Goldman, D. J., Worobec, S. W., Seigel, R. B., Hecker, R. V., & Ordal, G. W. (1982) *Biochemistry 21*, 915–920.
Goy, M. F., Springer, M. S., & Alder, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4964–4968.
Kehry, M. R., Doak, T. C., & Dahlquist, F. W. (1984) *J. Biol. Chem. 259*, 11828–11835.
Kleene, S. J., Toews, M. L., & Adler, J. (1977) *J. Biol. Chem. 252*, 3214–3218.
Kort, E. N., Goy, M. F., Larsen, S. H., & Adler, J. (1975) *Proc. Natl. Acad. Sci. U.S.A 72*, 3939–3943.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
Ordal, G. W. (1976) *J. Bacteriol. 126*, 72–79.
Ordal, G. W., & Goldman, D. J. (1975) *Science 189*, 802–805.
Ordal, G. W., & Villani, D. P. (1980) *J. Gen. Microbiol. 118*, 471–478.
Slocum, M. S., & Parkinson, J. S. (1985) *J. Bacteriol. 163*, 586–594.
Springer, M. S., Goy, M. F., & Adler, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 183–187.
Springer, W. R., & Koshland, D. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 533–537.
Thoelke, M. S., Bedale, W. A., Nettleton, D. O., & Ordal, G. W. (1987) *J. Biol. Chem. 262*, 2811–2816.
Thoelke, M. S., Parker, H. M., Ordal, E. A., & Ordal, G. W. (1988) *Biochemistry 27*, 8453–8457.
Towes, M. L., Goy, M. F., Springer, M. S., & Adler, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5544–5548.
Ullah, A. J. H., & Ordal, G. W. (1981) *J. Bacteriol. 145*, 958–965.
Van Der Werf, P., & Koshland, D. E. (1977) *J. Biol. Chem. 252*, 2793–2795.

Roles of Colchicine Rings B and C in the Binding Process to Tubulin†

F. Javier Medrano,‡ José M. Andreu,‡ Marina J. Gorbunoff,§ and Serge N. Timasheff*,§
Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Cientificas, 28006 Madrid, Spain, and Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Received December 29, 1988; Revised Manuscript Received March 14, 1989

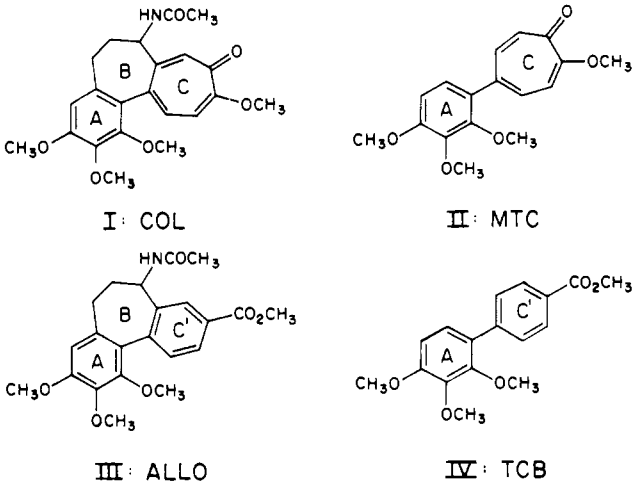
ABSTRACT: The interactions of tubulin with colchicine analogues in which the tropolone methyl ether ring had been transformed into a *p*-carbomethoxybenzene have been characterized. The analogues were allocolchicine (ALLO) and 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB), the first being transformed colchicine and the second transformed colchicine with ring B eliminated. The binding of both analogues has been shown to be specific for the colchicine binding site on tubulin by competition with colchicine and podophyllotoxin. Both analogues bind reversibly to tubulin with the generation of ligand fluorescence. The binding of ALLO is slow, the fluorescence reaching a steady state in the same time span as colchicine; that of TCB is rapid. The displacement of ALLO by podophyllotoxin proceeds with a half-life of ca. 40 min. Binding isotherms generated from gel filtration and fluorescence measurements have shown that both analogues bind to tubulin with a stoichiometry of 1 mol of analogue/mol of α–β tubulin. The equilibrium binding constants at 25 °C have been found to be $(9.2 \pm 2.5) \times 10^5$ M^{-1} for ALLO and $(1.0 \pm 0.2) \times 10^5$ M^{-1} for TCB. Binding of both analogues was accompanied by quenching of protein fluorescence, perturbation of the far-ultraviolet circular dichroism of tubulin, and induction of the tubulin GTPase activity, similarly to colchicine binding. Both inhibited microtubule assembly in vitro, ALLO substoichiometrically, and both induced the abnormal cooperative polymerization of tubulin, which is characteristic of the tubulin–colchicine complex. Analysis in terms of the simple bifunctional ligand binding mechanism developed for colchicine [Andreu, J. M., & Timasheff, S. N. (1982) *Biochemistry 21*, 534–543] and comparison with the binding of the colchicine two-ring analogue, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one [Andreu, J. M., Gorbunoff, M. J., Lee, J. C., & Timasheff, S. N. (1984) *Biochemistry 23*, 1742–1752], have shown that transformation of the tropolone methyl ether part of colchicine into *p*-carbomethoxybenzene weakens the standard free energy of binding to tubulin by 1.4 ± 0.1 kcal/mol, while elimination of ring B weakens it by 1.0 ± 0.1 kcal/mol. The roles of rings C and B of colchicine in the thermodynamic and kinetic mechanisms of binding to tubulin were analyzed in terms of these findings.

The binding of colchicine to tubulin is known to be a slow process that conforms to a two-step mechanism consisting of a fast and reversible bimolecular binding reaction, followed by a slow monomolecular reaction (Garland, 1978; Lambeir & Engelborghs, 1981). The binding of colchicine to tubulin induces a conformational change in the protein, which is manifested by a perturbation of the far-UV circular dichroism (Andreu & Timasheff, 1982c), the induction of assembly-independent GTPase activity (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981), and self-assembly into structures other than microtubules (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b; Andreu et al., 1983). Colchicine, which strongly inhibits microtubule assembly (Wilson & Bryan, 1974) at substoichiometric levels (Margolis & Wilson, 1977), is a three-ring structure (Chart I, structure I) that consists of a trimethoxyphenyl ring (ring A) linked to a tropolone

† Communication No. 1677 from the Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254. This work was supported in part by NIH Grants CA-16707 and GM-14603 and U.S.–Spain Joint Committee for Scientific and Technological Cooperation Grant CCB-8402/037. Preliminary reports of this work were presented at the 1st Spanish Biophysics Meeting (Sitges, Spain, 1986) (Medrano et al., 1986) and the 1st Meeting of the Spanish Biophysical Society (Valladolid, Spain, 1987).
* To whom correspondence should be addressed.
‡ Centro de Investigaciones Biologicas.
§ Brandeis University.

0006-2960/89/0428-5589$01.50/0

Chart I



methyl ether ring (ring C) by a seven-membered ring (ring B). A detailed examination of the binding process using single-ring analogues of the trimethoxyphenyl and tropolone parts of the colchicine molecule has led to a simple thermodynamic model of the binding in terms of a bidentate mechanism: the tropolone methyl ether ring (ring C) and the trimethoxyphenyl ring (ring A) bind to independent subsites on the protein; binding of the tropolone ring induces the conformational change (Andreu & Timasheff, 1982a,c). In this model, it has been proposed that the middle connecting ring (ring B) is the source of the peculiar kinetic characteristics of the interaction. This model has been confirmed by studies on the binding to tubulin of 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC)[1] (Chart I, structure II) (Andreu et al., 1984), which is a colchicine analogue consisting of only the trimethoxyphenyl and tropolone methyl ether rings.

In view of the conclusion that the conformational change in tubulin was induced by its interaction with ring C of colchicine (Andreu & Timasheff, 1982c), it was thought of interest to probe further this conclusion by the use of colchicine analogues in which the structure of ring C had been modified, while ring A was left intact. The analogue of this nature closest to colchicine is allocolchicine (ALLO), which is derived from colchicine by rearrangement of its tropolone methyl ether (ring C) to *p*-carbomethoxybenzene (ring C′) (Chart I, structure III). Following a demonstration that this compound, as well as some of its biphenyl analogues, i.e., compounds that do not contain ring B, are bound to tubulin and inhibit in vitro microtubule formation (Medrano et al., 1986), a detailed study has been undertaken of their interactions with tubulin. The results obtained with allocolchicine and its analogue from which ring B had been excised, 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB) (Chart I, structure IV), are reported in this paper. Comparison of these results with those on the interactions of tubulin with colchicine and MTC has permitted the definition of the nature of the thermodynamic contributions of rings C and B to the mechanism of binding.

Materials and Methods

Ligands and Other Materials. Colchicine and podophyllotoxin were from Aldrich Chemical Co. GTP dilithium salt was from Boehringer-Mannheim. ALLO was prepared by the base-catalyzed rearrangement of colchicine (Fernholtz, 1950). The synthesis of TCB is described below. To check their purity, the two products (ALLO and TCB) were subjected to thin-layer chromatography (TLC) on 0.25 mm thick silica gel plates (Fertigplatten, Merck), developed with chloroform–acetone–diethylamine (7:2:1) or dichloromethane–methanol (99:1). TCB gave a single spot under ultraviolet light; ALLO gave one major spot and two minor spots. The minor spots represented less than 5% of the total weight, their contribution to the absorbance was less than 3% at 288 nm, and they gave no fluorescence in 90% dioxane (see Binding Measurements). The interaction of every isolated component with tubulin was examined by fluorescence and difference absorption. Only the major spot was able to interact with tubulin, and ALLO was routinely used without further purification. The concentrations of ALLO and TCB were measured spectrophotometrically. The extinction coefficients were determined by weighing dry crystals of the compounds with a Mettler UM-3 electrobalance, dissolving the crystals gravimetrically in a final solution of PG buffer and recording the UV spectrum. Five independent determinations gave ϵ(315 nm) = 4680 ± 160 M^{-1} cm^{-1}, ϵ(288 nm) = 11 860 ± 130 M^{-1} cm^{-1}, and ϵ(276 nm) = 10 250 ± 140 M^{-1} cm^{-1} for ALLO and ϵ(315 nm) = 3825 ± 40 M^{-1} cm^{-1}, ϵ(284 nm) = 12 100 ± 200 M^{-1} cm^{-1}, and ϵ(276 nm) = 11 400 ± 25 M^{-1} cm^{-1} for TCB. Sephadex G-25 was from Pharmacia, and Bio-Gel P-4 was from Bio-Rad. [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid and sodium dodecyl sulfate were from Sigma. Glycerol was from Merck, analytical grade, and all other chemicals were of reagent grade.

