Washington.

Garland, D. L., and Teller, D. C. (1975), *Ann. N.Y. Acad. Sci. 253*, 232.
Gaskin, F., Cantor, C. R., and Shelanski, M. L. (1975), *Ann. N.Y. Acad. Sci. 253*, 133.
Harris, C. E., and Teller, D. C. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 540 (abstr).
Inoue, S. (1952), *Exp. Cell. Res., Suppl. 2*, 305.
Kirschner, M. W., Williams, R. C., Weingarten, M., and Gerhart, J. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 1159.
Margolis, R. L., and Wilson, L. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74*, 3466.
Margolis, R. L., and Wilson, L. (1978), *Cell 13*, 1.
McClure, W. O., and Paulson, J. C. (1977), *Mol. Pharmacol. 13*, 560.
Olmsted, J. B., and Borisy, G. G. (1973), *Biochemistry 12*, 4282.
Owellen, R. J., Owens, Jr., A. H., and Donigian, D. W. (1972), *Biochem. Biophys. Res. Commun. 47*, 685.
Penningroth, S. M., and Kirschner, M. W. (1977), *J. Cell Biol. 75*, 283a.
Rhee, S. G., and Chock, P. B. (1976), *Biochemistry 15*, 1755.
Schmitt, H., and Atlas, D. (1976), *J. Mol. Biol. 102*, 743.
Shelanski, M. L., and Taylor, E. W. (1968), *J. Cell Biol. 38*, 304.
Shelanski, M. L., Gaskin, F., and Cantor, C. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70*, 765.
Sherline, P., Leung, J. T., and Kipnis, D. M. (1975), *J. Biol. Chem. 250*, 5481.
Taylor, E. (1965), *J. Cell Biol. 25*, 145.
Weingarten, M. D., Suter, M. M., Littman, D. R., and Kirschner, M. W. (1974), *Biochemistry 13*, 5529.
Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968), *Biochemistry 7*, 4466.
Wilson, L. (1970), *Biochemistry 9*, 4999.
Wilson, L., and Bryan, J. (1974), *Adv. Cell Mol. Biol. 3*, 21.
Wilson, L., and Friedkin, M. (1967), *Biochemistry 6*, 3126.
Wilson, L., and Meza, I. (1973), *J. Cell Biol. 58*, 709.

Selective Methyl Esterification of Erythrocyte Membrane Proteins by Protein Methylase II†

Patrizia Galletti, Woon Ki Paik, and Sangduk Kim*

ABSTRACT: Methyl esterification of erythrocyte membrane proteins has been demonstrated by incubating the isolated membrane with purified protein methylase II (*S*-adenosylmethionine:protein-carboxyl *O*-methyltransferase, EC 2.1.1.24) and *S*-adenosyl-L-[*methyl*-^{14}C]methionine. Methyl esterification of membrane-bound proteins occurred selectively to proteins corresponding to bands 3 (mol wt 97 000), 4 (mol wt 75 000), and 4.5 (mol wt 48 000) [designated according to Steck, T. L. (1974), *J. Cell Biol. 62*, 1] as identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Mild alkali treated depleted vesicles which lacked bands 1, 2, 5, and 6 had a higher methyl accepting capacity; 500 pmol of methyl groups/mg of depleted vesicle proteins vs. 200 pmol of methyl groups/mg of intact membrane proteins. Alkali-extractable membrane components were not methylated.

Protein methylase II (*S*-adenosylmethionine:protein-carboxyl *O*-methyltransferase, EC 2.1.1.24) is an enzyme which methylates (esterifies) free carboxyl groups of polypeptides (Kim and Paik, 1970; Diliberto and Axelrod, 1974; Kim, 1977). The enzyme thus neutralizes negatively charged free carboxyl groups of protein substrates by the production of protein carboxyl methyl esters. The esters are unstable and spontaneously hydrolyzed at physiological pH and temperature, yielding methanol (Kim and Paik, 1976). The enzyme has been purified from various mammalian tissues (Kim, 1973, 1974; Kim et al., 1978; Edgar and Hope, 1976). The molecular weight of the rat erythrocyte enzyme was determined to be 25 000 (Kim, 1974).

Although the enzyme is widely distributed in eukaryotes (Kim, 1977; Kim et al., 1978) and prokaryotes (Kim et al., 1977; Springer and Koshland, 1977), the identity of the natural methyl acceptor protein(s) is unknown. Furthermore, the relationship of protein methylation to enzyme function is not understood. The fact that the soluble fraction of the erythrocyte possessed a substantial amount of the enzyme despite an absence of endogenous methyl acceptor substrate (Kim et al., 1975; Kim, 1974) was particularly enigmatic. In order to investigate the endogenous methyl acceptor protein(s) in erythrocyte, we considered erythrocyte membrane proteins as possible methyl acceptors.

This paper reports the enzymatic methyl esterification of erythrocyte membrane proteins with purified protein methylase II. $NaDodSO_4$[1]–polyacrylamide gel electrophoresis of the methylated membrane enabled us to identify a few specifically methylated protein bands.

Materials and Methods

Materials. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (specific activity, 55–60 mCi/mmol) was purchased from New England

† From the Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140. *Received March 14, 1978.* This work was supported by Research Grants AM09603 from the National Institute of Arthritis and Metabolic Diseases, CA12226 from the National Cancer Institute, and GM20594 from the National Institute of General Medical Sciences, U.S.A.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

0006-2960/78/0417-4272$01.00/0