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## Mechanism of Inhibition of Microtubule Polymerization by Colchicine: Inhibitory Potencies of Unliganded Colchicine and Tubulin-Colchicine Complexes<sup>†</sup>

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**ABSTRACT:** The tubulin-colchicine binding reaction appears to involve a number of intermediate steps beginning with rapid formation of a transient preequilibrium complex that is followed by one or more slow steps in which conformational changes in tubulin and colchicine lead to formation of a poorly reversible final-state complex. In the present study, we investigated the relative ability of unliganded colchicine and preformed final-stage tubulin-colchicine complex to incorporate at microtubule ends and to inhibit addition of tubulin at the net assembly ends of bovine brain microtubules in vitro. Addition of 0.1  $\mu$ M final-stage tubulin-colchicine complex to suspensions of microtubules at polymer-mass steady-state resulted in rapid incorporation of one to two molecules of tubulin-colchicine complex per microtubule net assembly end concomitant with approximately 50-60% inhibition of tubulin addition. Incorporation of colchicine-tubulin complex continued slowly with time, without significant additional change in the rate of tubulin addition. In contrast, addition of unliganded colchicine to microtubule suspensions resulted in incorporation of small numbers of colchicine molecules at microtubule ends and inhibition of tubulin addition only after periods of time that varied from several minutes to approximately 20 min depending upon the concentration of colchicine. Inhibition of tubulin addition beginning with unliganded colchicine increased slowly with time, concomitant with increases in the concentration of final-state tubulin-colchicine complex and the amount of colchicine bound per microtubule end. The results indicate that inhibition of tubulin incorporation at microtubule ends is caused by colchicine-liganded tubulin in the form of a final-state complex. Unliganded colchicine and the transient preequilibrium complex either do not bind at all to microtubule ends or bind substantially more weakly than final-state TC complex.

Colchicine binds to tubulin and disrupts a diverse array of cellular processes by acting on the polymerization dynamics of microtubules [reviewed by Dustin (1984)]. It is clear that colchicine perturbs the exchange of tubulin at microtubule ends (Olmsted & Borisy, 1973; Margolis & Wilson, 1977; Sternlicht & Ringel, 1979; Margolis et al., 1980; Farrell & Wilson, 1980, 1984; Deery & Weisenberg, 1981; Bergen & Borisy, 1983, 1986; Lambeirs & Engleborghs, 1981; Andreu et al., 1983; Wilson et al., 1985; Wilson & Farrell, 1986; Medrano et al., 1989). However, a number of interesting aspects of the

mechanism remain unresolved.

Extensive studies on the kinetics of colchicine binding to tubulin have indicated that the binding reaction occurs as a two-step process, with initial rapid formation of a transient, reversible, low-affinity preequilibrium complex followed by one or more slow steps in which conformational changes in the tubulin and in colchicine lead to formation of a very poorly reversible final-state tubulin-colchicine (TC)<sup>1</sup> complex [e.g.,

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<sup>1</sup> Abbreviations: EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid; GTP, guanosine 5'-triphosphate; GXP, guanine nucleotide in microtubules; MAP(s), microtubule-associated protein(s); MES, 2-(N-morpholino)ethanesulfonic acid; TC, tubulin-colchicine.

see Garland (1978), Lambier and Engelborghs (1981), Andreu and Timasheff (1982a,b), and Hastie et al. (1989)]. Conformational changes in tubulin upon binding to colchicine have been indicated by changes in the fluorescence of tubulin during TC complex formation (Garland, 1978; Lambier & Engelborghs, 1981), by perturbation of the far-UV circular dichroism of tubulin (Andreu & Timasheff, 1982a), by an increase in the GTPase activity of tubulin upon the binding of colchicine (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981), by a decrease in the dissociation constant between  $\alpha$ - and  $\beta$ -tubulin after binding to colchicine (Detrich et al., 1982), and by the self-assembly of TC complexes into nonmicrotubule polymeric structures (Andreu et al., 1983). Changes in the circular dichroic spectrum of colchicine upon its binding to tubulin indicate that a conformational change also takes place in the colchicine (Detrich et al., 1981).

Examination of the thermodynamics of binding of colchicine derivatives to tubulin has led to an attractive bidentate model for colchicine binding [Andreu & Timasheff, 1982a,b; also see Cortese et al. (1977)] in which the methoxytropolone moiety of colchicine (ring C) binds initially to its subsite. This in turn induces a slow conformational change in the tubulin bringing the trimethoxyphenyl (ring A of colchicine) binding subsite of tubulin into its proper position. The specific nature of the conformational changes in tubulin is not known, but the changes have been suggested to play an important role in the ability of colchicine to affect tubulin addition and loss at microtubule ends (Detrich et al., 1982; Andreu et al., 1983). An interesting possibility is that colchicine-induced conformational changes that occur in tubulin during formation of final-state TC complex in solution are affecting important conformational transitions in tubulin that occur normally when it adds to microtubule ends [e.g., see Stewart et al. (1990)] and that colchicine may be mimicking the action of a natural regulatory substance (Sherline et al., 1975) whose function is to modulate microtubule assembly dynamics by inducing "colchicine-like" conformational changes in tubulin.

Colchicine inhibits microtubule polymerization at concentrations well below the concentration of free tubulin in solution, an action called substoichiometric poisoning (Olmsted & Borisy, 1973; Margolis & Wilson, 1977; Margolis et al., 1980). These studies revealed that low concentrations of colchicine affected microtubule polymerization by interacting with the ends of the microtubules rather than with tubulin in soluble pools. Considerable evidence indicates that final-state TC complex can add to one or both microtubule ends through a polymerization-dependent pathway and perturb normal assembly dynamics (Margolis & Wilson, 1977; Sternlicht & Ringel, 1979; Farrell & Wilson, 1980, 1984; Bergen & Borisy, 1986). Early experiments also indicated that the most active form of colchicine was the final-state TC complex (Margolis & Wilson, 1977). In that study, incubation of microtubules on electron microscope grids with final-state TC complex followed by washing away unincorporated TC complex resulted in the inhibition of microtubule elongation, but incubation in a similar manner with unliganded colchicine did not inhibit elongation. However, these experiments did not rule out the possibility that unliganded colchicine might bind to microtubule ends but dissociate rapidly upon washing or that it might affect microtubule assembly dynamics weakly relative to the action of final-state TC complex.