Synthesis of 2,3,4-Trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB). TCB was synthesized by two standard methods. (i) Arylation (Rondestvedt & Blanchard, 1955) of 1,2,3-trimethoxybenzene at 50 °C with methyl *N*-nitroso-*N*-acetyl-4-aminobenzoate resulted in a 10% yield. (ii) The mixed Ullman reaction, described below, gave a 24% yield.

Methyl 4-iodobenzoate (0.02 M) and 0.05 M 1,2,3-trimethoxy-4-iodobenzene were thoroughly mixed with 40 g of activated copper (Kleiderer & Adams, 1933). The mixture was brought to 190 °C and heated for 3 h, the temperature not exceeding 290 °C. The reaction mixture was extracted three times with 100 mL of CH_2Cl_2. After washing with 5% $NaHCO_3$ and water, the combined extracts were dried and evaporated to a residue (4.9 g). The residue was kept at 250 μm and 120 °C for 2 h. The nonsublimable material was boiled with four 50-mL portions of medium petroleum ether (MPE). These extracts were chromatographed on a column of neutral alumina poured in low petroleum ether (LPE). The elution was carried out by a slow increase in C_6H_6 concentration. This gave an almost pure product. Recrystallization from MPE gave white needles, mp 78.0–79.0 °C (uncorrected). Elemental analysis, found: C, 67.54; H, 5.97. Theory: C, 67.53; H, 6.00. Mass spect: MW 302 (theory 302), with fragmentation pattern consistent with the structure of TCB. IR (CCl_4): 1733 (C=O); 1618 (aromatic); 1280 (C—O). ^1H NMR ($CDCl_3$): 8.07 [d, J = 8.7, H-C(3′), H-C(5′)]; 7.59 [d, J = 8.7, H-C(2′), H-C(6′)]; 7.06 [d, J = 8.7, H-C(6)]; 6.76 [d, J = 8.7, H-C(5)]; 3.94 (s, ring A OCH_3); 3.935 (s, ester OCH_3); 3.91 (s, ring A OCH_3); 3.67 (s, ring A OCH_3). ^{13}C NMR ($CDCl_3$): 167.0 (C=O); 153.7 (C-2); 151.4 (C-4); 142.9 (C-1′); 142.5 (C-3); 129.3 (C-3′, C-5′); 129.0 (C-2′, C-6′); 128.3 (C-4′); 127.4 (C-1); 124.7 (C-6); 107.5 (C-5); 61.0 (2 ring A OCH_3); 56.0 (ring A OCH_3); 52.0 (ester OCH_3).

[1] Abbreviations: ALLO, allocolchicine; TCB, 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; TME, tropolone methyl ether; PG buffer, 10 mM sodium phosphate, 0.1 mM GTP, pH 7.0, buffer; GTP, guanosine triphosphate; ANS, 8-anilinonaphthalene-1-sulfonate.

Solubility of Ligands. ALLO and TCB were dissolved in dimethyl sulfoxide and stored at −20 °C. For each experiment an aliquot of the stock solution in DMSO was added to the working buffer to give the desired concentration of the analogue. ALLO did not present major solubility problems provided there was a minimal quantity of the organic solvent in the solution. The maximum quantity used in these experiments was 1% dimethyl sulfoxide, at which more than 10^{-4} M ALLO can be dissolved. Between 0 and 0.05% dimethyl sulfoxide the solubility of ALLO is limited to ca. 2×10^{-5} M. Routinely less than 0.5% dimethyl sulfoxide was used in the experiments. The solubility of TCB is limited, independent of the quantity of dimethyl sulfoxide added. Above 3×10^{-5} M the observed effects were a decrease in the absorption of this ligand with time and the development of an anomalous fluorescence intensity, even though no visible precipitate was noticed. These effects were not observed below 3×10^{-5} M TCB.

Protein. Tubulin was purified from calf brains and stored in liquid nitrogen. It was prepared for use and its concentration was measured as described previously (Weisenberg et al., 1968; Lee et al., 1973; Andreu et al., 1984). The tubulin–colchicine complex was prepared as described (Andreu & Timasheff, 1982c). All experiments, other than assembly, were done in 10 mM sodium phosphate and 0.1 mM GTP, pH 7.0, buffer (PG buffer), at 25 °C except where indicated.

Spectroscopic Measurements. Light absorption spectra were obtained on a Cary 16 spectrophotometer. Difference absorption spectra were made with the automatic slit mechanism using mixing tandem cells of 0.438 + 0.438 cm light path (Hellma) mounted on holders thermostated at 25 ± 0.5 °C with a Lauda K2RD water bath. In the case of ALLO, the spectra were obtained as described by Andreu et al. (1984). In the case of TCB, which adsorbed to the Teflon plugs of the cells, the spectra were obtained by the procedure of Medrano and Andreu (1986).

Fluorescence measurements were made with a Fica 55 MK II double-beam spectrofluorometer that gives corrected excitation and emission spectra. Excitation and emission bandwidths were 2.5 and 7.5 nm, respectively. Fluorescence cells (0.5 × 0.5 cm) were mounted on thermostated holders, and the temperatures of the solutions inside the cell were measured with an Omega copper–constantan thermocouple connected to a Yew digital thermometer. The inner filter effect was minimized by using sample absorbances less than 0.05. When needed, it was corrected for by the graphical procedure of Mertens and Kägi (1979). Fluorescence was expressed in arbitrary units that correspond to the spectrofluorometer output (millivolts) with the reference potential set at 600 V and a gain of 50.

Circular dichroism spectra were obtained with a Rousell-Jouan Dichrograph II, in a 0.01-cm cell at 24 °C. The difference dichroic absorption $[A(L) - A(R)]$ was employed to calculate the mean residue molar ellipticity, $[\Theta]$, from the relation $[\Theta] = 3300\,[A(L) - A(R)]\,c^{-1}d^{-1}$, where c is the mean residue concentration and d the light path in decimeters (Adler et al., 1973). The mean residue weight used was 109 (Lee et al., 1978).

Binding Measurements. Binding measurements were performed by the equilibrium gel chromatography technique of Hummel and Dreyer (1962), as carefully applied to tubulin (Andreu, 1985), and by fluorometric titration. In gel chromatography, samples containing 8 nmol of tubulin and a known total concentration of ligand in a given buffer (final volume ≤ 0.8 mL) were prepared and applied immediately to columns equilibrated with the same buffer of identical ligand concentration. With ALLO, the binding measurements were made on 0.9 × 25 ± 1 cm Sephadex G-25 columns. Because TCB adsorbs to Sephadex G-25, Bio-Gel P-4 was used for this ligand, on which the adsorption is much weaker. The retardation in the elution time of TCB, however, led to the use of 0.9 × 10 ± 1 cm columns with this ligand. The ligand concentrations in the column effluents were measured fluorometrically. This was necessitated by the large overlap of the absorption spectra of tubulin and the ligands that rendered impossible measurements of the ligand concentration spectrophotometrically, as had been done with MTC (Andreu et al., 1984). The biphenyl compounds, ALLO and TCB, undergo a large increase of their fluorescence in organic solvents, and this property was used to measure the ligand concentrations in the column effluent. Dioxane was used as the organic solvent because of its miscibility with water and because tubulin precipitates in this solvent and does not interfere with the fluorescence measurements. In the case of ALLO, the final mixture was 1 part aqueous sample and 9 parts dioxane. For TCB, it was 5 parts aqueous sample, 5 parts 10% sodium dodecyl sulfate solution (to prevent the binding of this ligand to denatured tubulin), and 90 parts dioxane. The mixture was microfuged for 2 min at 10000*g*. The fluorescence intensity of the ligands in these solvents at 25 °C was 5.6 ± 0.3 units/10^{-6} M ALLO and 24.1 ± 0.5 units/10^{-6} M TCB (concentrations in the initial aqueous solution), as determined with standards run in each experiment. The excitation wavelengths were 295 and 290 nm and the emission wavelengths were 450 and 410 nm for ALLO and TCB, respectively. The protein concentration was measured by its absorption at 276 nm, taken as the difference between the total absorption and the contribution of the ligand at this wavelength (calculated from its extinction coefficient and the concentration measured by the solvent extraction procedure).

