Photoaffinity Labeling of Tubulin with (2-Nitro-4-azidophenyl)deacetylcolchicine: Direct Evidence for Two Colchicine Binding Sites†

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Received December 20, 1988; Revised Manuscript Received May 11, 1989

ABSTRACT: A new photoaffinity analogue of colchicine, (2-nitro-4-azidophenyl)deacetylcolchicine (NAPDAC), bound to two classes of sites on bovine renal tubulin and photolabeled both the α- and β-subunits. The apparent K_i for the photoaffinity analogue was 1.40 ± 0.17 μM (mean ± SD, n = 3) as measured by competition with [^{3}H]colchicine. Values of the apparent K_ds for the two sites, as measured by the direct binding of the [^{3}H]NAPDAC to tubulin, were 0.48 ± 0.11 μM and 11.6 ± 3.5 μM (mean ± SD, n = 6), and the corresponding stoichiometries of binding of the two sites were 0.25 ± 0.06 and 1.3 ± 0.4 mol/mol of tubulin (mean ± SD, n = 6). NAPDAC was a potent inhibitor of microtubule formation as detected by electron microscopy. When tubulin was photolabeled with NAPDAC at 25 °C, 15 ± 3 mol % (mean ± SD, n = 6) of the [^{3}H]NAPDAC was convalently bound to the α-subunit, and 67 ± 9 mol % (mean ± SD, n = 6) was covalently bound to the β-subunit. Since NAPDAC is a mixture of two interconvertible diastereomers, the photoincorporation of each was also examined. One diastereomer photolabeled both α- and β-tubulin; however, the other did not significantly photolabel either subunit. Tubulin photolabeled with NAPDAC (1:1 mole ratio) exhibited a 23% decrease in colchicine binding. Preblocking and prephotolysis experiments with colchicine, NAPDAC, or ANPAH-CLC [Williams et al. (1985) *J. Biol. Chem. 260*, 13794–13802] provided evidence for conformational changes in tubulin upon colchicine binding. Peptide maps of [^{3}H]NAPDAC-labeled α- and β-tubulin, using *Staphylococcus aureus* V8 protease, demonstrated the presence of NAPDAC in one peptide of the α-subunit and in five peptides of the β-subunit as detected by autoradiography. NAPDAC provides the first direct evidence for two colchicine binding sites on tubulin.

Knowledge of the location and properties of the colchicine binding site on tubulin is essential for understanding the interaction of colchicine with tubulin. We have initiated experimentation with photoreactive analogues of colchicine to study the colchicine binding site by photoaffinity labeling. [6-[(4-Azido-2-nitrophenyl)amino]hexanoyl]colchicine (ANPAH-CLC)[1] photolabels the primary colchicine binding site on the α-subunit of bovine renal tubulin with a stoichiometry of photoincorporation of 1 mol of ANPAH-CLC/mol of tubulin (Williams et al., 1985). More than 97% of the ANPAH-CLC is photoincorporated into the α-subunit, and the photolabeled tubulin exhibits a greater than 90% loss of colchicine binding activity (Williams et al., 1985). The distance between the photoreactive aryl azido moiety and the nitrogen on the B-ring of the colchicine moiety in ANPAH-CLC is about 20 Å (Williams et al., 1985). Studies by other investigators are in agreement that the α-subunit is the location of the primary colchicine binding site (Schmitt & Atlas, 1976; Serrano et al., 1984). However, the β-subunit has also been proposed as the high-affinity colchicine binding site (Luduena & Roach, 1981). To further study the colchicine binding site, we have synthesized a second photoaffinity analogue of colchicine, (2-nitro-4-azidophenyl)deacetylcolchicine (NAPDAC).[2] NAPDAC was synthesized with two primary goals in mind.

The first goal was to reduce the distance between the photoreactive aryl azido moiety and the B-ring nitrogen of the deacetylcolchicine moiety to 10 Å. This represents the shortest distance possible for an aryl azido photoreactive group without locating it on the A-ring of colchicine. The second goal was to examine the binding and photolabeling of tubulin by a colchicine analogue that had the carbonyl of the amide on the B-ring removed. This structural alteration makes NAPDAC more closely related to colcemid than to colchicine. Our preliminary studies with fluorescent and spin-label derivatives of colchicine have demonstrated that alterations in the B-ring substituents yield analogues that still bind very effectively to tubulin (Barnes et al., 1980, 1981). Recently, our approach has been affirmed by studies that suggest that the B-ring domain critically affects the kinetics of binding and the reversibility of the ligand–tubulin interaction (Bhattacharyya et al., 1986; Ray et al., 1981).

Furthermore, the *n*-acyl group on the B-ring appears to be required for enhanced fluorescence of the analogue in the

†Support from National Institutes of Health (Grants R01-GM-30665 and RR-08194 to R.F.W.) is gratefully acknowledged. Portions of this work are for partial fulfillment of the requirements for a doctoral degree (L.J.F.).

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[1] Abbreviations: NAPDAC, (2-nitro-4-azidophenyl)deacetylcolchicine; DAC, deacetylcolchicine; FNPA, 4-fluoro-3-nitrophenyl azide; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; K_d, apparent dissociation constant; K_i, apparent inhibition constant; ANPAH-CLC, [6-[(4-azido-2-nitrophenyl)amino]hexanoyl]-colchicine; CLC, colchicine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; GTP, guanosine 5′-triphosphate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DIAS1, NAPDAC diastereomer 1 (αR,7*S*); DIAS2, NAPDAC diastereomer 2 (αS,7*S*).

[2] Manuscript in preparation.

0006-2960/89/0428-8515$01.50/0

presence of tubulin (Bhattacharyya et al., 1986). The carbonyl may, through hydrogen bonding or other dipolar mechanisms, be assisting a conformational change in tubulin that leads to the reduction in the angle between the biaryl rings (Bane et al., 1984; Dietrich et al., 1981), thus promoting fluorescence. Consequently, analogues with *n*-acyl groups should be expected to interact differently with tubulin than analogues lacking the carbonyl.

Several previous studies have detected multiple colchicine binding sites on tubulin. We employed an equilibrium binding technique using a HPLC-modified Hummel–Dreyer method with [^{3}H]colchicine to detect two classes of colchicine binding sites (Williams et al., 1983). Ringel and Sternlicht (1984), using [^{13}C]colchicine, detected two different colchicine sites by NMR. Structurally related analogues of colchicine have also evidenced multiple binding sites. For example, Ray et al. (1984) found that colcemid bound to two classes of sites on tubulin. Deinum and Lincoln (1986) used a spin-labeled analogue of allocolchicine to detect two classes of sites by equilibrium EPR titration. In all of these studies, either equilibrium binding assays or B-ring-modified colchicine derivatives or a combination of both was necessary to detect the low-affinity binding site.