In the present study using microtubule-associated protein (MAP)-rich bovine brain microtubule suspensions at steady state, we analyzed the effects of colchicine on tubulin addition to microtubule net assembly ends when the drug was added

in unbound form and when it was added in the form of final-state TC complex. The data indicate that unliganded colchicine and the rapidly formed transient preequilibrium complex do not become incorporated detectably at microtubule ends and do not inhibit tubulin exchange but that incorporation of low numbers of final-state TC complex per microtubule end strongly inhibits tubulin addition. The results are consistent with previous data from several laboratories indicating that inhibition of tubulin addition at microtubule ends by colchicine is due to the polymerization-mediated incorporation of final-state colchicine-liganded tubulin molecules at microtubule ends.

## MATERIALS AND METHODS

**Preparation and Polymerization of Bovine Brain Microtubule Protein.** Bovine brain microtubule protein consisting of approximately 70% tubulin and 30% microtubule-associated proteins (MAPs) was isolated by three cycles of depolymerization and polymerization in vitro in the absence of glycerol by the procedure of Asnes and Wilson (1979) as modified by Farrell and Wilson (1984). The microtubules were centrifuged through sucrose cushions and stored frozen as pellets at  $-80^{\circ}\text{C}$ . Pellets were resuspended by Dounce homogenization in 100 mM MES, 1 mM EGTA, and 1 mM  $\text{MgSO}_4$ , pH 6.8 (reassembly buffer), and after incubation for 10 min at  $0^{\circ}\text{C}$ , suspensions were centrifuged at  $39500g$  for 20 min at  $4^{\circ}\text{C}$  to remove aggregated or denatured protein (SS-34 rotor; Sorvall RC-5 centrifuge). All polymerization experiments were carried out in the presence of a GTP-regenerating system consisting of 10 mM acetyl phosphate and 0.1 IU/mL acetate kinase.

**Preparation of Final-State Unlabeled and Tritium-Labeled TC Complexes.** Unlabeled and radiolabeled TC complexes were prepared in a mixture of tubulin and MAPs as described by Farrell and Wilson (1984). Briefly, microtubule protein (2–3 mg/mL) in MEM reassembly buffer (no GTP) was incubated with  $30\text{ }\mu\text{M}$  [ $^3\text{H}$ ]colchicine (or unlabeled colchicine) for an initial 15 min at  $30^{\circ}\text{C}$ , then at  $0^{\circ}\text{C}$  for 15 min to dissociate any residual polymers, and finally at  $30^{\circ}\text{C}$  for an additional 1 h. Unreacted colchicine was removed by gel filtration at  $4^{\circ}\text{C}$  using  $1\text{ cm} \times 18\text{ cm}$  columns of Bio-Gel P-10. Approximately 0.8–0.9 mol of colchicine was bound per mole of tubulin in the tubulin–MAP mixtures, as determined by analysis of radiolabel incorporation into the tubulin (Farrell & Wilson, 1984). In all experiments, the concentration of final-state TC complex is based upon the concentration of bound colchicine. TC complexes were drop-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The TC complex solution was thawed rapidly and centrifuged at  $39500g$  for 10 min at  $4^{\circ}\text{C}$  prior to use in experiments to remove any aggregated or denatured protein. Final-state TC complex is defined operationally as the tubulin–colchicine complex which forms slowly at  $37^{\circ}\text{C}$  and which is stable when cooled to  $0^{\circ}\text{C}$  and separated from unliganded colchicine by gel filtration (Wilson, 1970; Bamberg et al., 1973).

**Incubation of Unliganded Colchicine and Final-State TC Complexes with Steady-State Microtubules: Analysis of Tubulin and Colchicine Incorporation.** Microtubule protein (between 2 and 3 mg/mL) in reassembly buffer containing 0.2 mM GTP and a GTP-regenerating system was polymerized to polymer-mass steady-state at  $30^{\circ}\text{C}$ . Microtubule suspensions at polymer-mass steady-state were added to test tubes containing dried [ $^{14}\text{C}$ ]GTP (final specific activity 14.6 Ci/mmol) and either no colchicine (controls), unliganded [ $^3\text{H}$ ]colchicine (final specific activity 22.8 Ci/mmol), or [ $^3\text{H}$ ]colchicine–TC complexes and gently mixed. Incorporation of

Table I: Addition of Unliganded Colchicine or TC Complex to Steady-State MAP-Rich Bovine Brain Microtubules: Effects on Microtubule Polymer Mass, Mean Microtubule Length, and Microtubule Number Concentration<sup>a</sup>

colchicine or TC complex concn ( $\mu$ M)	polymer mass				mean length		no. concn	
	sedimentation		turbidimetry					
	mg/mL	% of control	OD, 350 nm	% of control	$\mu$ m	% of control	$\times 10^{-10}$ M	% of control
Colchicine								
control	2.18	100	0.612	100	19.4 ( $\pm 0.8$ )	100	4.25 ( $\pm 0.2$ )	100
0.3	2.03	93	0.597	98	19.0 ( $\pm 0.8$ )	98	4.02 ( $\pm 0.2$ )	95
0.5	2.02	92	0.596	97	16.6 ( $\pm 0.8$ )	86	4.59 ( $\pm 0.2$ )	108
0.8	2.02	92	0.594	97	16.9 ( $\pm 0.7$ )	87	4.50 ( $\pm 0.2$ )	104
TC Complex								
control	2.29	100	0.655	100	27.6 ( $\pm 1$ )	100	3.12 ( $\pm 0.1$ )	100
0.1	2.23	97	0.627	96	25.8 ( $\pm 1$ )	94	3.26 ( $\pm 0.1$ )	104

<sup>a</sup> Colchicine or TC complex was added to suspensions of MAP-rich microtubules (2.71 or 2.76 mg/mL total protein, respectively) at polymer-mass steady-state 60 min after initiation of polymerization (Materials and Methods). The quantity of total protein remaining as microtubule polymer 100 min after drug addition was determined by turbidimetry at 350 nm and by sedimentation (40 min, 48000g, Sorvall RC5 centrifuge). Microtubule lengths and number concentrations were determined as described in Materials and Methods. Values in parentheses represent the standard error.

<sup>14</sup>C-labeled nucleotide primarily reflects the net addition of tubulin at A ends of the microtubules (i.e., growth at A ends), while [<sup>3</sup>H]colchicine incorporation reflects the uptake of colchicine or TC complexes at the A ends (Margolis et al., 1980). At desired times, 100- $\mu$ L aliquots were removed and the microtubules stabilized by dilution into 4 mL of 30% glycerol, w/v, 10% dimethyl sulfoxide, w/v, and 2.5 mM ATP in MEM reassembly buffer (stabilizing buffer) at 30 °C. A rapid glass-fiber filtration assay was used to quantitate incorporation of radiolabeled tubulin and colchicine into the microtubules at steady-state (Wilson et al., 1982; Jordan & Wilson, 1990).