Binding measurements by fluorometric titration were made as follows. The fluorescence of the free ligand was measured, and its value, practically negligible, was subtracted from all further measurements. The fluorescence intensity of bound ligand was determined by titrating with tubulin a ligand solution of a concentration that gave no appreciable inner filter effect, until the fluorescence due to bound ligand reached saturation. The obtained values were 109 ± 3 units/10^{-6} M bound ALLO and 195 ± 4 units/10^{-6} M bound TCB in PG buffer, pH 7.0 at 25 °C, with excitation at 315 nm and emission at 390 and 373 nm for ALLO and TCB, respectively. The ligand excitation wavelength was chosen so as to avoid excitation of intrinsic protein fluorescence. Aliquots of a protein solution were then titrated with known total concentrations of ligand. The bound ligand was measured by its corrected fluorescence, and the free ligand concentration was taken as the difference between total and bound concentrations. TCB fluorescence data needed no inner filter correction because of the low concentrations employed (less than 3×10^{-5} M). The values of the binding equilibrium constant and the number of sites were obtained from Scatchard plots of the data. Quenching of the intrinsic protein fluorescence by ligand was also employed to estimate the binding affinity. The maximal fluorescence quenching by excess ligand was measured. Then the fraction of sites occupied, α, was taken as equal to the fraction of the maximal quenching effect at a given total ligand concentration. The binding equilibrium constant was determined by employing the relationship $\alpha/(1 - \alpha) = K_b[A]$ (Lehrer & Fasman, 1966), where [A] is the free-ligand concentration taken as the difference between known total and

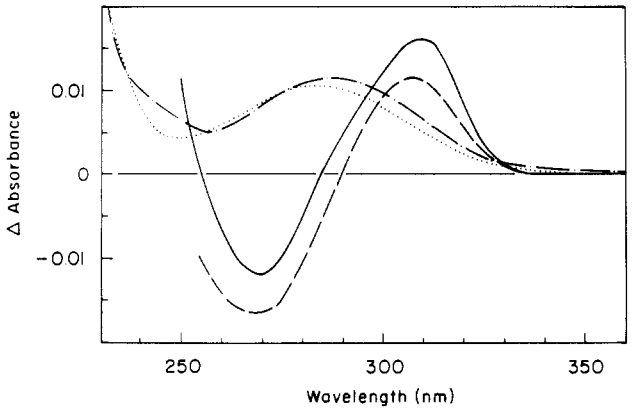


FIGURE 1: Difference absorption spectra generated by the interaction of tubulin with ALLO and TCB in PG buffer, pH 7.0 at 25 °C. (Solid line) Difference spectrum of 8.1×10^{-6} M ALLO and 9.2×10^{-6} M tubulin; (dashed line) difference spectrum of 2.34×10^{-5} M TCB and 1.03×10^{-5} M tubulin vs ligand and protein in separate solutions; (dot-dash line) direct absorption spectrum of 3.0×10^{-5} M ALLO; (dotted line) direct absorption spectrum of 2.8×10^{-5} M TCB in PG buffer, pH 7.0. The direct spectra are reduced 31 times.

bound ligand. Bound ligand was measured in the same experiment from its corrected fluorescence or calculated from the protein concentration and α, assuming a 1:1 binding stoichiometry.

Miscellaneous Procedures. Colchicine binding to tubulin was followed by the fluorescence of bound colchicine (Bhattacharyya & Wolff, 1974; Andreu & Timasheff, 1982a,c). The GTPase activity developed in tubulin by addition of colchicine, ALLO, and TCB was measured as previously described (Andreu & Timasheff, 1981). Sedimentation velocity experiments were performed in a Beckman Model E analytical ultracentrifuge. Identical samples, with and without ligand, were run simultaneously in double-sector cells in an An-D rotor at 60 000 rpm. The in vitro assembly of microtubules was performed in 10 mM sodium phosphate, 0.1 mM GTP, 0.5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 6 mM $MgCl_2$, and 3.4 M glycerol, pH 6.5, buffer (assembly buffer) at 37 °C in a thermostated cuvette, and the mass of polymer formed was followed turbidimetrically at 350 nm (Lee & Timasheff, 1977) with a Varian 635 spectrophotometer. The self-assembly of tubulin into abnormal polymers in the presence of ALLO and TCB was followed turbidimetrically at 350 nm at 37 °C in PG–16 mM $MgCl_2$ buffer, pH 7.0. The polymers formed in both cases were fixed with 0.5% glutaraldehyde, adsorbed to formvar carbon-coated grids, negatively stained with 2% uranyl acetate, and examined under a Philips EM 300 electron microscope.

RESULTS

Determination of the Interaction of Biphenyl Colchicine Analogues with Tubulin by Difference Absorption and Fluorescence Spectroscopy. The interaction of tubulin with ALLO and TCB (structures III and IV, Chart I) was first examined by difference absorption spectroscopy, and the results are displayed in Figure 1. The solid line in Figure 1 is the difference spectrum generated by a solution of 8.1×10^{-6} M ALLO and 9.2×10^{-6} M tubulin vs ligand and protein in separate solutions; the dashed line is the difference spectrum generated by 2.34×10^{-5} M TCB and 1.03×10^{-5} M tubulin. Interaction between tubulin and ALLO generated a spectrum characterized by a maximum at 310 nm and a minimum at 270 nm. Similarly, the interaction of tubulin and TCB generated a spectrum characterized by a maximum at 306 nm and a minimum at 269 nm. These wavelengths were established from several spectra obtained at different concentrations

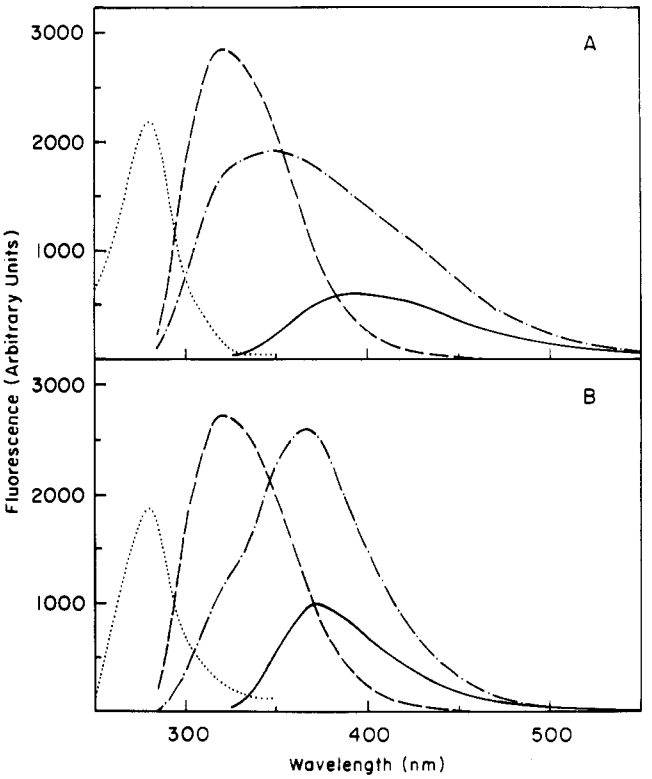


FIGURE 2: Fluorescence changes produced by the interaction of ALLO (A) and TCB (B) with tubulin in PG buffer, pH 7.0, 25 °C. (A) (Dotted line) Fluorescence excitation spectrum of 2.0×10^{-6} M ALLO with 1.0×10^{-6} M tubulin (emission analyzed at 480 nm; amplified 50 times); (dashed line) fluorescence emission spectrum of 6.7×10^{-6} M tubulin (excitation at 280 nm); (dot-dash line) same with 2.01×10^{-5} M ALLO (excitation at 280 nm); (solid line) same solution as dot-dash line, but excitation at 315 nm. (B) (Dotted line) Fluorescence excitation spectrum of 2.0×10^{-6} M TCB with 1.0×10^{-6} M tubulin (emission analyzed at 480 nm; amplified 250 times); (dashed line) fluorescence emission spectrum of 6.1×10^{-6} M tubulin (excitation at 280 nm); (dot-dash line) same with 4.0×10^{-5} M TCB (excitation at 280 nm); (solid line) same solution as dot-dash line, but excitation at 315 nm. It should be noted that the dot-dash-line emission spectra contained contributions from both quenched protein and bound ligand spectra. The fluorescence of free ligand (ALLO and TCB) solutions was negligible on this scale.

of protein and ligand. The direct spectra of ALLO and TCB, shown by the dot-dash and the dotted lines, respectively, gave maxima at 288 and 284 nm and minima at 257 and 250 nm for ALLO and TCB, respectively. When ALLO and TCB were placed in organic solvents, the absorption maximum underwent a red shift of 3–7 nm and there was an increase of 25–50% in the extinction coefficient. On the basis of the effects of the organic solvents on ALLO and TCB, the perturbations of the spectra of these ligands in the presence of tubulin can be ascribed to transfer from a more polar to a less polar environment inside the binding site.

The fluorescence of ALLO and TCB is very weak in aqueous solution, while in organic solvents these ligands display a high fluorescence, the fluorescence maximum undergoing a blue shift with a decrease of the dielectric constant of the solvent. Binding of ALLO and TCB to tubulin also leads to a high fluorescence. As shown in Figure 2, the excitation spectra of tubulin and the ligands had a large overlap. Due to this, an excitation wavelength of 315 nm was used for the ligands, since at that wavelength there was no significant excitation of the protein. The excitation spectra of the ligands bound to tubulin had to be recorded at an emission wavelength of 480 nm at which there was no emission of the protein, but only of the bound ligand. The emission maxima of ALLO and

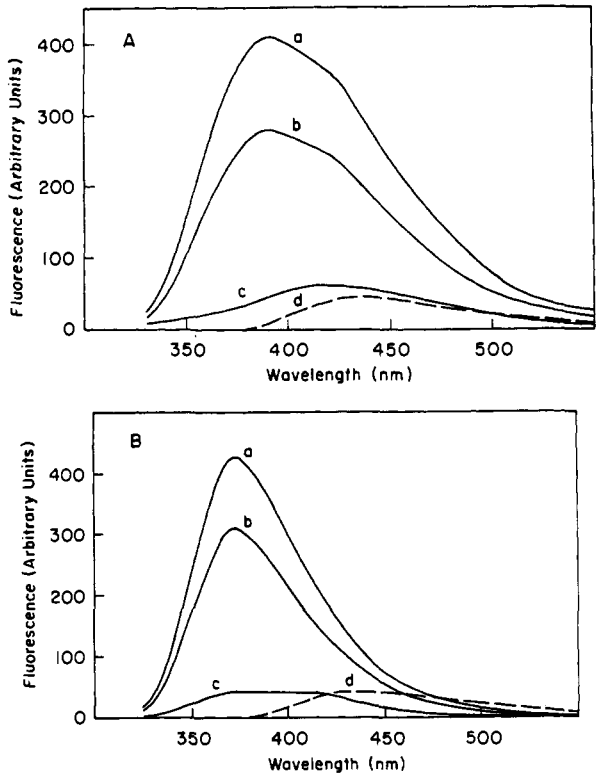


FIGURE 3: Inhibition by colchicine of the binding of ALLO (A) and TCB (B) to tubulin. Fluorescence emission spectra (excitation at 320 nm) are shown of ALLO and TCB added to tubulin (tracings a) and to the tubulin–colchicine complex (tracings c). Tracings d are the fluorescence emission spectra (excitation at 365 nm) of colchicine bound to tubulin prior to the addition of the analogues; excitation of these samples at 320 nm gave ca. 40% of the intensity shown in tracings d. Tracings b are the fluorescence emission spectra of ALLO and TCB added to the tubulin–colchicine complex after irradiation with UV light for 15 min. Concentrations: [tubulin] = 4.0×10^{-6} M; [tubulin–colchicine] = 4.0×10^{-6} M; [ALLO] = 2.0×10^{-5} M; and [TCB] = 2.0×10^{-5} M.