This report describes the binding, inhibition of polymerization, and the photolabeling of bovine renal tubulin by NAPDAC, a new photoaffinity analogue of colchicine. Limited proteolysis of tubulin photolabeled with NAPDAC and with ANPAH-CLC demonstrates that the two photoaffinity analogues interact with different sets of amino acids in tubulin. The results of the photolabeling and binding experiments with NAPDAC provide direct evidence for two classes of colchicine binding domains on tubulin. A preliminary account of part of this work has been reported (Floyd et al., 1985).

Experimental Procedures

Materials. [4-^{3}H]Colchicine (specific activity 7.0–8.1 Ci/mmol) was purchased from Amersham and unlabeled colchicine from Aldrich Chemical Co. Purity of [4-^{3}H]-colchicine was at least 98% as determined by thin-layer chromatography (TLC) on silica gel (EM Laboratories, Inc.) in chloroform/acetone/diethylamine (5:4:1) (Ertel & Wallace, 1970). [^{3}H]Deacetylcolchicine (specific activity 4.6 Ci/mmol) was prepared by custom labeling of deacetylcolchicine by Amersham, and it was purified as previously described (Williams et al., 1985). NAPDAC was synthesized by reacting 4-fluoro-3-nitrophenyl azide with deacetylcolchicine under conditions of phase-transfer catalysis as described elsewhere.[2] In all experiments, a mixture of NAPDAC diastereomers was used unless stated otherwise.

Ultrasphere C_{18} HPLC columns were supplied by ES Industries. All solvents for HPLC were HPLC grade, glass-distilled, and were purchased from Burdick and Jackson. 2-(*N*-Morpholino)ethanesulfonic acid (MES) and guanosine 5′-triphosphate (GTP) were supplied by Sigma. Sodium dodecyl sulfate (SDS) and 18-crown-6 ether were purchased from Pierce Chemical Co. and Aldrich Chemical Co., respectively. Arylamide and bis(acrylamide) (electrophoresis grades) were obtained from Polysciences, Inc. ME agarose was obtained from FMC Corp. *N,N,N′,N′*-Tetramethylenediamine (TEMED) (electrophoresis purity), ammonium persulfate, Coomassie brilliant blue R-250, and bromophenol blue were purchased from Bio-Rad. X-Omat AR photographic film was obtained from Eastman Kodak Co. Fluoro-Hance was purchased from Research Products International Corp. Beef kidneys were obtained fresh from a local slaughterhouse.

Synthesis of [^{3}H]NAPDAC. [^{3}H]DAC (5 mL, 2 mCi/mL) was placed in a vial equipped with a Teflon-faced septum. The solvent was evaporated to dryness under a stream of nitrogen. DAC (0.02 mmol), FNPA (0.24 mmol), 18-crown-6 ether (0.06 mmol), K_2CO_3 (0.09 mol), and 0.5 mL of benzene were added to the vial. The solution was kept at 80 °C with stirring for 16 h. The [^{3}H]NAPDAC diastereomers were isolated by preparative thin-layer chromatography as described for the purification of NAPDAC.[2] The [^{3}H]NAPDAC was purified to 99% by reverse-phase HPLC on a C_{18} Ultrasphere column with a mobile phase of methanol/water, 70:30, and a flow rate of 1 mL/min. The diastereomers, eluting at 12.8 and 18.9 min, were collected from 10 injections, combined, evaporated to dryness, and reinjected to check purity. The [^{3}H]NAPDAC mixture was dissolved in ethanol to give a concentration of 0.54 mM and a specific activity of 0.76 Ci/mmol. Separation of the two diastereomers of NAPDAC was accomplished under the same HPLC conditions as described above. Individual NAPDAC isomers were used in photolysis experiments immediately after their separation. The physical and chemical properties were identical with those described elsewhere.[2]

Preparation of Tubulin. Tubulin was purified from bovine renal medulla by four cycles of in vitro assembly and disassembly of microtubules (Barnes & Roberson, 1979). Renal medullary tubulin was polymerized in the presence of dimethyl sulfoxide and glycerol and did not contain microtubule-associated proteins. Renal medulla tubulin used for all of the binding and photoincorporation experiments was at least 95% pure as determined by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (Barnes & Roberson, 1979). Five different tubulin preparations were used in the present experiments.

Binding of NAPDAC and Colchicine to Tubulin. Binding of NAPDAC to tubulin was measured indirectly by competition with [^{3}H] colchicine. [^{3}H]Colchicine was incubated with tubulin (20 μg) in the absence and presence of NAPDAC in 50 mM sodium phosphate, 1 mM $MgSO_4$, and 0.1 mM EGTA, pH 6.8, for 3 h at 37 °C in a covered water bath. The assay volume was 200 μL, and the concentration of [^{3}H]-colchicine ranged from 0.1 to 5.0 μM with a specific activity of 0.45 Ci/mmol. Concentrations of NAPDAC ranged from 1.0 to 25.0 μM. The [^{3}H]colchicine–tubulin complex was isolated by retention on DEAE-cellulose paper disks, and the radioactivity of the complex was measured as previously described (Barnes & Roberson, 1979). Stock [^{3}H]colchicine was stored at −20 °C in ethanol and diluted into water for use in the assay. Concentrations of colchicine and NAPDAC were calculated from absorbances at 350 nm. The maximum concentration, 0.7% (v/v), of ethanol in the assay did not affect binding of [^{3}H]colchicine (Barnes & Roberson, 1979).

Binding of the photoaffinity analogue to tubulin was also measured directly by using [^{3}H]NAPDAC. The assay conditions and the separation of bound and free [^{3}H]NAPDAC were the same as in the competitive binding assay. The final assay concentrations of [^{3}H]NAPDAC were from 0.3 to 5.0 μM with a specific activity of 0.67 Ci/mmol.

The binding of [^{3}H]NAPDAC was inhibited by competition with colchicine. Concentrations of [^{3}H]NAPDAC were varied from 0.15 to 22.0 μM in the presence of 0 and 2.0 μM colchicine. The assay conditions and the separation of bound and free [^{3}H]NAPDAC are described above.