**Colchicine Binding to Tubulin in Steady-State Microtubule Suspensions.** In order to determine the amount of [<sup>3</sup>H]-colchicine that was bound to tubulin as final-state TC complex at various times after adding colchicine, 0.5  $\mu$ M [<sup>3</sup>H]colchicine (final specific activity 2.84 Ci/mmol) was added to a microtubule suspension at polymer-mass steady-state. At the desired times, 0.5-mL aliquots were removed, cooled to 0 °C, and incubated for 15 min to stop the colchicine binding reaction and to depolymerize the microtubules. Bound colchicine was determined after separation from unbound colchicine by gel filtration using 1 cm  $\times$  18 cm columns of Bio-Gel P10 (Bamburg et al., 1973).

**Reagents and Miscellaneous Procedures.** Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Microtubule number concentration, reported in terms of the molar concentration of microtubules, was determined as described previously (Farrell et al., 1987; Jordan & Wilson, 1990). Briefly, microtubule suspensions were placed on electron microscope grids, negatively stained, and photographed on a Philips EM300 electron microscope at a magnification of between 900 $\times$  and 1200 $\times$ . A Zeiss MOP-3 image processor was utilized to accumulate and process the microtubule length data. Between 500 and 600 microtubules were counted for each mean length determination. The quantity of tubulin in the form of microtubule polymer was determined using a value of 70% tubulin and 30% MAPs after sedimenting the microtubule suspensions to obtain a microtubule pellet. The concentration of microtubules (for convenience, reported as moles per liter of microtubules rather than number of microtubules per liter) was calculated from the total tubulin content of the microtubules, the mean microtubule length, and a value of 1690 tubulin dimers per micrometer. Colchicine was obtained from Sigma Chemical Co. [ring C, methoxy-<sup>3</sup>H]Colchicine (33.4 Ci/mmol) was purchased from New England Nuclear Corp., Inc. [<sup>14</sup>C]GTP was purchased from Research Products International, Mt

Prospect, II. Bio-Gel P10 was obtained from Bio-Rad Laboratories. All chemicals were reagent grade.

## RESULTS

**Effects of Unliganded Colchicine and Final-State TC Complex on Microtubule Length Distributions and on Polymer Mass.** In order to be able to investigate the inhibition of tubulin addition to microtubule ends in relation to the binding of colchicine at the ends, it was necessary to know accurately the microtubule number concentration after incubation with unliganded colchicine and with final-state TC complex. We chose for this investigation a MAP-rich microtubule preparation which maintains stable microtubule polymer-mass plateaus and length distributions in vitro for at least several hours after suspensions of the microtubules attain steady-state (Farrell et al., 1987; Jordan & Wilson, 1990). One hundred minutes after unliganded colchicine or final-state TC complex at the concentrations used in the present study was added to suspensions of the microtubules at polymer-mass steady state, microtubule polymer mass had declined less than 10% even at the highest colchicine concentration used (0.8  $\mu$ M), and the microtubule number concentration remained unchanged (Table I).

**Differential Inhibition of Tubulin Addition at Microtubule A Ends by Free Colchicine and TC Complex.** Tubulin incorporation at the ends of these microtubules at steady state occurs predominantly by a flux or treadmilling mechanism, with tubulin addition at the operationally defined net assembly or A ends and tubulin loss at the net disassembly or D ends occurring at a rate of approximately 1.1 tubulin dimers/s. Dynamic instability behavior is not easily detectable [described in detail previously; see Wilson and Farrell (1986), Farrell et al. (1987), and Jordan and Wilson (1990)]. Net incorporation of radiolabeled tubulin into the microtubules when the microtubules are pulsed at steady state with [<sup>3</sup>H]GTP occurs in an apparent biphasic fashion. An initial burst of incorporation at one or both microtubule ends which may represent a small amount of dynamic instability behavior that is not detectable by computer-enhanced video microscopy is followed by an approximately linear incorporation of tubulin that represents mainly the net rate of microtubule elongation at the A ends (Farrell et al., 1987; Jordan & Wilson, 1990).

The onset of inhibition and the attainment of maximal inhibition of net tubulin addition at A ends occurred quickly after 0.2  $\mu$ M final-state TC complex was added to microtubules at polymer-mass steady-state (Figure 1a,b, closed circles). Maximal inhibition of the tubulin addition rate occurred within 10–15 min of adding TC complex, and little change occurred

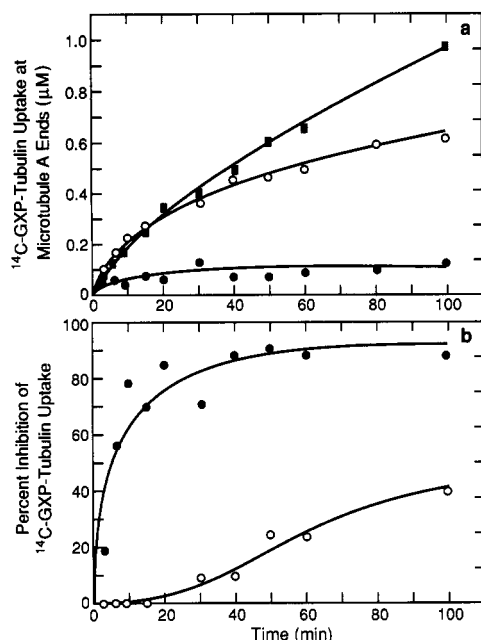


FIGURE 1: Inhibition of [ $^{14}\text{C}$ ]GXP-tubulin incorporation at A ends of microtubules after addition of colchicine or TC complexes. Microtubule protein (2.3 mg/mL) was polymerized to polymer-mass steady-state in reassembly buffer. At zero time (70 min after initiation of polymerization), the suspension was divided into three portions and, each portion was pulsed with a trace quantity of [ $^{14}\text{C}$ ]GTP (see Materials and Methods). One portion served as the control suspension. A solution of TC complex at a final concentration of 0.2  $\mu\text{M}$  was added to a second portion, and colchicine at a final concentration of 0.2  $\mu\text{M}$  was added to the third portion. (a) At the indicated times, aliquots of each suspension were processed by the glass fiber filter assay to determine tubulin incorporation at A ends. Squares, control; open circles, unliganded colchicine; closed circles, TC complex. (b) Data from (a) replotted as the percentage inhibition of GXP uptake relative to untreated (control) uptake. Open circles, unliganded colchicine; closed circles, TC complex.

in the degree of inhibition during the next 90 min of incubation. In contrast, addition to the microtubules of 0.2  $\mu\text{M}$  unliganded colchicine inhibited tubulin addition only after a delay of more than 20 min, and the degree of inhibition developed slowly with time thereafter (Figure 1a,b, open circles).