TCB bound to tubulin were 390 and 373 nm, respectively.

The interaction of these compounds with tubulin resulted also in the quenching of the intrinsic fluorescence of the protein, as shown clearly by comparison of the unliganded and liganded protein emission spectra (Figure 2, dashed and dot-dash lines, respectively). This quenching effect is similar to the quenching of protein fluorescence in the tubulin–colchicine complex (Andreu & Timasheff, 1982c) and by complexation with MTC (Andreu et al., 1984; Bane et al., 1984).

Specificity and Kinetics of the Interactions. Once it had been established that ALLO and TCB interact with tubulin, generating difference absorption and fluorescence spectra, it was of interest to ask whether the bindings of these compounds were specifically directed to the colchicine binding site in tubulin. First, it was ascertained that ALLO and TCB did not generate any difference absorption signal when mixed with colchicine. The concentrations used were similar to those of the binding experiments. To determine whether these ligands bind to the tubulin–colchicine complex, ligand fluorescence emission spectra were obtained after ALLO or TCB was mixed with unliganded tubulin and with the tubulin–colchicine complex. The results, shown in Figure 3, indicate that there is essentially no binding to the tubulin–colchicine complex, as the fluorescence of the analogues in the presence (tracings c) of the tubulin–colchicine complex is very weak. On the other hand, in the absence of colchicine, mixing of tubulin with ALLO or TCB generated a strong spectrum (tracings a). When colchicine was released from the tubulin–colchicine

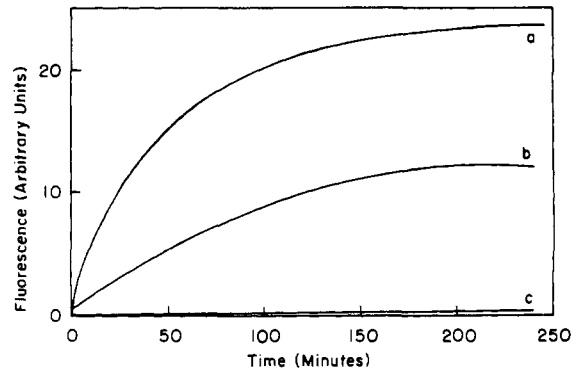


FIGURE 4: Inhibition of colchicine binding to tubulin by ALLO and TCB. Fluorescence time courses are shown of the binding of 10^{-5} M colchicine to 1.1×10^{-5} M tubulin in the absence of analogues (a) or in the presence of 2.0×10^{-5} M TCB (b) or 1×10^{-4} M ALLO (c). The excitation wavelength was 365 nm, and the emission wavelength was 430 nm. The colchicine and ALLO or TCB were consecutively added to the protein solution in the fluorometer cuvette at 25 °C as small volumes of concentrated solution. They were mixed simultaneously, starting the reaction.

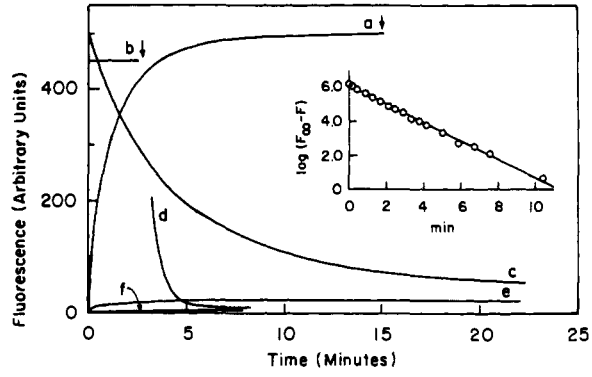


FIGURE 5: Fluorescence time course of the binding of ALLO and TCB to tubulin. The reactions were started by addition of the ligands to the protein solution: (a) 3.0×10^{-5} M ALLO and 7.8×10^{-6} M tubulin; (b) 2.0×10^{-5} M TCB and 7.9×10^{-6} M tubulin. After attainment of the plateau, 10^{-4} M podophyllotoxin was added to samples a and b, producing tracings c and d, respectively. (The time scale of curve c indicates time after addition of podophyllotoxin at arrow (15 min) of curve a; the break between curves b and d corresponds to the time of podophyllotoxin addition and mixing of the solution.) Tracings e and f are the same as a and b, but podophyllotoxin was added 2 min before ALLO and TCB. The fluorescence scale of tracings b, d, and f is reduced by a factor of 2. The time scale of tracings c and e is reduced 10 times. (Inset) Logarithmic (Naperian) plot of tracing a.

complex by photoconversion to lumicolchicine (Andreu & Timasheff, 1983), the tubulin recovered a partial binding capacity for ALLO and TCB, with fluorescence characteristics similar to those of nonirradiated tubulin (tracings b). The simplest interpretation of these results is that neither ALLO nor TCB binds significantly to the tubulin–colchicine complex but they do bind to the sites vacated when colchicine is converted to lumicolchicine; i.e., the two analogues bind to the same site as colchicine.

To test further the identity of the binding sites for colchicine and the two analogues, the effects of ALLO and TCB on the fluorescence time course of colchicine binding were examined. The results are presented in Figure 4, where tracing a is the fluorescence time course generated by the binding of 10^{-5} M colchicine to 1.1×10^{-5} M tubulin at 25 °C. Addition of 2.0×10^{-5} M TCB or 1×10^{-4} M ALLO produced tracings b and c, respectively. At this level, ALLO inhibits almost completely the binding of colchicine, while the inhibition by TCB is weaker. This is fully consistent with a slow rate of dissociation for the tubulin–ALLO complex and a very fast one for the

TCB complex, as shown in Figure 5.

The binding time course of the two ligands to tubulin is shown in Figure 5, where tracing a was obtained on addition of 3.0×10^{-5} M ALLO to 7.8×10^{-6} M tubulin and tracing b was obtained with 2.0×10^{-5} M TCB and 7.9×10^{-6} M tubulin. It is evident that the binding of ALLO to tubulin is slow, similar to that of colchicine (Figure 5a), with the important difference that the binding of ALLO is pseudo first order over the time span measured,[2] while that of colchicine is biphasic (Garland, 1978; Lambeir & Engelborghs, 1981). Application to the data of the kinetic equation $F_\infty - F_t = Ae^{-at}$, where F_t is fluorescence at time t, gave the straight line shown in the inset of Figure 5. The apparent rate constant calculated from its slope was 0.009 s^{-1} at 3×10^{-5} M ALLO. In the case of TCB the binding time course could not be followed in a conventional fluorometer, since the emission intensity reached more than 95% of its maximal value in less than 30 s, similar to the binding of MTC to tubulin (Andreu et al., 1984).

The rate of dissociation of the tubulin–ALLO and tubulin–TCB complexes was determined by displacement with podophyllotoxin. The results are shown in Figure 5, where at the point indicated by the arrow 1×10^{-4} M podophyllotoxin was added, and the displacement of the analogues was followed (tracings c and d). The results indicate a very rapid displacement of TCB, but slow displacement of ALLO, with a half-life of ca. 40 min, while that of MTC is ca. 3 min (Andreu et al., 1984). In the samples recorded as tracings e and f, tubulin was incubated for 2 min with 1×10^{-4} M podophyllotoxin prior to the addition of 3.0×10^{-5} M ALLO or 2.0×10^{-5} M TCB. The lack of generation of fluorescence shows blocking of the ALLO and TCB binding site by this competitor that shares the trimethoxybenzene ring with colchicine.

As a further test of specificity, a qualitative survey was made of the binding of the analogues to proteins other than native tubulin. It was found that TCB can bind to thermally denatured tubulin (data not shown). It binds to bovine serum albumin, displaying fluorescence and difference absorption signals, and to ovalbumin, displaying a weak fluorescence, and it competes with ANS for the binding site on serum albumin but does not bind to the ANS site on tubulin. These effects were not observed with ALLO.

Binding Equilibrium Parameters. The quantitative characterization of the binding equilibrium of ALLO and TCB to tubulin was performed by the equilibrium gel chromatography technique (Hummel & Dreyer, 1962). Typical elution profiles are shown in Figure 6. Since the binding of ALLO to tubulin is slow (Figure 5, tracing a), to assure attainment of equilibrium the elution times used were twice the time necessary to reach equilibrium in the fluorescence experiments under the same conditions. This was further verified by varying the flow rate. The ligand concentrations were measured by their fluorescence in dioxane–water mixtures (Figure 6, insets), as described under Materials and Methods. The appearance of a distinct peak and trough in the elution pattern indicates that equilibrium had been attained. When the tubulin–colchicine complex was used instead of tubulin, there was no detectable binding of either ALLO or TCB (squares of Figure 6).