The assay incubation time of 3 h was chosen so decay of colchicine binding activity of tubulin in the presence of colchicine was less than 13% (Barnes & Roberson, 1979). Assays were done in duplicate or triplicate for each experiment; the

direct and competitive binding experiments were each done three times, and representative data are presented. The Scatchard-like plots (Scatchard, 1949) were analyzed by linear regression for each phase of the plot and corrected for overlap by solution of the appropriate simultaneous equations (Feldman, 1972). Data were also computer fit to a two-site model by using the programs of Yamoka et al. (1981).

Photolabeling of Tubulin with Photoaffinity Analogues. Tubulin was incubated with NAPDAC, ANPAH-CLC, or FNPA in 100 mM MES, 1 mM $MgSO_4$, 0.1 mM GTP, and 0.1 EGTA, pH 6.8, at room temperature or 4 °C for 15 min in a quartz test tube before photolysis. The solution was then irradiated for 1 h with a low-pressure Hg lamp in a Farrand Model A4 fluorometer equipped with a 420-nm cutoff filter. After photolysis, the irradiated solutions were dialyzed against the photolysis buffer at 4 °C for 20–24 h. All samples were protected from light when not being irradiated. UV/vis absorption spectra of the samples obtained before and after irradiation were measured with a Gilford Model 2600 XY or a Hewlett-Packard 8451 A spectrophotometer.

In some experiments the mixture of NAPDAC diastereomers were separated by HPLC using a Beckman Ultrasphere C_{18} 5-μm octadecylsilane column (4.5 mm i.d. × 25 cm). Immediately after separation and collection of the individual diastereomers, the solutions were frozen at −80 °C to minimize reequilibration. The solvent was removed under vacuum prior to the addition of tubulin and buffer. The solutions were photolyzed for 1 h at 25 °C as previously described. Tubulin photolabeled with each NAPDAC diastereomer was electrophoretically separated into α- and β-subunits as described below.

Electrophoretic Analyses. [^{3}H]NAPDAC-labeled tubulin was separated into α- and β-subunits by preparative SDS–acrylamide slab gels. Dissociated protein samples were analyzed on standard gels composed of 10% (w/v) acrylamide cross-linked with 0.08% (w/v) methylenebis(acrylamide) and containing 0.1% SDS, prepared according to Laemmli (1970) as modified by Studier (1983). A stacking gel of 3% (w/v) acrylamide and 0.08% (w/v) methylenebis(acrylamide) was used. Tubulin samples were reduced and carboxymethylated with sodium iodoacetate in 8 M urea (Hirs, 1967) to improve the electrophoretic separation of α- and β-tubulin (Little et al., 1981). Prior to electrophoresis the samples were dialyzed against 10 mM sodium phosphate, pH 7.2, and dissociated in 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol at 80–90 °C for 15 min. About 1 mg of photolabeled tubulin was subjected to preparative slab gel electrophoresis per analysis. The electrophoresis was conducted at approximately 25 °C at a constant current of 15 mA for migration through the stacking gel and 30 mA for migration through the separating gel.

After electrophoresis, the bands of α- and β-tubulin were visualized by immersion of the gel in 4 M sodium acetate (Higgins & Dahmus, 1979). The gel slices containing α- and β-tubulin were washed with water to remove sodium acetate and then placed in a fixing solution of methanol/acetic acid/water (5:1.5:3.5) overnight. The gel slices were again washed with water, and the tubulin subunits were electroeluted from the gel by using an ISCO electrophoresis concentrator, Model 1750. The inner buffer was 5 mM Tris-HCl, pH 8.7, and 0.1% SDS, and the outer buffer was 40 mM Tris-HCl, pH 8.7, and 0.1% SDS. The electroelution was conducted at 3 W for 3–4 h at 4 °C. After electroelution, the protein mass was measured by the method of Lowry et al. (1951), and the amount of [^{3}H]NAPDAC incorporated into each subunit was calculated from the specific activity.

Photolabeled tubulin was also separated into α- and β-subunits by using hybrid acrylamide–agarose tube gels (Horowitz et al., 1984). Hybrid acrylamide–agarose tube gels were prepared by casting a warm solution of 1% (w/v) ME agarose and 14.8% (w/v) acrylamide in 0.2 M Tris-HCl, pH 8.9, 10 mM EDTA, and 0.1% (w/v) SDS into 5 mm i.d. × 12 cm glass tubes. A stacking gel of 3% (w/v) acrylamide and 0.08% (w/v) methylenebis(acrylamide) was used. Protein samples were dissociated as previously described for SDS gels. Electrophoresis was conducted at approximately 25 °C at constant current of 1 mA per gel for migration through the stacking gel and 2 mA per gel for migration through the separating gel. Analytical gels were stained with Coomassie brilliant blue R-150 (Fairbanks et al., 1971). Radioactivity of tubulin covalently photolabeled with ANPAH-CLC or NAPDAC was quantitatively measured by slicing the gel into 1-mm segments immediately after electrophoresis. The segments were dissolved in 1 mL of water by heating at 85 °C for 1 h. Samples were counted in 12 mL of Safety Solve scintillation cocktail.

Proteolysis of [^{3}H]NAPDAC-Labeled α- and β-Tubulin. [^{3}H]NAPDAC-labeled tubulin was separated into α- and β-subunits by preparative slab gel electrophoresis. The subunits were subjected to limited proteolysis with *Staphylococcus aureus* V8 protease (Little et al., 1981) and separated into fragments by using a 12–20% (w/v) exponential gradient acrylamide gel cross-linked with 0.08% (w/v) methylenebis(acrylamide) and containing 0.1% SDS. A stacking gel of 3% (w/v) acrylamide and 0.08% (w/v) methylenebis(acrylamide) was used. Different masses of α- or β-tubulin with varied masses of protease were layered on the gel, and the electrophoresis was conducted at approximately 25 °C at a constant current of 15 mA for 30 min through the stacking gel. The electrophoresis was stopped for a 2-min digestion, and then electrophoresis was continued at a constant current of 30 mA for approximately 3 h for migration through the separating gel. The gel was fixed in methanol/acetic acid/water (5:1.2:3.8) and stained with Coomassie blue (Fairbanks et al., 1971). Slab gels were analyzed by fluorography (Bonner & Laskey, 1974) after treatment with Fluoro-Hance and drying on Whatman 3MM paper. Dried gels were placed in an X-ray film holder container preflashed Kodak X-Omat AR film. Films were exposed at −70 °C for 4–8 weeks.