On the basis of these and other preliminary data, we chose a concentration of 0.5  $\mu\text{M}$  unliganded colchicine and a 5-fold lower concentration of final-state TC complex (0.1  $\mu\text{M}$ ) to examine the relationship between inhibition of tubulin addition at microtubule A ends and incorporation of colchicine at the ends. These concentrations of the two forms of colchicine yielded similar extents of inhibition approximately 1 h after addition to the microtubule (Figure 2). Inhibition of tubulin uptake by the two forms of colchicine and the extent of colchicine incorporation at microtubule ends are shown in Figures 2 and 3 using a double-labeling protocol in which the rate of tubulin addition at microtubule ends was monitored by incorporation of [ $^{14}\text{C}$ ]GTP (Figure 2a,b) and colchicine incorporation at A ends was monitored by uptake of [ $^3\text{H}$ ]colchicine (Figure 3).

Similar to results with 0.2  $\mu\text{M}$  unliganded colchicine, the onset of inhibition developed slowly with 0.5  $\mu\text{M}$  unliganded colchicine, and the extent of inhibition continued to increase with time for the duration of the experiment (open circles). This is shown more clearly in Figure 2b where the percentage inhibition of tubulin incorporation as a function of time is plotted for the two forms of colchicine. In contrast, the onset of inhibition with 0.1  $\mu\text{M}$  TC complex was rapid, and a plateau of maximal inhibition was attained within approximately 10 min of incubation, even though the concentration of TC

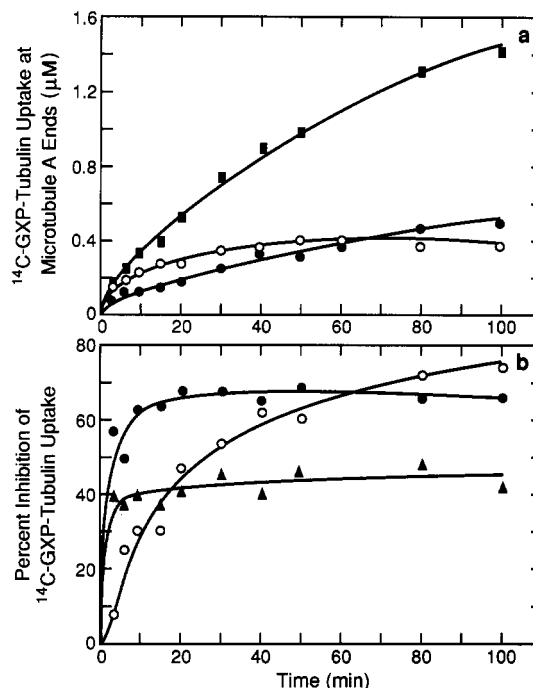


FIGURE 2: Inhibition of [ $^{14}\text{C}$ ]GXP-tubulin incorporation at A ends of microtubules after addition of colchicine or TC complexes. Microtubule protein (2.5 mg/mL) was polymerized to polymer-mass steady-state in reassembly buffer. At zero time (60 min after initiation of polymerization), the suspension was divided into three portions, and each portion was pulsed with a trace quantity of [ $^{14}\text{C}$ ]GTP (Materials and Methods). One portion served as the control suspension. A solution of [ $^3\text{H}$ ]colchicine-TC complex at a final concentration of 0.1  $\mu\text{M}$  was added to a second portion, and [ $^3\text{H}$ ]colchicine at a final concentration of 0.5  $\mu\text{M}$  was added to the third portion. The mean microtubule length in the control suspension was 10.1  $\mu\text{m}$ . The mean lengths in the colchicine-treated and TC complex treated microtubule suspensions were within 10% of the mean length of the microtubules in the control suspension at approximately 60 min after drug addition, and, therefore, the microtubule number concentrations in the colchicine-treated and TC complex treated suspensions were not distinguishable from the control suspension (data not shown; see Materials and Methods). (a) At the indicated times, aliquots of each suspension were processed by the glass fiber filter assay to determine tubulin incorporation at A ends. Uptake of 1.0  $\mu\text{M}$  [ $^{14}\text{C}$ ]GXP-tubulin is equivalent to 1143 tubulin dimers per microtubule. Squares, control; open circles, unliganded colchicine; closed circles, TC complex. (b) Data from (a), replotted as the percentage inhibition of GXP uptake relative to untreated (control) uptake. Open circles, unliganded colchicine; closed circles, TC complex. Triangles, 0.05  $\mu\text{M}$  TC complex added to a microtubule suspension similar to that described in (a) in a separate experiment [GXP-tubulin uptake data are not shown for this concentration in (a)].

complex was 5-fold lower than the concentration of unliganded colchicine (closed circles). Similar rapid onset of inhibition and rapid attainment of a plateau of maximal inhibition were obtained with 0.05  $\mu\text{M}$  final-state TC complex, but with lower overall inhibition than with 0.1  $\mu\text{M}$  TC complex (Figure 2b, triangles). These data suggest that the form of colchicine required to inhibit tubulin addition at microtubule ends is the final-state TC complex rather than unliganded colchicine (see below).

**Incorporation of [ $^3\text{H}$ ]Colchicine at Microtubule Ends in Relation to Inhibition of Tubulin Addition to Microtubule Ends.** Previously we found that when low concentrations of unliganded colchicine were added to MAP-rich bovine brain microtubules under conditions similar to those used in this study, the colchicine became incorporated into the microtubules specifically at the A ends, and not at the D ends or along the length of the microtubules (Margolis et al., 1980). Carrying out the preceding experiment in a double-label fashion

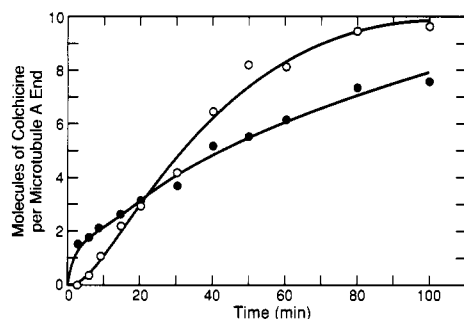


FIGURE 3: Incorporation of [ $^3\text{H}$ ]colchicine at A ends of steady-state microtubules. The [ $^3\text{H}$ ]colchicine incorporation data from the experiment described in Figure 2 are expressed in terms of the number of molecules of colchicine bound per microtubule. When TC complex was added, 6.6% of the total added TC complex was incorporated into the microtubules at 100 min of incubation, and the ratio of total tubulin incorporated to total TC complex incorporated per microtubule was 205 to 1. The ratio of soluble tubulin to TC complex in solution was 37.2 to 1 ( $3.72\ \mu\text{M}$  tubulin,  $0.1\ \mu\text{M}$  TC complex). Therefore, TC complex added to the microtubule ends approximately 5 times less efficiently than free tubulin. When unliganded colchicine was added,  $0.054\ \mu\text{M}$  TC complex had formed 100 min after addition of the colchicine, and 17.7% of the complex that formed had become incorporated into the microtubules. Closed circles,  $0.1\ \mu\text{M}$  TC complex; open circles,  $0.5\ \mu\text{M}$  colchicine.

enabled us to determine the amount of colchicine bound at microtubule A ends in relation to the degree of inhibition of tubulin uptake at the ends produced by the two forms of colchicine.