The binding isotherms of ALLO and TCB to tubulin in PG buffer at pH 7.0 and 25 °C are shown in Figure 7, tracings a and b, respectively, where the solid symbols are the individual column technique measurements and the solid lines are the isotherms that correspond to binding equilibrium constants, $K_b = 6.3 \times 10^5$ M^{-1} and $K_b = 1.01 \times 10^5$ M^{-1}, and 1.04 and 0.96 sites per tubulin dimer for ALLO and TCB, respectively.

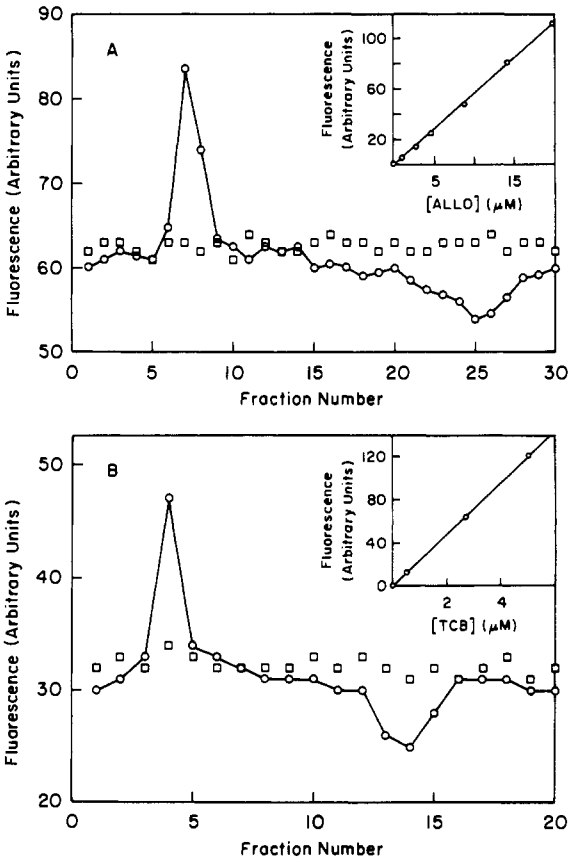


FIGURE 6: Gel chromatography elution profiles of tubulin in columns equilibrated with ALLO (A) and TCB (B). A total of 8 nmol of tubulin (circles) or tubulin–colchicine complex (squares) was chromatographed in a column equilibrated with (A) 1.07×10^{-5} M free ALLO or (B) 2.25×10^{-6} M TCB in PG buffer, pH 7.0, 25 °C. The measurements correspond to the ligand elution profiles after extraction of the fractions in dioxane (see Materials and Methods). The protein eluted in fractions 6–8 in (A) and in fractions 3–5 in (B). The insets show the calibration plots of the fluorescence intensity of the ligands in dioxane.

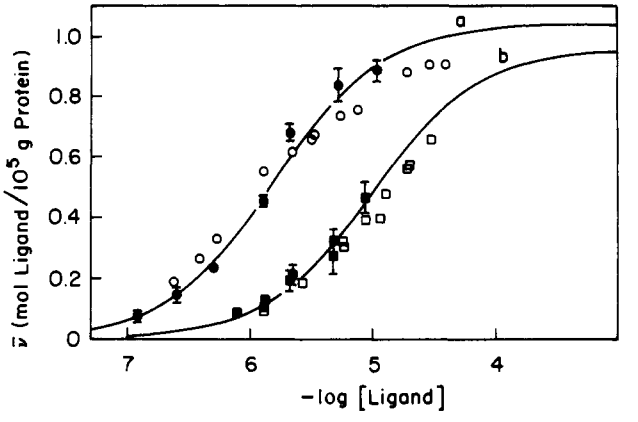


FIGURE 7: Binding isotherms of ALLO (circles) and TCB (squares) to tubulin in PG buffer, pH 7.0, 25 °C. The solid symbols are column measurements (see Materials and Methods and Figure 6) at protein concentrations of $2–5 \times 10^{-6}$ M. The open symbols are ligand fluorescence binding measurements (see Materials and Methods) at 1.2×10^{-6} M (circles) and 7.0×10^{-6} M (squares) protein. The solid lines are fits to the column measurements [(a) $K_b = 0.63 \times 10^6$ M^{-1}, $n = 1.04$; (b) $K_b = 1.01 \times 10^5$ M^{-1}, $n = 0.96$] obtained from Scatchard plots of these data.

[2] There is a suggestion that the fluorescence development at times less than 10–20 s may be more rapid than that measured in the current experiments. This time range, however, is not accessible to our measurements.

Table I: Binding of ALLO and TCB to Tubulin at 25 °C

	ALLO				TCB			
method	protein concn (M)	K_b (M^{-1})	$\Delta G°$ (kcal/mol)	mol of ligand/10^5 g of tubulin	protein concn (M)	K_b (M^{-1})	$\Delta G°$ (kcal/mol)	mol of ligand/10^5 g of tubulin
gel chromatography	2.0×10^{-6} to 5.0×10^{-6}	$(6.3 \pm 2.1) \times 10^5$	−7.91	1.04 ± 0.06	2.5×10^{-6} to 5.0×10^{-6}	$(1.01 \pm 0.20) \times 10^5$	−6.83	0.96 ± 0.05
ligand fluorescence	1.2×10^{-6} to 4.1×10^{-5}	$(12.2 \pm 2.6) \times 10^5$	−8.30	0.87 ± 0.08	1.2×10^{-6} to 3.3×10^{-5}	$(1.15 \pm 0.27) \times 10^5$	−6.90	0.92 ± 0.09
protein fluorescence quenching	3.2×10^{-6}	$(9.1 \pm 2.5) \times 10^5$	−8.13		8.5×10^{-6}	$(0.88 \pm 0.22) \times 10^5$	−6.74	

Table II: Temperature Dependence of the Binding of ALLO and TCB to Tubulin Measured by Ligand Fluorescence

	ALLO		TCB	
temp (°C)	K_b ($\times 10^5$) (M^{-1})	$\Delta G°$ (kcal/mol)	K_b ($\times 10^5$) (M^{-1})	$\Delta G°$ (kcal/mol)
0	12.1 ± 1.2	−7.60		
4			1.43 ± 0.25	−6.54
10	13.4 ± 1.0	−7.92	1.66 ± 0.14	−6.77
25	12.2 ± 2.6	−8.28	1.15 ± 0.27	−6.91
35	12.9 ± 1.3	−8.60	1.19 ± 0.15	−7.14
$\Delta H°$ (kcal/mol)	−0.05 ± 0.2		0.8 ± 0.5	
$\Delta S°$ (eu)	27.7 ± 0.8		26.2 ± 2.0	

These had been determined from Scatchard plots of the data. The results of the ligand fluorescence titrations (see Materials and Methods), performed under the same conditions with 1.2×10^{-6} or 7.0×10^{-6} M tubulin, for ALLO and TCB, respectively, are shown by the open symbols in Figure 7. The protein fluorescence quenching method resulted in comparable equilibrium constants. The value of K_b obtained with ALLO is consistent with the inhibition constant of colchicine binding reported by Deinum et al. (1981). A summary of the results obtained by means of the different procedures is presented in Table I. Varying the protein concentration had no detectable effects on the binding equilibrium parameters of both ligands, indicating the absence of linked protein self-association. The apparent standard free energy changes, calculated from the binding equilibrium constants and averaged over the various techniques used in their measurement, resulted in values of $\Delta G° = -8.1 \pm 0.2$ kcal mol^{-1} for the interaction of ALLO with tubulin and -6.8 ± 0.1 kcal mol^{-1} for the interaction of TCB with tubulin in PG buffer, pH 7.0 at 25 °C.

The binding of ALLO and TCB to tubulin, as measured by fluorometric titration, was found to be weakly dependent on temperature, as shown in Table II. The apparent standard free energy changes varied between −7.6 and −8.6 kcal mol^{-1} over the temperature range studied for the interaction of ALLO with tubulin and between −6.5 and −7.2 kcal mol^{-1} for the interaction of TCB with tubulin. This resulted in $\Delta H°$ values not far from zero and in $\Delta S°$ values of 28 and 26 eu for ALLO and TCB, respectively.

Effects of Ligand Binding on the Conformation and Self-Associations of Tubulin. In view of the binding of the two analogues to tubulin in the same site as colchicine, and since colchicine induces a structural change in tubulin manifested by a small perturbation of the protein circular dichroism at 217 nm and the appearance of GTPase activity (Andreu & Timasheff, 1982c), these properties were examined in tubulin liganded to ALLO or TCB. Figure 8 shows the far-ultraviolet circular dichroism spectra of tubulin in the absence (tracing a) and in the presence (tracings b and c) of the respective ligands and the calculated difference spectra (tracings d and e). In both cases, there is a small but significant increase in

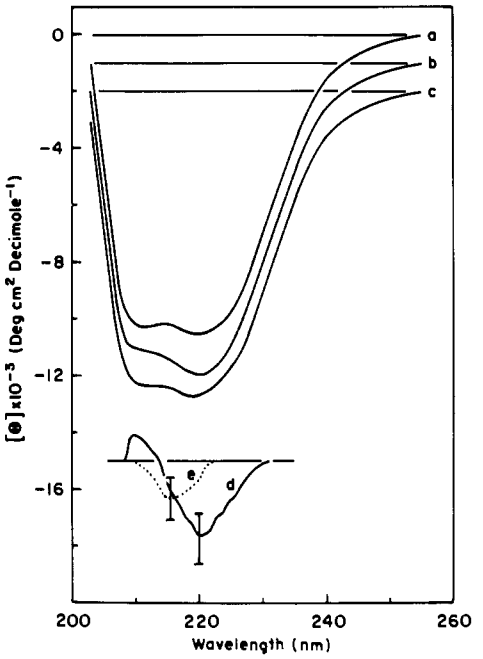


FIGURE 8: Effect of ligands on the circular dichroism spectra of tubulin, in PG buffer, pH 7.0 at 24 °C. (a) Spectrum of $(1.88–2.45) \times 10^{-5}$ M tubulin; (b) spectrum of 2.45×10^{-5} M tubulin and 2.99×10^{-5} M ALLO; (c) spectrum of 1.88×10^{-5} M tubulin and 1.96×10^{-5} M TCB. Each spectrum is the average of duplicate samples. Tracings d and e are the result of subtracting (a) from (b) and (c), respectively, amplified 5 times.

negative ellipticity similar to that observed in the presence of colchicine. For ALLO the ellipticity increment had a magnitude of −530 ± 180 deg cm^2 dmol^{-1} near 220 nm, and for TCB its magnitude was −260 ± 150 deg cm^2 dmol^{-1} near 217 nm. The ratio [Θ](220)/[Θ](210) changed from 1.034 ± 0.010 for unliganded tubulin to 1.088 ± 0.009 and 1.053 ± 0.009 in the presence of ALLO and TCB, respectively, and the negative peak at 220.5 nm was shifted to 219 nm. These small changes are very similar to those previously reported for tubulin liganded to colchicine (Andreu & Timasheff, 1982c), to MTC (Andreu et al., 1984), and to tropolone methyl ether (Andreu & Timasheff, 1982c).