Analysis of in Vitro Polymerization. Polymerization of purified bovine renal tubulin was measured by the scattering of light at 350 nm (Barnes & Roberson, 1979). Turbidity was detected with a Gilford Model 2600 spectrophotometer equipped with an internal cuvette heater thermostatically regulated at 37 °C and interfaced with a Hewlett-Packard 7225 B plotter. Tubulin, 0.8–1.2 mg/mL, was polymerized in 100 mM MES, pH 6.7, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP, and 10% (v/v) Me_2SO. Polymerization was initiated by the addition of Me_2SO and was monitored by the change in absorbance at 350 nm as a function of time. In some experiments colchicine or NAPDAC were added to final concentrations of 5 μM before the addition of Me_2SO.

At different times during polymerization, 5-μL samples were transferred from the cuvette to 200-mesh copper grids coated with formvar and carbon (Ted Pella, Inc.). The samples were stained with cytochrome *c* and 1% uranyl acetate (Barnes & Roberson, 1979; Olmsted & Borisy, 1973). The air-dired samples were examined with a Zeiss 9A electron microscope operated at 60 kV, 20 mA, and a 7K magnification.

Results

Binding of NAPDAC to Tubulin. NAPDAC behaved as an apparent competitive inhibitor of the binding of [^{3}H]-

Table I: Binding Parameters of NAPDAC, Colchicine, and ANPAH-CLC to Renal Tubulin[a]

ligand	n	n_1[b]	K_{d^1} (μM)	n_2	K_{d^2} (μM)
		Computer Fit			
NAPDAC	4	0.18 ± 0.07	0.24 ± 0.05	2.3 ± 0.2	32.0 ± 8.0
colchicine	3	0.29 ± 0.16	0.45 ± 0.03	ND	ND
ANPAH-CLC	3	0.36 ± 0.12	0.53 ± 0.21	ND	ND
		Resolved Scatchard-like Plot[c]			
NAPDAC	6	0.25 ± 0.06	0.48 ± 0.11	1.3 ± 0.4	11.6 ± 3.5
colchicine[d]	4	0.46 ± 0.04	0.42 ± 0.04	ND	ND
ANPAH-CLC	3	0.37 ± 0.05	0.52 ± 0.11	ND	ND

[a] The stoichiometries and apparent dissociation constants were obtained by computer fit of the data to a two-site model for NAPDAC and a one-site model for colchicine and ANPAH-CLC according to Yamaoka et al. (1981). n_1 and n_2 are stoichiometries of the high- and low-affinity sites, respectively, expressed as mol of ligand/mol of tubulin. K_{d^1} and K_{d^2} are corresponding apparent dissociation constants for the two sites. ND, not detectable. [b] Values are expressed as means ± standard deviations; n = number of determinations. [c] Parameters were calculated by resolution of the two components by solving simultaneous equations for the two lines for NAPDAC according to Feldman (1972). Parameters for colchicine and ANPAH-CLC were calculated from linear Scatchard-like plots. [d] Data from Barnes et al. (1983).

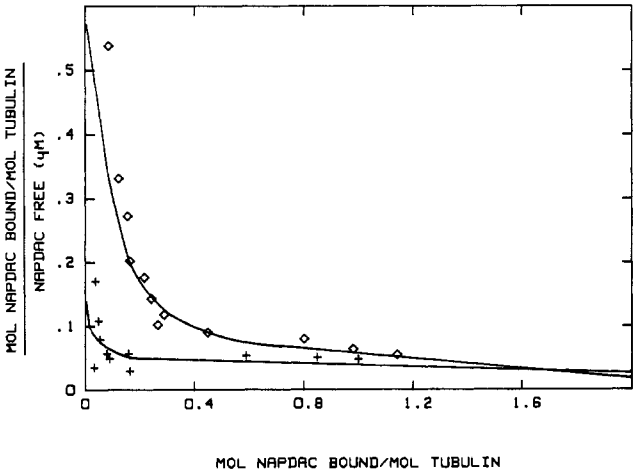


FIGURE 1: Colchicine inhibition of [^{3}H]NAPDAC binding to renal tubulin. Tubulin (0.1 mg/mL) was incubated at 37 °C for 3 h with NAPDAC concentrations ranging from 0.20 to 22.0 M with 0 μM (◇) and 2.0 μM (+) colchicine. The lines represent a computer fit of the data to a two-site binding model.

colchicine on the basis of both a linear double-reciprocal graphical analysis of the data and a modified Dixon plot (Dixon, 1953) of the reciprocal of the mass of colchicine bound as a function of the NAPDAC concentration (data not shown). The value of the apparent K_i calculated from the modified Dixon plot was 1.25 ± 0.2 μM (mean ± SD, n = 3), and that from the double-reciprocal analysis was 1.4 ± 0.17 μM (mean ± SD, n = 3) in the colchicine concentration range of 0.25–3.5 μM. Secondary plots of the apparent K_d for colchicine versus the concentration of NAPDAC and the slope of the primary double-reciprocal plot versus NAPDAC concentration were nonlinear, which indicated that the binding is not a simple bimolecular interaction (data not shown).

[^{3}H]NAPDAC bound to renal tubulin and exhibited apparent K_d values, calculated by linear regression of biphasic Scatchard-like plots for the two sites, equal to 0.48 ± 0.11 μM and 11.6 ± 3.5 μM (mean ± SD, n = 6) as shown in Figure 1. Corresponding stoichiometries for the two sites were 0.25 ± 0.06 and 1.3 ± 0.4 mol/mol of tubulin (mean ± SD, n = 6). The data for the direct binding of [^{3}H]NAPDAC to tubulin were also computer fit by using a two-site model with the program of Yamoka et al. (1981). The values obtained for the apparent dissociation constants were 0.24 ± 0.05 μM and 32 ± 8 μM (mean ± SD, n = 4). The corresponding stoichiometries for the two sites obtained from the computer fit were 0.18 ± 0.07 and 2.3 ± 0.2 mol/mol of tubulin (mean ± SD, n = 4). For comparison, the apparent dissociation constants and stoichiometries for both colchicine and ANPAH-CLC (Williams et al., 1985) were determined by computer fit of the data to one-site models and are given in Table I. The apparent dissociation constants and stoichiometries obtained for colchicine and ANPAH-CLC are in good agreement with those values obtained for NAPDAC at the high-affinity site.