The [ $^3\text{H}$ ]colchicine incorporation data from the experiment described in Figure 2, expressed as the number of colchicine molecules incorporated per microtubule, are plotted as a function of time in Figure 3. When  $0.5\ \mu\text{M}$  unliganded colchicine was added, no detectable colchicine was incorporated at the A ends of the microtubules for the first several minutes (the first data point is at 3 min, open circles). Some incorporation was detected at 6 min (approximately 0.3 molecule of colchicine per microtubule). Beyond 6 min, the number of molecules of colchicine incorporated per microtubule increased relatively steeply for the next hour and then apparently less steeply for the duration of the experiment. The relatively low incorporation of [ $^3\text{H}$ ]colchicine at microtubule A ends during the first few minutes after unliganded colchicine was added correlated well with the absence of inhibition of tubulin incorporation with this form of the drug (Figure 2b).

In contrast to results with unliganded colchicine, an initial one to two molecules of colchicine were incorporated per microtubule A end within 3 min of adding final-state TC complex. The quantity of incorporated colchicine increased in a curvilinear fashion with time for the duration of the experiment (Figure 3). With unliganded colchicine, the extent of colchicine incorporation per microtubule was below that observed with final-state TC complex during the initial 15 min of incubation, consistent with the relatively lower inhibition of GXP-tubulin incorporation with unliganded colchicine as compared with TC complex (Figure 2a,b). Approximately 1 h after adding the two forms of colchicine, the stoichiometries of colchicine incorporation per microtubule with unliganded colchicine and final-state TC complex were reasonably similar, with incorporation beginning with unliganded colchicine exceeding incorporation beginning with TC complex by 25–30% (see below).

*Relationship between the Rate of TC Complex Formation, Addition of TC Complexes to Microtubule Ends, and Inhibition of Tubulin Incorporation at Microtubule Ends.* We wanted to determine the rate of final-state TC complex for-

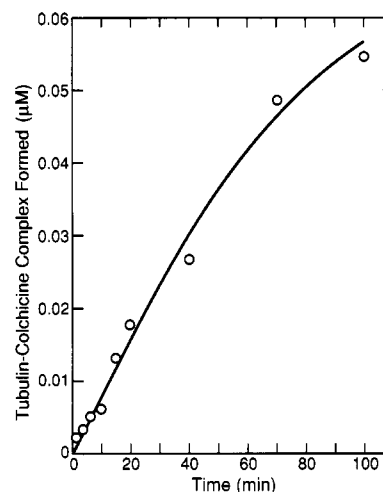


FIGURE 4: Formation of final-state TC complexes with time after addition of  $0.5\ \mu\text{M}$  colchicine to a microtubule suspension at steady state. Microtubule protein ( $2.5\ \text{mg/mL}$ ) was polymerized to polymer-mass steady-state in reassembly buffer. [ $^3\text{H}$ ]Colchicine,  $0.5\ \mu\text{M}$ , was added at zero time (45 min after initiation of polymerization). Bound colchicine was determined at the times indicated (Materials and Methods).

mation after adding  $0.5\ \mu\text{M}$  unliganded colchicine to steady-state microtubules under the conditions used in the previous experiment to examine the relationship between formation of final-state TC complex, binding of TC complex to microtubule ends, and inhibition of tubulin addition at microtubule ends. Thus,  $0.5\ \mu\text{M}$  [ $^3\text{H}$ ]colchicine was added to a steady-state microtubule suspension, and at desired intervals, aliquots of the suspension were cooled to  $0\ ^\circ\text{C}$  to depolymerize the microtubules and to stop any further binding of colchicine, and the quantity of final-state TC complex was quantitated by gel filtration (Materials and Methods).

As expected, the rate of final-state TC complex formation was relatively slow, increasing continuously for the 100-min duration of the experiment (Figure 4). The increase in formation of final-state TC complex with time with  $0.5\ \mu\text{M}$  unliganded colchicine was approximately parallel to the increase with time in the incorporation of colchicine at microtubule ends (Figure 3) and the increase with time in the inhibition of tubulin addition at the ends (Figure 2a,b). Immediately after addition of colchicine when little inhibition of tubulin uptake at microtubule ends was observed, the amount of TC complex formed was low, and the amount of colchicine bound to the microtubules was low. For example, the concentration of TC complex formed 3 min after adding  $0.5\ \mu\text{M}$  unliganded colchicine was  $0.004\ \mu\text{M}$ , and no colchicine was detected bound to the microtubules. Less than 5% inhibition of tubulin incorporation occurred at this time (Figure 2b). In contrast, 3 min after adding final-state TC complex, approximately 60% inhibition of GXP-tubulin incorporation into microtubules occurred (Figure 2b), concomitant with incorporation of 1.5 molecules of TC complex per microtubule. These data suggest that inhibition of the rate of tubulin addition to microtubule ends is caused by incorporation at the ends of final-state TC complex.

Interestingly, when TC complex was added, the ratio of total tubulin incorporated to total TC complex incorporated per microtubule was 205 to 1. However, the ratio of soluble tubulin to TC complex in solution was 37.2 to 1 ( $3.72\ \mu\text{M}$  tubulin,  $0.1\ \mu\text{M}$  TC complex). Therefore, TC complex became incorporated at microtubule ends approximately 5 times less efficiently than free tubulin [see also Sternlicht and Ringel (1979)].

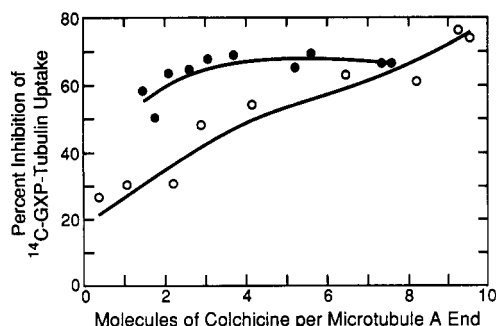


FIGURE 5: Inhibition of GXP-tubulin uptake at A ends of microtubules in relation to the number of molecules of colchicine bound per microtubule. Data are from the experiment described in Figures 2 and 3. Closed circles, 0.1  $\mu$ M TC complexes; open circles, 0.5  $\mu$ M unliganded colchicine.