Both ALLO and TCB elicited a GTPase activity in tubulin. The maximal rates obtained at 37 °C on addition of 100 μM ligand to 18 μM protein in PG buffer and 16 mM $MgCl_2$, pH 7.0, containing 1% dimethyl sulfoxide were 0.012 and 0.0084 mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1} for ALLO and TCB, respectively. The value for colchicine at identical conditions was 0.015 mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1} both in the presence and in the absence of 1% dimethyl sulfoxide. The enzyme activities induced in tubulin by the binding of ALLO and TCB were reproducibly 80% and 56% of that induced by colchicine. These somewhat lower values are consistent with the progressively weaker binding of the two analogues to tubulin.

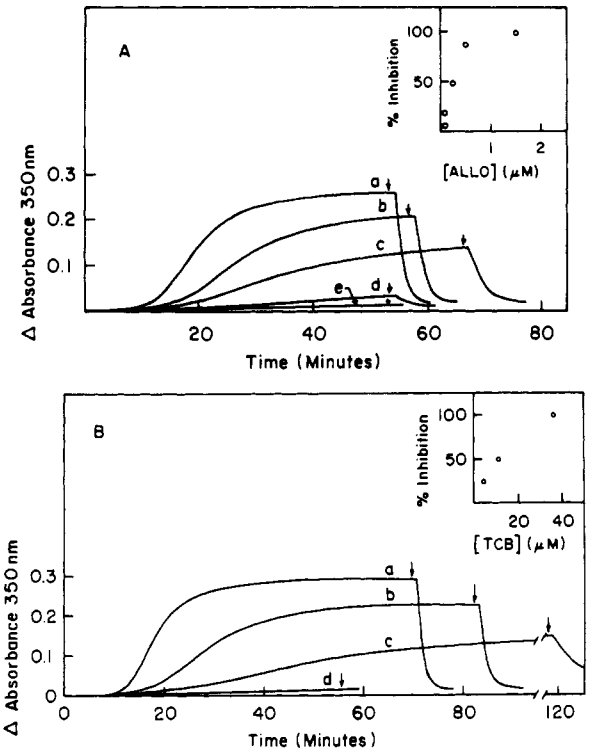


FIGURE 9: Effects of ALLO and TCB on the turbidity time course of the in vitro microtubule assembly. The reaction was started by warming the solution in the assembly buffer from 10 to 37 °C; the arrows indicate cooling of the samples. (A) (a) 1.12×10^{-5} M tubulin; (b–e) same, with 10^{-7} M, 2.5×10^{-7} M, 5.0×10^{-7} M, and 1.6×10^{-6} M ALLO. (B) (a) 1.03×10^{-5} M tubulin; (b–d) same, with 4.25×10^{-6} M, 1.11×10^{-5}, and 3.57×10^{-5} M TCB. The insets show the percentage of inhibition as a function of ligand concentration. The critical concentration of microtubule assembly under these conditions was 0.4 ± 0.1 mg/mL tubulin.

The effects of ALLO and TCB binding on tubulin self-association reactions were also examined; 1.41×10^{-4} M ALLO or 1.40×10^{-4} M TCB did not induce any significant changes in the sedimentation velocity of 10.28 or 7.76 mg/mL tubulin, respectively, in PG buffer, pH 7.0 at 26 °C. This indicates the absence of ligand-induced tubulin self-association under these conditions, which is in agreement with the observation that the ligand binding isotherms are independent of protein concentration (see above).

Substoichiometric amounts of ALLO were found to inhibit the in vitro microtubule assembly. As shown in Figure 9A, the turbidity generated by the self-assembly of 10^{-5} M pure tubulin was reduced by 50% by 2.5×10^{-7} M ALLO, similar to its inhibition of the polymerization of mouse brain supernatant (Fitzgerald, 1976). TCB also inhibited microtubule assembly (Figure 9B), but its concentration had to be of the same order of magnitude as that of tubulin because of the weaker binding. This was verified by measuring the binding of TCB to unassembled tubulin in parallel experiments in PG and assembly buffers at 25 °C at a protein concentration of 2×10^{-6} M. The values of the parameters obtained, $K_b = 0.90 \times 10^5$ M^{-1} and 0.92×10^5 M^{-1} and $n = 1.05$ and 1.15 for PG and assembly buffers, respectively, indicated that the strength and extent of binding had not been changed by the assembly conditions. The morphology of the polymerization products under inhibition conditions was indistinguishable from that of the microtubule controls without drugs, as viewed by electron microscopy (not shown). An excess of these ligands (7.5×10^{-5} M) induced an increase in turbidity in PG buffer and 16 mM $MgCl_2$, pH 7.0, when the solution was heated to 37 °C, as shown in Figure 10, but no microtubules were seen

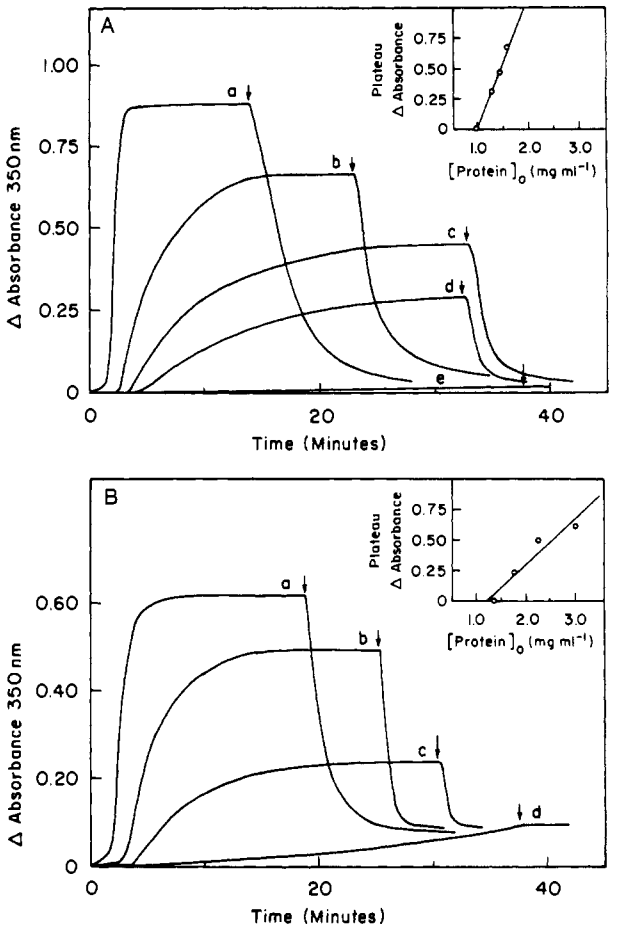


FIGURE 10: Turbidity time course of the abnormal polymerization of tubulin induced by ALLO and TCB in PG–16 mM $MgCl_2$ buffer, pH 7.0. The reaction was started by warming the solution from 10 to 37 °C; the arrows indicate cooling of the samples. (A) Polymerization of tubulin induced by 7.5×10^{-5} M ALLO. The concentrations of tubulin were (a–e), 3.15, 1.58, 1.42, 1.26, and 0.95 mg mL^{-1}. (B) Polymerization of tubulin induced by 7.5×10^{-5} M TCB. The concentrations of tubulin were (a–d) 3.00, 2.25, 1.78, and 1.34 mg mL^{-1}.

under the electron microscope. This polymerization was reversed by cooling to 10 °C. The process was characterized by a lag time and a critical concentration, characteristic of nucleated cooperative self-assembly (Oosawa & Asakura, 1975). The values of the critical concentrations measured, 1.0 and 1.2 mg/mL of tubulin, in the presence of ALLO and TCB, respectively, were similar to those found for colchicine and MTC (Andreu et al., 1983, 1984). The formation of such anomalous tubulin polymers by stoichiometric binding is characteristic for the complexation of tubulin with colchicine (Andreu & Timasheff, 1982c, 1983; Saltarelli & Pantaloni, 1982) and further supports the conclusion that both ALLO and TCB bind in the colchicine site on tubulin and mimic closely its behavior.