The binding of [^{3}H]NAPDAC to tubulin was inhibited by competition with colchicine. Figure 1 shows the Scatchard-like plot for NAPDAC binding to tubulin in the presence of 0 and 2.0 μM colchicine. NAPDAC concentrations ranged from 0.2 to 22.0 μM. Colchicine inhibited the binding of [^{3}H]NAPDAC to tubulin at both sites. The stoichiometry of [^{3}H]-NAPDAC incorporation into tubulin was significantly decreased at both sites by the presence of colchicine such that the determination of accurate dissociation constants was difficult.

In Vitro Polymerization of Tubulin. Bovine renal tubulin was subjected to conditions for in vitro polymerization in the absence and presence of colchicine and NAPDAC. For the control polymerization, the increase in absorbance at 350 nm was about 0.24 after 15 min at 0.87 mg of tubulin/mL, and microtubules were detectable by electron microscopy. In the presence of 5 μM NAPDAC, the increase in absorbance at 350 nm after 15 min was about 0.18. But the absorbance increased linearly in the presence of NAPDAC in contrast to the asymptotic absorbance increase in the absence of NAPDAC. Electron micrographs indicated the presence of aggregates and a few, short microtubules in the presence of NAPDAC. In the presence of 5 μM colchicine, there was no change in absorbance at 350 nm after 15 min, and no microtubules were detectable by electron microscopy.

Photolabeling of Tubulin with [^{3}H]NAPDAC. Renal tubulin and [^{3}H]NAPDAC, at mole ratios of NAPDAC to tubulin from 0.2:1 to 2.8:1, were irradiated and subsequently dialyzed as described under Experimental Procedures. The stoichiometry of incorporation of [^{3}H]NAPDAC into tubulin as a function of the initial mole ratios is shown in Figure 2. A maximum of about 1.4 mol of NAPDAC was photoincorporated per mole of tubulin. When a mole ratio of NAPDAC to tubulin was 1:1 or less, the percent incorporation was 81 ± 7% (mean ± SD, n = 6) for the photoincorporations conducted at 25 °C.

The nonspecific photoincorporation of [^{3}H]FNPA was measured by photolabeling tubulin for 1 h at 25 °C with mole ratios of [^{3}H]FNPA to tubulin of 1:1 and 2.2:1. With a 1:1 molar ratio, 1% of the [^{3}H]FNPA was incorporated. With a 2.2:1 molar ratio, 2.7% of the [^{3}H]FNPA was incorporated. This percent incorporation was very low compared to the percent incorporation of NAPDAC. The nonspecific incorporation of [^{3}H]colchicine was also measured and found to be only 2–3% under conditions similar to those employed for

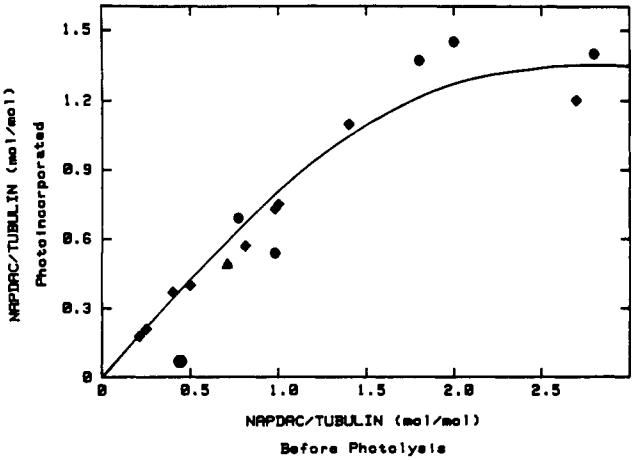


FIGURE 2: Stoichiometry of photoincorporation of [^{3}H]NAPDAC into tubulin. The initial mole ratio of [^{3}H]NAPDAC to tubulin was varied from 0.2 to 2.8 in 16 different experiments with three tubulin preparations. The tubulin and [^{3}H]NAPDAC solutions were irradiated for 1 h at either 25 °C (■) or 4 °C (●). In one experiment tubulin was photolabeled for 1 h at 25 °C with the separated NAPDAC stereoisomers: DIAS1 (●) and DIAS2 (▲).

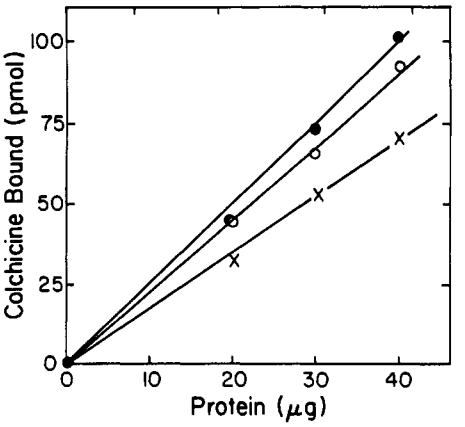


FIGURE 3: [^{3}H]Colchicine binding to tubulin photolabeled with NAPDAC. Tubulin was photolabeled with NAPDAC for 1 h at 4 °C. Mass of [^{3}H]colchicine bound is expressed as a function of mass of tubulin. Control tubulin (●); tubulin photolyzed without NAPDAC (○); tubulin photolyzed with NAPDAC (1:1 mol:mol) (×).

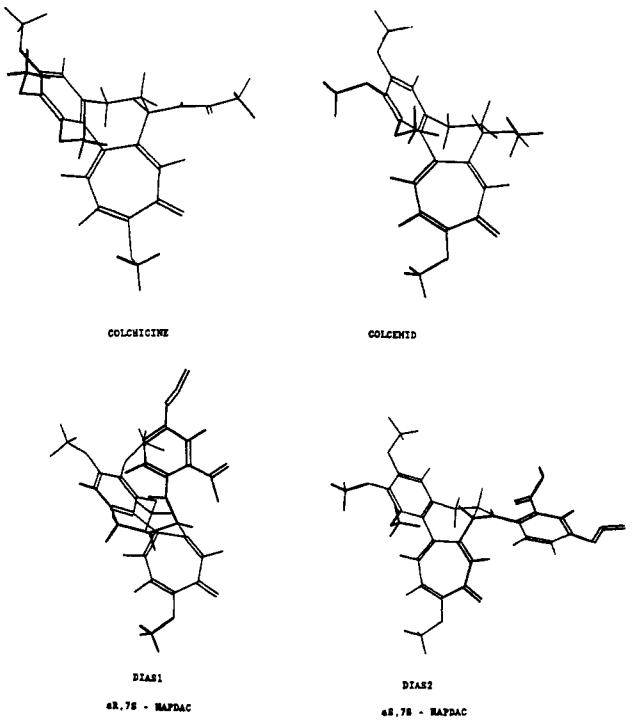
the photoincorporation of [^{3}H]NAPDAC and [^{3}H]ANPAH-CLC (Williams et al., 1985). Approximately 9 mol % of [^{3}H]colchicine could be incorporated into tubulin by using a high-intensity mercury lamp; however, the tubulin aggregated during the photolysis. Gel analysis of the photolyzed material showed nonspecific labeling, with neither the α nor β-tubulin remaining intact after photolysis.