The amount of colchicine incorporated per microtubule end required to produce a specific degree of inhibition of tubulin addition was somewhat greater beginning with unliganded colchicine as compared with preformed final-state TC complexes, giving the appearance that the inhibitory potency of the final-state TC complex might be greater than intermediate form(s) of the complex (Figure 5). For example, when final-state TC complex was used, the lowest number of molecules of TC complex per microtubule A end resulting in approximately 55% inhibition of tubulin addition at that end was approximately 1.5–2 molecules of TC complex per microtubule. However, 55% inhibition of tubulin addition to microtubule ends required 4–5 molecules of TC complex per microtubule when inhibition was produced beginning with unliganded colchicine. A likely explanation for the apparent difference in potency is that incorporation of TC complex to microtubule ends at low ratios of TC complex to tubulin reduces the rate of tubulin addition to the ends but does not inactivate the ends and that both TC complex and free tubulin can continue to add to the ends with time (Sternlicht & Ringel, 1979; Farrell & Wilson, 1980, 1984; see Discussion). The fact that with increasing periods of time incorporation of TC complex continued with no significant change in the percentage inhibition of tubulin addition to the ends is consistent with this interpretation (Figures 2, 3, and 5). However, it is possible that newly formed TC complex might add efficiently to microtubule ends but that it might not inhibit tubulin addition or it might inhibit addition weakly as compared with final-state TC complex.

Formation of the initial transient TC complex and formation of possible intermediate-state complexes would occur rapidly as compared with the rate of formation of final-state TC complex at any given colchicine concentration. Further, the rate of formation of the preequilibrium complex and possible intermediate-state complexes should increase with increasing concentration of colchicine. To examine whether newly formed TC complex might incorporate efficiently at microtubule ends but be less able than final-state TC complex to inhibit addition of tubulin to the ends, we determined the stoichiometry of [ $^3$ H]colchicine incorporation at 25% and 50% inhibition of [ $^{14}$ C]GXP-tubulin addition at five different unliganded colchicine concentrations. If relatively weak or inactive newly formed TC complex can become incorporated at MT ends, higher stoichiometries of colchicine should be incorporated beginning with high free colchicine as compared with low free colchicine concentration at a specific degree of inhibition.

Data on the relationship between inhibition of tubulin addition to microtubule ends and colchicine incorporation at ends for five unliganded colchicine concentrations are shown in

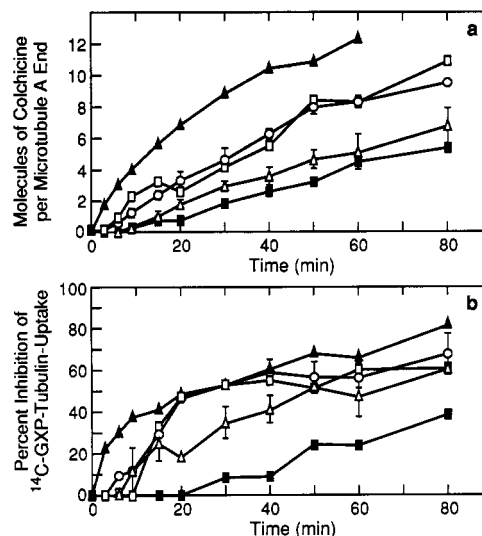


FIGURE 6: Relationship between inhibition of GXP-tubulin incorporation and the quantity of TC complex incorporation at microtubule A ends for five concentrations of free colchicine. Microtubule protein solutions (2.5 mg/mL) were polymerized to polymer-mass steady-state and divided into portions as described in the legend to Figure 1. At zero time (60 min after initiation of polymerization), a trace quantity of [ $^{14}$ C]GTP was added to all portions. For each individual experiment, one portion served as the control suspension, and the desired concentration of [ $^3$ H]colchicine was added to sample portions. Closed squares, 0.2  $\mu$ M colchicine; open triangles, 0.3  $\mu$ M colchicine (mean results from three independent experiments); open circles, 0.5  $\mu$ M [ $^3$ H]colchicine (mean results from three independent experiments); open squares, 0.6  $\mu$ M [ $^3$ H]colchicine; closed triangles, 0.8  $\mu$ M [ $^3$ H]colchicine. (a) Molecules of TC complex bound per microtubule A end. (b) Percent inhibition of GXP-tubulin incorporation relative to control incorporation. Error bars represent standard error of the mean.

Table II: Number of Colchicine Molecules per Microtubule End at 25% and 50% Inhibition of Tubulin Addition to Microtubule Ends<sup>a</sup>

colchicine concn ( $\mu$ M)	time (min)	molecules of colchicine per microtubule end
25% Inhibition		
0.2	58	3.9
0.3	21	1.6
0.5	12	1.8
0.6	13	2.5
0.8	4	2.1
50% Inhibition		
0.2	96 <sup>b</sup>	6.9 <sup>b</sup>
0.3	55	4.9
0.5	27	4.3
0.6	26	3.9
0.8	22	7.4

<sup>a</sup> Data from Figure 6a,b. <sup>b</sup> Extrapolated values.

Figure 6a,b, and the quantities of colchicine incorporated at 25% and 50% inhibition of tubulin incorporation from the data in Figure 6 for these concentrations are shown in Table II. As can be seen from the data (Figure 6a), the rate of TC complex incorporation into the microtubules increased substantially as the unliganded colchicine concentration increased. In addition, the time of onset of inhibition of tubulin-GXP addition decreased and the rate of development of inhibition after the delay period increased substantially with increasing colchicine concentration (Figure 6b). However, the quantity of colchicine incorporated at the time inhibition developed to 25% or to 50% was reasonably similar for all five colchicine concentrations (Table II). Thus, if weak or inactive newly formed TC complex intermediates exist, they do not appear to be incorporated efficiently at microtubule ends as compared with final-state TC complexes.



## DISCUSSION

*Substoichiometric Inhibition of Tubulin Incorporation by Final-State TC Complex: Lack of Detectable Inhibition by Unliganded Colchicine and the Preequilibrium TC Complex.* We found that the onset of inhibition of tubulin addition at microtubule A ends occurred rapidly after adding final-state TC complex to microtubules which had previously been polymerized to polymer-mass steady-state (Figures 1 and 2). Inhibition of tubulin addition coincided with incorporation of small numbers of TC complexes at the microtubule ends (Figures 3–5). However, when unliganded colchicine was used, inhibition of tubulin addition at the microtubule ends occurred only after a characteristic time delay. For example, with 0.2  $\mu\text{M}$  unliganded colchicine, inhibition was not detected until approximately 20 min after adding the drug, and with 0.5  $\mu\text{M}$  unliganded colchicine, inhibition did not develop for 3–5 min after drug addition (Figures 1, 2, and 6). Little or no colchicine could be detected bound to the microtubules during the delay period. For example, during the 3–5-min period of delay in the experiment shown in Figure 2 with 0.5  $\mu\text{M}$  unliganded colchicine (30 to 1 ratio of total tubulin to total colchicine), the concentration of newly formed final-state TC complex was very low (3–5 nM TC complex, ratio of total tubulin to TC complex of approximately 4000 to 1, Figure 4), and little or no colchicine was detected at the ends of the microtubules (Figure 3).