Discussion

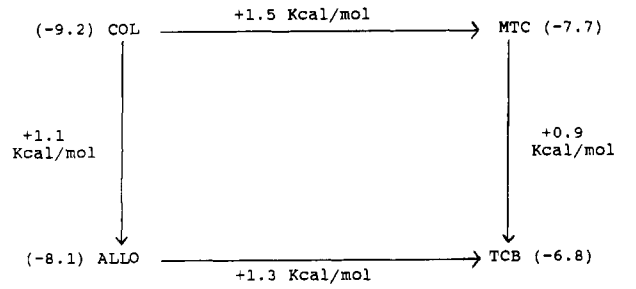
Binding to Tubulin of the Colchicine Analogues ALLO and TCB. The interactions of ALLO and TCB with tubulin have been firmly established by difference absorption spectroscopy, fluorescence, and equilibrium gel chromatography. Both inhibit the binding of colchicine, and the binding of both analogues is inhibited by colchicine and podophyllotoxin. It is possible to conclude, therefore, that both colchicine analogues in which the tropolone ring C had been transformed into a *p*-carbomethoxybenzene ring (ring C′) bind to the colchicine

binding site of tubulin. Since the trimethoxyphenyl ring (ring A of colchicine) is preserved in both ALLO and TCB and, therefore, interacts with the trimethoxyphenyl-specific subsite on tubulin, it is reasonable to conclude that the tropolone methyl ether binding subsite on tubulin can accommodate the carbomethoxy six-membered aromatic ring as well. That this is indeed so is clearly demonstrated by the fact that both ALLO and TCB induce in tubulin the same small change in CD at 217 nm as is induced by colchicine (Andreu & Timasheff, 1982c), which has been demonstrated to be a consequence of the binding of tropolone methyl ether to tubulin (Andreu & Timasheff, 1982c), while the binding of podophyllotoxin, which shares the trimethoxyphenyl ring with colchicine, had no effect on the protein CD. In the present study, both ligands with the modified ring C have been shown to induce in tubulin effects very similar to those that accompany colchicine binding, i.e., the perturbation of the protein circular dichroism, quenching of the intrinsic protein fluorescence, and generation of GTPase activity. It can be concluded, therefore, that the conformational state of tubulin liganded to ALLO or TCB is the same as that within the tubulin–colchicine complex and that this state is induced by the binding of the carbomethoxybenzene moiety of the drugs to the protein. This is supported by the finding that the stoichiometric binding of both ALLO and TCB induced, under microtubule-promoting conditions, the same reversible polymerization of tubulin into nonmicrotubule polymers as had been found for colchicine (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b; Andreu et al., 1983) and MTC (Andreu et al., 1984). Furthermore, both analogues with altered ring C inhibit microtubule assembly in vitro. The inhibition of ALLO is substoichiometric, just as for colchicine (Margolis & Wilson, 1977) and MTC (Andreu et al., 1984), while TCB is required at concentrations similar to that of tubulin, because its binding affinity for tubulin ($K_d = 10^{-5}$ M) is of the same order of magnitude as the protein concentration used in the experiments (ca. 10^{-5} M). Preliminary experiments, made at a tubulin concentration of 9×10^{-5} M, suggest stoichiometric inhibition by TCB.

Thermodynamic Consequences of the Modification of Ring C and the Role of Rings B and C. The binding of colchicine to tubulin has been interpreted in terms of a bifunctional ligand binding to two subsites on the protein (Andreu & Timasheff, 1982a). This model seems also to describe the bindings of ALLO and TCB to tubulin: both bind to the trimethoxyphenyl binding subsite on tubulin, as evidenced by podophyllotoxin inhibition, and both bind to the tropolone methyl ether subsite, as discussed above. What are the consequences of the structural departures of these two ligands from colchicine? This question seems addressed best by comparison of the four ligands, colchicine, ALLO, MTC, and TCB, given in Chart II. The first two are three-ring structures with different rings C, the second pair are derivatives of the first two from which ring B had been excised. Three striking results emerge: (i) Alteration of ring C to ring C′ increases the free energy of binding to tubulin by identical amounts ($\delta\Delta G^\circ = +1.0 \pm 0.1$ kcal/mol), whether the overall structure is composed of two or three rings. (ii) Elimination of ring B increases the free energy of binding by identical amounts ($\delta\Delta G^\circ = +1.4 \pm 0.1$ kcal/mol), whether ring C is a tropolone methyl ether or a *p*-carbomethoxybenzene. (iii) Elimination of ring B changes the binding kinetics from slow to rapid.

The first two results confirm the earlier conclusion (Andreu & Timasheff, 1982c) that the binding of the two ends of colchicine to tubulin are independent of each other and indicate

Chart II[a]



[a] The values in parentheses are the standard free energies of binding of the ligand to tubulin at 25 °C. Those for MTC, ALLO, and TCB have been measured directly. The value of $-\Delta G^\circ$ for COL has been calculated from the kinetics data of Garland (1978) and enthalpy values reported in the literature [see Andreu and Timasheff (1982a)]. The listed value (−9.2 kcal/mol) is the most probable value subject to the uncertainties of this calculation (±0.3 kcal/mol).

that ring B is not involved in the productive binding contacts with the protein. What is the role of ring B? Ring B confers rigidity on the ligand molecule. This has two consequences, one thermodynamic, the other kinetic. Thermodynamically, the rigidity of the ligand results in an increase in the negative free energy of binding as a consequence of the reduction of free rotation between rings A and C (or C′) relative to the two-ring structures. This eliminates the loss of rotational entropy associated with the immobilization of the two-ring structure upon binding, which makes a positive contribution to the standard free energy of binding, estimated to be 1–3 kcal mol^{-1} (Glasstone, 1940; Andreu et al., 1984). The thermodynamic role of ring B in the binding of colchicine to tubulin, therefore, appears to be totally nonspecific. It need not participate directly in the binding mechanism, but its role can be simply the reduction of the unfavorable entropy change that accompanies the freezing in one configuration in the binding site the two ends of the ligand that do the actual binding. This is fully supported by a calorimetric examination of the binding of MTC, ALLO, and colchicine to tubulin (Menendez, Laynez, Medrano, and Andreu, unpublished results). The second contribution of ring B is kinetic. It retards the binding process as followed by the development of fluorescence. Colchicine can assume four possible conformations, only one of which has been proposed as capable of forming the tight complex with tubulin (Detrich et al., 1981). Its binding to tubulin requires, therefore, a conformational rearrangement to the one that can fit sterically into the bidentate site on the protein (Detrich et al., 1981). Such processes may be slow (Kende et al., 1976). In the two-ring analogues, this process is fast due to the high degree of free rotation. Indeed, both two-ring analogues reach fluorescence equilibrium within seconds [see Figure 5 and Bane et al. (1984)], while colchicine and ALLO require minutes [see Figure 5 and Garland (1978)]. On the other hand, it is also conceivable that the ring-B substituent may constitute a transient steric impediment both to the approach of rings A and C to their binding subsites and to their dissociation from the protein.

The finding that the development of fluorescence in the presence of MTC and TCB is rapid need not necessarily mean that the complete binding process is also rapid. The binding of free tropolone methyl ether (ring C of colchicine) to tubulin is slow, and the extent of its inhibition of microtubule formation has been shown to be a function of the duration of tubulin preincubation with this ligand (Andreu & Timasheff, 1982c). Tropolone methyl ether is known to bind to the ring

C binding subsite on tubulin, yet its binding is accompanied neither by generation of ligand fluorescence nor by quenching of protein fluorescence. This suggests that slow steps, not detected by changes in fluorescence, may be present along the reaction pathway. This question is currently under investigation in our laboratories.

What is the role of ring C? The tropolone methyl ether ring of colchicine has been shown to bind to tubulin by a slow process and to generate a conformational change in the protein (Andreu & Timasheff, 1982a,c). A similar conformational change occurs on binding of ALLO or TCB, in which the structure of ring C had been altered (see Figure 8). The negative standard enthalpy change characteristic of the binding of tropolone methyl ether to tubulin (Andreu & Timasheff, 1982a) suggests hydrogen-bond formation between the oxygens of the ligand and pertinent donor groups in the protein binding site. Comparison between the binding affinities of the tropolone containing drugs (colchicine and MTC) and the carbomethoxybenzene analogues (ALLO and TCB) shows a decrease in binding affinity by 1 kcal/mol. Since the rest of the molecules have identical structures, this entire difference can be attributed to the binding in the ring-C protein subsite. The binding of tropolone methyl ether to tubulin proceeds with a standard free energy change, ΔG°, of -3.2 kcal mol^{-1}. When this is corrected for the cratic contribution, the intrinsic standard free energy change, ΔG°_{int}, of binding of the tropolone methyl ether moiety when it is part of the colchicine molecule becomes $\Delta G^\circ_{int} = -5.4$ kcal mol^{-1}. Then, when the structure is changed to ALLO, ΔG°_{int} of the binding of ring C′ is -4.4 kcal mol^{-1}. What is the source of this decrease in binding affinity? Rossi (M. Rossi, private communication) has computed the displacement of the ring C′ oxygens in ALLO and TCB relative to the spatial positions of the ring C oxygens of the three-dimensional structure of colchicine when the trimethoxyphenyl rings are superimposed exactly. For both analogues, the positions in space of each of the oxygens (carbonyl and methoxy, respectively) were displaced from those found in colchicine by 0.6–0.7 Å. If it is assumed that $\delta\Delta G^\circ$ is the consequence only of the change of the electrostatic component of the hydrogen bond due to an increased separation by 0.6–0.7 Å between the ligand oxygen and the proton-donating group in the protein, all other factors remaining the same, the ΔG°_{int} values would correspond to a change of this separation of from 2.6–3.0 to 3.2–3.7 Å. While these values are quite reasonable for hydrogen bonds, it must be emphasized that this calculation is only a gross qualitative estimate. It would not be meaningful to attempt any more rigorous calculations in the absence of knowledge of the exact contacts and the three-dimensional spatial configuration of the binding site. It is significant, however, that this small change in the spatial positioning of ring C′ relative to ring C is sufficient to abolish neither the specificity of binding nor the generation of a conformational change in the protein. On the other hand, the stacking of ring C with aromatic protein residues may be also contributing to the binding (Andreu & Timasheff, 1982c; Rava et al., 1987).