Subunit Distribution of Photoincorporated [^{3}H]NAPDAC. To determine the subunit distribution of photoincorporated [^{3}H]NAPDAC, the tubulin was subjected to either preparative slab gel electrophoresis or hybrid acrylamide–agarose tube gels as described under Experimental Procedures. The subunit localization of [^{3}H]NAPDAC incorporation into tubulin was studied under various conditions. The effect of temperature, initial molar ratio of [^{3}H]NAPDAC to tubulin, and preincubation of [^{3}H]NAPDAC with tubulin were investigated.

Tubulin photolabeled at 4 °C incorporated 56 ± 7% (mean ± SD, n = 3) of the [^{3}H]NAPDAC. The photolabeled tubulin was found to contain the label distributed equally in both α- and β-subunits. Tubulin photolabeled at 25 °C incorporated 82 ± 7% (mean ± SD, n = 6) of the [^{3}H]NAPDAC. The modified tubulin contained 15 ± 3 mol % of the [^{3}H]NAPDAC in the α-subunit and 67 ± 9 mol % in the β-subunit. The percent distribution into α- and β-subunits was the same whether a 3-h 25 °C incubation of tubulin with [^{3}H]NAPDAC prior to photolysis or a protocol with no preincubation of tubulin with [^{3}H]NAPDAC was employed.

Photoincorporation of [^{3}H]NAPDAC Diastereomers. NAPDAC is a mixture of two diastereomers.[2] The mixture used in the experiments described above was found to contain a 17:83 ratio of diastereomer 1 (DIAS1) to diastereomer 2 (DIAS2) based on separation by HPLC.[2]. The structures of the two diastereomers are shown in Chart I. Tubulin photolabeled with the mixture of NAPDAC diastereomers was found to have 18 ± 3% of the incorporated [^{3}H]NAPDAC in the α-subunit and 82 ± 7% of the incorporated NAPDAC in the β-subunit. To determine if there was a differential labeling of tubulin by the individual diastereomers of NAPDAC, they were separated by HPLC prior to photolysis as described under Experimental Procedures. The concentration of each diastereomer was calculated by UV/vis absorbance at 350 nm assuming the extinction coefficient of each was the same as measured for the mixture. The incorporation of

Chart I



DIAS1 was 17%, and the incorporation of DIAS2 was 69%. Since the total incorporation of DIAS1 was 17% and because it comprised only 17% of the mixture of diastereomers, the contribution of DIAS1 to the incorporation of the mixture is less than 3% and therefore negligible. This low incorporation of DIAS1 probably represents nonspecific incorporation into tubulin. The [^{3}H]NAPDAC-photolabeled tubulin samples were separated into α- and β-tubulin by acrylamide agarose tube gels as previously described. The subunit distribution of DIAS2 exhibited the same trend as observed for the mixture: the majority of the label was incorporated into the β-subunit, but a significant amount was also incorporated into the α-subunit. DIAS1 showed a low and equal distribution into both subunits.

Colchicine Binding to Photolabeled Tubulin. Tubulin was photolabeled at 4 °C with NAPDAC at a mole ratio of 1:1 and dialyzed as described under Experimental Procedures. Control tubulin preparations were obtained by dialysis treatment alone and by photolysis without NAPDAC followed by dialysis. The capacity of each dialyzed tubulin preparation

Table II: Photoincorporation and Subunit Distribution of [^{3}H]NAPDAC into Renal Tubulin That Has Been Preincubated with a Ligand[a]

preincubn [analogue/tubulin (mol/mol)]	photolysis temp (°C)	[^{3}H]NAPDAC/tubulin (mol/mol)		total incorpn (mol %)	subunit incorpn			
		initial	incorpd		NAPDAC/α-tubulin mol/mol	NAPDAC/α-tubulin mol %	NAPDAC/β-tubulin mol/mol	NAPDAC/β-tubulin mol %
[^{3}H]NAPDAC	25	0.81	0.57	70	0.10	13	0.47	57
[^{3}H]NAPDAC	4	1.60	0.80	50	0.46	23	0.34	21
colchicine (0.84)	25	0.84	0.38	45	0.19	22	0.19	22
colchicine (0.78)	4	0.78	0.33	42	0.16	20	0.17	22

[a] Tubulin was preincubated for 3 h at 25 °C with [^{3}H]NAPDAC or colchicine. [^{3}H]NAPDAC was added if not initially present, and the solutions were irradiated for 1 h at the indicated temperatures.

Table III: Photoincorporation and Subunit Distribution of [^{3}H]NAPDAC-Labeled Tubulin That Has Been Prephotolabeled with ANPAH-CLC and of [^{3}H]ANPAH-CLC-Labeled Tubulin That Has Been Prephotolabeled with NAPDAC[a]

analogue	temp (°C)	prephotolyzed NAPDAC/tubulin or ANPAH-CLC/tubulin (mol/mol)	ligand/tubulin (mol/mol)		total incorpn (mol %)	incorpn (mol %)	
			initial ratio	incorpn ratio		α-tubulin	β-tubulin
[^{3}H]NAPDAC	25	none			81 ± 9[b]	15 ± 3[c]	67 ± 9[c]
	4	none			53 ± 3[d]	25 ± 3[d]	25 ± 4[d]
		ANPAH-CLC					
	25	0.81	0.82	0.31	38	23	15
	4	0.99	0.99	0.39	39	18	21
[^{3}H]ANPAH-CLC	25	none			68 ± 4[e]	65 ± 4[e]	2 ± 3[e]
		NAPDAC					
	25	0.82	0.82	0.40	49	43	6

[a] Tubulin was photolabeled at the indicated temperatures for 1 h with ANPAH-CLC or NAPDAC and then photolabeled for 1 h with [^{3}H]-NAPDAC or [^{3}H]ANPAH-CLC, respectively. [b] Mean ± SD, $n = 8$. [c] Mean ± SD, $n = 6$. [d] Mean ± SD, $n = 3$. [e] Mean ± SD, $n = 5$.

to bind [^{3}H]colchicine was measured as a function of the tubulin mass. The results are shown in Figure 3. Tubulin photolabeled with NAPDAC lost 23% of the colchicine binding capacity compared to the control tubulin preparation. In addition, irradiation of tubulin alone did not significantly affect its colchicine binding capacity.