Thus, unliganded colchicine did not bind or become incorporated at microtubule ends to a significant degree relative to incorporation of final-state TC complex, and it did not appreciably inhibit addition of tubulin to the microtubule ends under the conditions examined. The data further solidify interpretation of previous results carried out with a nonequilibrium approach indicating that unliganded colchicine does not inhibit microtubule elongation (Margolis & Wilson, 1977). The absence of significant inhibition of tubulin incorporation into the microtubules as well as the absence of detectable colchicine incorporation at the ends of the microtubules during the first several minutes after adding unliganded colchicine indicates that the preequilibrium complex also did not inhibit microtubule assembly nor did it become incorporated at the microtubule ends to any detectable extent. Thus, the affinity of unliganded colchicine and of the initial transient preequilibrium complex for microtubule ends, if any, must be very low relative to the affinity of the final-state TC complex.

While the likelihood seems low, the possibility that unliganded colchicine can bind to microtubule ends and that under appropriate conditions final-state TC complexes could form and inhibit addition of tubulin to the ends cannot be eliminated entirely. If the binding of colchicine to its site on tubulin at microtubule ends can occur in similar fashion to the binding of colchicine to its site on soluble tubulin, then a low-affinity preequilibrium complex might form transiently at a microtubule end. The presence of such a TC complex at the microtubule end even for short periods of time could transiently reduce the rate of tubulin addition. The fact that no colchicine became stably incorporated at microtubule ends for the first few minutes after addition of unliganded colchicine while tubulin addition continued indicates that if such transient low-affinity complexes do form at microtubule ends, the colchicine must dissociate before final-state TC complexes have an opportunity to form.

Some data were obtained which could be interpreted as indicating that intermediate TC complexes that may exist during the slow formation of the final-state TC complex may be incorporated at microtubule ends and either not inhibit

tubulin addition or inhibit tubulin addition less effectively than final-state TC complexes. For example, at 50–60% inhibition of tubulin addition to microtubule ends beginning with 0.1  $\mu\text{M}$  final-state TC complex (Figure 5, closed circles), approximately two molecules of colchicine were bound per microtubule, while the same degree of inhibition beginning with unliganded colchicine occurred with approximately five to six molecules of colchicine bound per microtubule (open circles). We examined this possibility by incubating microtubule suspensions with five concentrations of unliganded colchicine and determining the stoichiometry of colchicine incorporation at microtubule ends in relation to inhibition of tubulin addition for the different concentrations. No significant differences in the extent of colchicine incorporation were found at 25% or 50% inhibition of tubulin addition. Thus, no evidence for the ability of weak or inactive newly formed TC complex to efficiently incorporate at the microtubule ends was obtained. Therefore, the differences in the stoichiometry of colchicine incorporation with unliganded colchicine and final-state TC complex are most likely related to the “end-conserving” mechanism by which TC complex and tubulin continuously add to microtubule ends (see below).

*Does Colchicine Act at Microtubule Ends by an “End-Blocking” or an “End-Conserving” Mechanism?* It seems clear that colchicine in the form of a final-state TC complex becomes incorporated at microtubule ends and perturbs the dynamics of tubulin addition and loss at the ends. However, the questions of how much colchicine is required to exert its inhibitory activity and how inhibition is actually exerted are unresolved [e.g., see Sternlicht and Ringel (1979), Margolis et al. (1980), Farrell and Wilson (1980, 1984), and Wilson et al. (1985)]. In early studies on the ability of colchicine to block tubulin addition at microtubule ends, data from this laboratory were obtained indicating that at 0.13  $\mu\text{M}$  colchicine, an average of 0.5 molecule of colchicine bound per microtubule A end reduced the rate of tubulin addition at the end by 50% (Margolis et al., 1980). The data were interpreted in terms of an “end-blocking” mechanism (i.e., the binding of one molecule of colchicine per microtubule A end was sufficient to reduce the rate of tubulin addition to zero or near zero).

However, when tubulin was polymerized into microtubules in vitro in the presence of final-state TC complex at high ratios of tubulin to TC complex, TC complexes became incorporated into the microtubules along with the tubulin in the form of TC-complex-tubulin copolymers (Sternlicht & Ringel, 1979; Farrell & Wilson, 1980, 1984). These results were interpreted in terms of an “end-conserving” mechanism [see Sternlicht and Ringel (1979)] in that addition of TC complex to microtubule ends does not completely prevent further tubulin addition to the ends. Rather, TC complex acts by reducing the rate of tubulin addition, but the tubulin can continue to add to the end along with TC complex.

The data in this report fully support the end-conserving mechanism at low ratios of TC complex to tubulin. In the present study, when final-state TC complex was added to microtubules at steady state, the rate of tubulin addition at A ends was maximally reduced by the TC concentration used after only a few minutes. Approximately 50–60% inhibition of tubulin incorporation was induced rapidly by the incorporation of one to two molecules of colchicine bound per microtubule net A end. However, TC complex continued to be incorporated with time with very little change in the rate of inhibition of tubulin addition (Figure 5). In the present work, when inhibition of tubulin addition was induced beginning with 0.5  $\mu\text{M}$  unliganded colchicine, inhibition of tubulin addition



at A ends increased continuously with time (Figure 2b), concomitant with an increase in the number of molecules of colchicine incorporated per A end (Figure 3), and with the increased formation of final-state TC complex (Figure 4). Thus, in the previous study from this laboratory in which MAP-rich microtubules at steady state were incubated with low concentrations of unliganded colchicine for 1 h at 30 °C (Margolis et al., 1980), if longer incubation times had been used, more colchicine would have been found incorporated per microtubule.