A final intriguing result is the difference between the kinetics of the development of fluorescence in the presence of colchicine and ALLO. With colchicine, the fluorescence changes with biphasic kinetics that have been interpreted as the parallel binding to two different tubulin conformers in slow equilibrium (Garland, 1978; Lambeir & Engelborghs, 1981). In the case of ALLO, the fluorescence develops with pseudo-first-order kinetics with an apparent rate constant of the order of magnitude of the less slow phase of colchicine binding. While no explanation can be given at present to this qualitative difference between the two processes, one possibility is that the binding of ALLO mediates shifts in both the equilibrium constant and the rate of the conformational conversion so that one tubulin conformer becomes predominant. Another possibility is that the observed fluorescence development corresponds to the colchicine slow phase, the less slow phase in the case of ALLO being of small amplitude and completed within a few seconds. The resolution of this question, however, will require further investigation.

Acknowledgments

We thank Kang-Min Lee for doing the GTPase activity measurements and Rebecca Meyers for her performance of the NMR and mass spectroscopy experiments.

Registry No. I, 64-86-8; II, 60423-21-4; III, 641-28-1; IV, 120666-35-5; GTPase, 9059-32-9; 1,2,3-trimethoxybenzene, 634-36-6; methyl *N*-nitroso-*N*-acetyl-4-aminobenzoate, 24355-33-7; methyl 4-iodobenzoate, 619-44-3; 1,2,3-trimethoxy-4-iodobenzene, 25245-37-8.

References

Adler, A. J., Greenfield, N. J., & Fasman, G. D. (1973) *Methods Enzymol. 27*, 675–735.

Andreu, J. M. (1985) *Methods Enzymol. 117*, 346–354.

Andreu, J. M., & Timasheff, S. N. (1981) *Arch. Biochem. Biophys. 211*, 151–157.

Andreu, J. M., & Timasheff, S. N. (1982a) *Biochemistry 21*, 534–543.

Andreu, J. M., & Timasheff, S. N. (1982b) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6753–6756.

Andreu, J. M., & Timasheff, S. N. (1982c) *Biochemistry 21*, 6465–6476.

Andreu, J. M., Wagenknecht, T., & Timasheff, S. N. (1983) *Biochemistry 22*, 1556–1566.

Andreu, J. M., Gorbunoff, M. J., Lee, J. C., & Timasheff, S. N. (1984) *Biochemistry 23*, 1742–1752.

Bane, S., Puett, D., MacDonald, T. L., & Williams, R. C., Jr. (1984) *J. Biol. Chem. 259*, 7391–7398.

Bhattacharyya, B., & Wolff, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2627–2631.

David-Pfeuty, T., Simon, C., & Pantaloni, D. (1979) *J. Biol. Chem. 254*, 11696–11702.

Deinum, J., Lincoln, P., Larsson, T., Lagercrantz, C., & Erkell, L.-J. (1981) *Acta Chem. Scand. Ser. B*, 677–681.

Detrich, H. W., III, Williams, R. C., Jr., MacDonald, T. L., Wilson, L., & Puett, D. (1981) *Biochemistry 20*, 5999–6005.

Fernholz, H. (1950) *Justus Liebigs Ann. Chem. 568*, 63–72.

Fitzgerald, T. J. (1976) *Biochem. Pharmacol. 25*, 1383–1387.

Garland, D. L. (1978) *Biochemistry 17*, 4266–4272.

Glasstone, S. (1940) *Textbook of Physical Chemistry*, p 875, Van Nostrand, Princeton, NJ.

Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta. 63*, 530–532.

Kende, A. S., Liebeskind, L. S., Kubiak, C., & Eisenberg, R. (1976) *J. Am. Chem. Soc. 98*, 6389–6391.

Kleiderer, E. C., & Adams, R. (1933) *J. Am. Chem. Soc. 55*, 4219–4225.

Lambeir, A., & Engelborghs, Y. (1981) *J. Biol. Chem. 256*, 3279–3282.

Lee, J. C., & Timasheff, S. N. (1977) *Biochemistry 16*, 1754–1764.

Lee, J. C., Frigon, R. P., & Timasheff, S. N. (1973) *J. Biol. Chem. 248*, 7253–7262.

Lee, J. C., Corfman, D., Frigon, R. P., & Timasheff, S. N. (1978) *Arch. Biochem. Biophys. 185*, 4–14.

Lehrer, S. S., & Fasman, G. D. (1966) *Biochem. Biophys. Res. Commun. 23*, 133–138.
Margolis, R. L., & Wilson, L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3466–3470.
Medrano, F. J., & Andreu, J. M. (1986) *Eur. J. Biochem. 158*, 63–69.
Medrano, F. J., Andreu, J. M., Gorbunoff, M. J., & Timasheff, S. N. (1986) presented at the 1 Reunión Española de Biofísica, Oct 1986, Sitges, Spain, Abstract EM-15.
Mertens, M. L., & Kagi, J. H. R. (1979) *Anal. Biochem. 96*, 448–455.
Oosawa, F., & Asakura, S. (1975) in *Thermodynamics of the Polymerization of Proteins*, Academic Press, London.
Rava, R. P., Hastie, S. B., & Myslik, J. C. (1987) *J. Am. Chem. Soc. 109*, 2202–2203.
Rondestvedt, C. S., Jr., & Blanchard, H. S. (1955) *J. Am. Chem. Soc. 77*, 1769–1774.
Saltarelli, D., & Pantaloni, D. (1982) *Biochemistry 21*, 2996–3006.
Weisenberg, R. C., Borisy, G. G., & Taylor E. (1968) *Biochemistry 7*, 4466–4479.
Wilson, L., & Bryan, J. (1974) *Adv. Cell. Mol. Biol. 3*, 21–72.

Topographical Characterization of the Domain Structure of the Bovine Adrenal Atrial Natriuretic Factor R_1 Receptor†

Bin Liu,‡ Sylvain Meloche,§ Normand McNicoll, Christine Lord, and André De Lêan*,‖
Laboratory of Molecular Pharmacology, Clinical Research Institute of Montreal, and Department of Pharmacology, University of Montreal, Montreal H2W 1R7, Canada

Received January 19, 1989; Revised Manuscript Received March 10, 1989

ABSTRACT: We have studied the structure and function of the membrane atrial natriuretic factor R_1 (ANF-R_1) receptor using limited proteolysis and exoglycosidase treatment. Limited digestion with trypsin of the receptor from bovine adrenal zona glomerulosa membranes resulted in the conversion of the native 130-kDa receptor into a single membrane-associated ANF-binding proteolytic fragment of 70 kDa. The 70-kDa fragment bound ANF with enhanced binding affinity but retained intact ANF-R_1 pharmacological specificity and was still sensitive to modulation by amiloride. Trypsin treatment of the membranes produced a dual effect on ANF binding. Low concentrations of trypsin ($\leq$25 μg/mg of protein) increased ANF binding while higher concentrations dose dependently reduced the binding of the hormone. The increase of ANF-binding activity was associated with the formation of the 70-kDa fragment while the loss of ANF binding paralleled the degradation of the 70-kDa fragment. Low concentrations of trypsin drastically decreased the ANF-sensitive guanylate cyclase activity of the membrane fraction. This loss of catalytic activity strongly correlated with the formation of the 70-kDa tryptic fragment. We also evaluated the effect of ANF binding on the susceptibility of the receptor to proteolytic cleavage. The occupied receptor exhibited a greater sensitivity to trypsin digestion than the unoccupied protein, consistent with the hypothesis that hormone binding induces an important conformational change in the receptor structure. On the other hand, the 70-kDa fragment was much more resistant to proteolysis when occupied by ANF, suggesting that the ANF-binding domain forms a very compact structure. Treatment of the membrane receptor with α-chymotrypsin and endoproteinase Glu-C also converted the native protein to a fragment of approximately 70 kDa. The exoglycosidase neuraminidase reduced by $\approx$4 kDa the molecular mass of both the intact receptor and the 70-kDa fragment, indicating that the fragment contains at least one oligosaccharide chain of complex type. These carbohydrate residues appear to reside in the close vicinity of the ANF-binding site as suggested by the inhibiting effect of wheat germ agglutinin on ANF binding. Taken together, these results demonstrate that the ANF-R_1 receptor is a transmembrane protein which consists of at least two functional domains: an extracellular ANF-binding domain and a cytoplasmic guanylate cyclase domain. Those two domains are separated by a protease-sensitive region, distal to the membrane-spanning sequence and which might be involved in receptor metabolism.

Atrial natriuretic factor (ANF)[1] elicits its potent vasodilatory, natriuretic, and diuretic properties by activating highly specific membrane receptors which have been identified in target tissues such as kidney (Napier et al., 1984; De Lêan et al., 1985), adrenal cortex (De Lêan et al., 1984), arteries (Napier et al., 1984; Hirata et al., 1984; Schenk et al., 1985a), and brain (Quirion et al., 1986; Saavedra et al., 1986). Results obtained from detailed pharmacological studies in cultured aortic cells (Leitman & Murad, 1986a; Leitman et al., 1986b;

† This work was supported by a grant from the Medical Research Council of Canada to the Multidisciplinary Research Group in Hypertension.
* Author to whom correspondence should be addressed.
‡ Permanent address: Cardiovascular Institute and Fu Wai Hospital, Beijing, China.
§ Recipient of a studentship from the Medical Research Council of Canada.
‖ Scientist of the Medical Research Council of Canada.

[1] Abbreviations: ANF, atrial natriuretic factor; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; ScGMP-TME, 2′-*O*-monosuccinylguanosine 3′,5′-cyclic monophosphate tyrosyl methyl ester; SDS, sodium dodecyl sulfate; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-ph enylalanine chloromethyl ketone; BS^3, bis(sulfosuccinimidyl) suberate; ED_{50}, 50% efficient dose.

0006-2960/89/0428-5599$01.50/0