Preblocking Experiments. Photoincorporation of ANPAH-CLC into tubulin specifically labels the α-subunit of renal tubulin (Williams et al., 1985). NAPDAC was photoincorporated into both the α- and β-subunits. Because the subunit labeling patterns of these two photoaffinity derivatives are different, the following preblocking experiments were done.

Preincubation of tubulin with ANPAH-CLC at 25 °C and a ratio of 0.92 (ANPAH-CLC to tubulin) reduced the amount of [^{3}H]NAPDAC incorporated into tubulin by 23% compared to tubulin photolabeled with [^{3}H]NAPDAC in the absence of ANPAH-CLC. When tubulin was preincubated with ANPAH-CLC, the subunit distribution of [^{3}H]NAPDAC was 32 mol % into α-tubulin and 30 mol % into β-tubulin. Preincubation with colchicine reduced the amount of [^{3}H]-NAPDAC photoincorporated by 44% at both 25 and 4 °C (Table II). When tubulin was preincubated with colchicine, the subunit distribution of [^{3}H]NAPDAC into α- and β-tubulin was essentially identical at either 4 or 25 °C as shown in Table II. This is compared to the incorporation of [^{3}H]-NAPDAC in the absence of either ANPAH-CLC or colchicine, which results in incorporation of 15 mol % into α-tubulin and 67 mol % into β-tubulin (Table III).

To prevent the possible exchange of the preblocking compound with [^{3}H]NAPDAC during the photolysis, tubulin was prephotolyzed with unlabeled ANPAH-CLC and then photolyzed with [^{3}H]NAPDAC at both 4 and 25 °C (Table III). The reverse was also done, where tubulin was prephotolyzed with NAPDAC and then photolyzed with [^{3}H]ANPAH-CLC. When tubulin was prelabeled with NAPDAC, the amount of [^{3}H]ANPAH-CLC incorporated was reduced 28%. The subunit distribution of [^{3}H]ANPAH-CLC is shown in Table III. When tubulin was prelabeled with ANPAH-CLC, the amount of [^{3}H]NAPDAC incorporated was reduced 53%. The subunit localization in the prephotolysis experiments for 4 and 25 °C is shown in Table III.

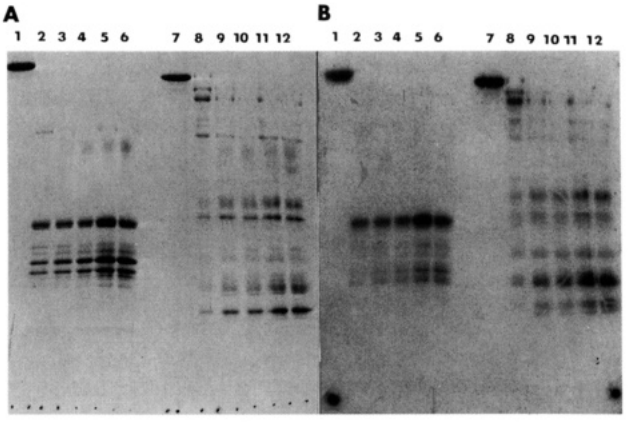


FIGURE 4: Proteolysis of [^{3}H]NAPDAC labeled α- and β-tubulin. Tubulin was photolabeled with [^{3}H]NAPDAC and separated into α- and β-subunits by preparative SDS–polyacrylamide gel electrophoresis. The individual subunits were subjected to limited proteolysis with *S. aureus* V8 protease. (Panel A) Coomassie stained; (panel B) corresponding autoradiogram. (Lane 1) 10 μg α-tubulin; (lane 2) 10 μg α-tubulin + 0.02 μg protease; (lane 3) 10 μg α-tubulin + 0.1 μg protease; (lane 4) 10 μg α-tubulin + 0.2 μg protease; (lane 5) 20 μg α-tubulin + 0.1 μg protease; (lane 6) 20 μg α-tubulin + 0.2 μg protease; (lane 7) 10 μg β-tubulin; (lane 8) 10 μg β-tubulin + 0.02 μg protease; (lane 9) 10 μg β-tubulin + 0.1 μg protease; (lane 11) 20 μg β-tubulin + 0.01 μg protease; (lane 12) 20 μg β-tubulin + 0.2 μg protease.

Proteolysis of [^{3}H]NAPDAC-Photolabeled Tubulin and of [^{3}H]ANPAH-CLC-Photolabeled Tubulin. [^{3}H]NAPDAC- and [^{3}H]ANPAH-CLC-labeled tubulin samples were separated into α- and β-subunits by preparative SDS gel electrophoresis. The α- and β-tubulins were subjected to limited proteolytic digestion using *S. aureus* V8 protease. The resulting peptides were separated by using SDS gel electrophoresis. Figure 4 is the peptide map of [^{3}H]NAPDAC-labeled tubulin, and Figure 5 is the peptide map of [^{3}H]AN-