*Possible Relationship between the Conformational Changes Occurring upon the Binding of Colchicine to Tubulin and Conformational Changes Occurring during Microtubule Polymerization.* The events which occur at microtubule ends during addition and loss of tubulin are not yet understood. It is likely that a complex series of reactions occurs that involves hydrolysis of tubulin-liganded GTP to GDP and  $P_i$  and that the involvement of GTP hydrolysis is responsible for the phase changes at microtubule ends that give rise to growing and shortening dynamics (Carlier & Pantaloni, 1981; O'Brien et al., 1987; Farrell et al., 1987; Carlier, 1989; Stewart et al., 1990; Melki et al., 1990). GTP-liganded tubulin has been postulated to exist at microtubule ends and to confer stability to the ends (a GTP cap), although evidence has been obtained in this laboratory (L. Wilson, K. W. Farrell, and H. P. Miller, unpublished data) and in Carlier's laboratory (Melki et al., 1990) that the stabilizing cap may consist of tubulin-liganded GDP- $P_i$  at the microtubule ends rather than GTP. The idea is that the rate constants for association and dissociation of tubulin-GTP (or tubulin-GDP- $P_i$ ) and tubulin-GDP at microtubule ends differ significantly and that when GDP-tubulin is exposed at microtubule ends the ends are relatively unstable as compared to when tubulin-GTP or tubulin GDP- $P_i$  is exposed at the ends. A relatively slow first-order transition in tubulin from a GTP-liganded or GDP- $P_i$ -liganded to a GDP-liganded conformation might occur after GTP hydrolysis and  $P_i$  release, and it seems highly plausible that it is the conformational state of the tubulin that determines the stability of the microtubule ends (Stewart et al., 1990).

The results described in this study are consistent with the hypothesis that the effects of colchicine on the dynamics of tubulin addition and loss at microtubule ends are caused by the colchicine-liganded tubulin itself and involve a modification of the tubulin resulting from binding of colchicine to its binding site. Inhibition of tubulin addition at microtubule ends could occur by steric hindrance due to the presence of the drug at an important interface between tubulin dimers in the microtubule lattice. However, colchicine can also inhibit the rate of tubulin dissociation at microtubule ends (Margolis et al., 1980; Farrell & Wilson, 1984; Wilson et al., 1985; Bergen & Borisy, 1986), and such a simple steric blocking mechanism would not be expected to affect the rate of dissociation of tubulin at the microtubule ends.

An intriguing possibility is that the conformational change induced in tubulin upon binding of colchicine is related in an important way to conformational changes that occur normally when unliganded tubulin becomes incorporated at the ends of microtubules. When colchicine binds to its tubulin binding site, it might induce a conformational change in the tubulin that is sufficiently different than the conformational change which occurs normally during polymerization that the ability of the microtubule end to accept additional tubulin and the ability of the tubulin to dissociate from the end are both reduced. In this regard, it may be significant that the binding of colchicine to tubulin increases the GTPase activity of tubulin

(David-Pfeuty et al., 1979; Andreu & Timasheff, 1981). Thus, the normal involvement of GTP or GTP hydrolysis at microtubule ends might be altered when TC complex is incorporated at the ends.

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## Ca<sup>2+</sup> Dependence of the Interactions between Protein C, Thrombin, and the Elastase Fragment of Thrombomodulin. Analysis by Ultracentrifugation<sup>†</sup>

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**ABSTRACT:** The two-way and three-way interactions among active-site-blocked bovine thrombin, bovine protein C, and the elastase fragment of rabbit thrombomodulin (eTM) were examined by analytical ultracentrifugation at 23.3 °C in 100 mM NaCl, 50 mM Tris (pH 7.65), and 1 mM benzamidine, in the presence of 0 to 5 mM calcium chloride. Thrombin and eTM form a tight ( $K_d < 10^{-8}$  M) 1:1 complex in the absence of Ca<sup>2+</sup> that weakens with the addition of Ca<sup>2+</sup> ( $K_d \approx 4 \mu\text{M}$  in 5 mM Ca<sup>2+</sup>). Without Ca<sup>2+</sup>, thrombin and protein C form a 1:1 complex ( $K_d \approx 1 \mu\text{M}$ ) and what appears to be a 1:2 thrombin-protein C complex. The  $K_d$  for the 1:1 complex weakens over 100-fold in 5 mM CaCl<sub>2</sub>. Protein C and eTM form a Ca<sup>2+</sup>-independent 1:1 complex ( $K_d \approx 80 \mu\text{M}$ ). Nearly identical binding to thrombin and eTM is observed when active-site-blocked activated bovine protein C is substituted for protein C. Thrombin inhibited by diisopropyl fluorophosphate and thrombin inhibited by a tripeptide chloromethyl ketone exhibited identical behavior in binding experiments, suggesting that the accessibility of protein C to the substrate recognition cleft of these two forms of thrombin is nearly equal. Human protein C binds with lower affinity than bovine protein C. Ternary mixtures also were examined. Protein C, eTM, and thrombin form a 1:1:1 complex which dissociates with increasing [Ca<sup>2+</sup>]. In the absence of Ca<sup>2+</sup>, protein C binds to the eTM-thrombin complex with an apparent  $K_d \approx 1 \mu\text{M}$ . Nearly identical binding to eTM-thrombin was observed for activated protein C and protein C, suggesting that there is little discrimination between substrate and product. This was confirmed kinetically. Activated protein C is a potent inhibitor of its own formation both by thrombin alone ( $K_i \approx 0.5 \mu\text{M}$ ) and by thrombin bound to eTM ( $K_i \approx 4 \mu\text{M}$ ). Bovine protein C lacking the 41 amino acid  $\gamma$ -carboxyglutamic acid containing peptide (Gla domain) exhibited virtually no interaction with either eTM or eTM-thrombin, whereas the Gla domain competed effectively and specifically with protein C in these interactions. Finally, binding of the Gla domain to eTM-thrombin was observed. These results indicate that the Gla domain may be involved directly in the binding of protein C to both thrombin and eTM.

**T**hrombomodulin (TM),<sup>1</sup> an endothelial cell surface protein, is a cofactor for the activation of protein C by thrombin [for

a review, see Esmon (1989)]. TM retains activity following solubilization with detergents, but the soluble form exists predominantly in the form of large aggregates (Winnard et al., 1989), making physical analyses difficult. However, limited proteolysis of TM by elastase yields a soluble fragment, termed eTM, which consists of the six epidermal growth factor-like domains of thrombomodulin (residues 234-486; Stearns et al.,

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<sup>1</sup> Abbreviations: EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Gla,  $\gamma$ -carboxyglutamic acid; PPACK, D-phenyl-L-prolyl-L-arginine chloromethyl ketone; DEGR, dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone; TM, rabbit thrombomodulin; eTM, elastase fragment of rabbit thrombomodulin; eTM-thrombin, 1:1 complex of the elastase fragment of rabbit thrombomodulin and bovine thrombin; APC, activated bovine protein C; gdPC, protein C lacking the N-terminal 42 amino acids (Gla domain);  $M_z$ , z-average molecular weight; EGF, epidermal growth factor.