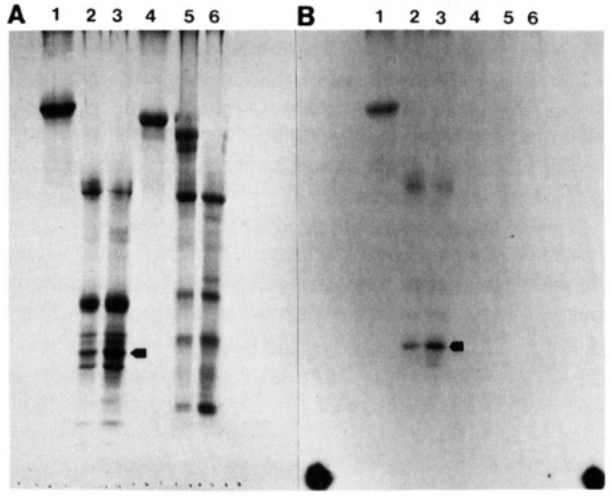


FIGURE 5: Proteolysis of [^{3}H]ANPAH-CLC labeled α- and β-tubulin. Tubulin was photolabeled with [^{3}H]ANPAH-CLC and separated into α- and β-subunits by preparative SDS–polyacrylamide gel electrophoresis. The individual subunits were subjected to limited proteolysis with *S. aureus* V8 protease. (Panel A) Coomassie stained; (panel B) corresponding autoradiogram. (Lane 1) 10 μg α-tubulin; (lane 2) 25 μg α-tubulin + 0.05 μg protease; (lane 3) 25 μg α-tubulin + 0.25 μg protease; (lane 4) 10 μg β-tubulin; (lane 5) 25 μg β-tubulin + 0.05 μg protease; (lane 6) 25 μg β-tubulin + 0.25 μg protease. The peptide labeled by ANPAH-CLC is indicated by the arrow in the Coomassie-stained gel (panel A) and in the autoradiogram (panel B).

PAH-labeled tubulin. NAPDAC photolabeled five peptides of the β-subunit (Figure 4, panel B, lanes 8–12). NAPDAC was found to photolabel one major peptide in the α-subunit (Figure 4, panel B, lanes 2–6) with minor amounts of the analogue in smaller peptides. ANPAH-CLC was also found to photolabel one major *S. aureus* V8 protease peptide in the α-subunit. The peptide labeled by ANPAH-CLC is indicated by the arrow in the Coomassie-stained gel (Figure 5, panel A) and in the autoradiogram (Figure 5, panel B). NAPDAC did not photolabel the same peptide in the α-subunit. ANPAH-CLC did not label the β-subunit [Figure 5 and Williams et al. (1985)].

Discussion

We conclude that NAPDAC binds and photolabels two classes of colchicine sites on tubulin, one on the α-subunit and one on the β-subunit. The two classes of sites were detected by direct binding of [^{3}H]NAPDAC, and colchicine inhibited the binding of NAPDAC to both sites. Conversely, NAPDAC inhibited the binding of colchicine to the high-affinity site, which is the one usually detected by using the standard filter assay for tubulin–ligand binding. These results point to the inherent problem associated with the standard filter assay technique for measuring colchicine binding or inhibition of colchicine binding by other ligands because the assay technique is based upon a kinetic event and does not measure equilibrium binding. The ability to detect both the high- and low-affinity sites with NAPDAC in the filter assay is due to the B-ring substituent of NAPDAC in comparison to the colchicine structure (Chart I). Nevertheless, the inhibition of NAPDAC binding to both sites by colchicine clearly indicates that the low-affinity site is a second colchicine binding site.

Binding Parameters. The data presented in Table I showed excellent agreement among the binding constants for NAPDAC, ANPAH-CLC, and colchicine. Previous studies that report two binding sites are in general agreement with the magnitude of binding constants obtained for NAPDAC (Deinum & Lincoln, 1986; Williams et al., 1983; Ringel & Sternlicht, 1984; Ray et al., 1984). The similarity of these binding constants for the three ligands at the high-affinity site (Table I) suggests that the binding domain on the α-subunit is the high-affinity site, with the binding domain on the β-subunit representing the low-affinity site. Renal tubulin photolabeled at 4 °C with a 1:1 mole ratio of ANPAH-CLC to tubulin had more than 90% of the label in the α-subunit and exhibited a greater than 90% loss of colchicine binding capacity (Williams et al., 1985) while tubulin photolabeled at 4 °C with NAPDAC under the same conditions lost about 23% of its colchicine binding capacity with 0.26 mol of NAPDAC incorporated per mole of α-subunit. Thus, the observed loss of colchicine binding activity is consistent with the location of the high-affinity site on the α-subunit. Furthermore, this assignment of the colchicine high-affinity binding site located on the α-subunit is in agreement with other reported studies (Schmitt & Atlas, 1976; Serrano et al., 1984).

A criticism of the previously employed photoaffinity analogue, ANPAH-CLC, was that the 20-Å distance between the aryl azido and deacetylcolchicine moieties might allow the ANPAH-CLC to bind to the β-subunit as a primary interaction site, yet span a cleft between the subunits, and thus photolabel the α-subunit (Roach et al., 1985). This argument is weakened by the fact that the six-carbon spacer is comprised of mobile carbon–carbon single bonds, and consequently, a very large number of conformations are accessible to the aryl azido moiety. If the colchicine binding site, assumed to be on the β-subunit, is close enough to the interface of the subunits for ANPAH-CLC to span a cleft and label the α-subunit, the conformational flexibility of ANPAH-CLC suggests that the β-subunit also be photolabeled; yet this was not observed (Williams et al., 1985). NAPDAC directly addresses this criticism by shortening the spacer chain to 10 Å, thus reducing the conformational mobility of the photoreactive group. The distance can be further reduced only by placing a photoreactive azido group directly on one of the rings of colchicine. However, aside from the stability considerations of azides, the structural requirements needed to ensure high-affinity binding are stringent. Modification of the A- and C-rings would likely yield poor binding analogues; however, the B-ring can be successfully modified without significant loss of binding activity (Zweig & Chignell, 1973; Fitzgerald, 1976; Cortese et al., 1977; Rosner et al., 1981). In addition, photolysis of an azido functional group, whether aryl, alkyl, or vinyl, located on one of the colchicine rings would require irradiation at wavelengths and intensities that would either convert the colchicine derivative into an inactive lumicolchicine form that would be rapidly released from the tubulin (Williams et al., 1985; Borisy et al., 1972; Amhein & Filner, 1973) or cause photodestruction of the protein. Both ANPAH-CLC and NAPDAC, as B-ring derivatives of colchicine, exhibit high-affinity binding characteristics to bovine renal tubulin that are similar to those of colchicine, itself, thus clearly indicating their specificity.

Specificity of NAPDAC/Tubulin Interaction. The covalent labeling of tubulin with the photoaffinity analogue NAPDAC on the α- and β-subunits provides the first direct, physicochemical evidence for two classes of colchicine binding sites. Several control experiments were done to rule out nonspecific photoincorporation. Both [^{3}H]fluoronitrophenyl azide and [^{3}H]colchicine were photoincorporated into tubulin at an insignificant level. Furthermore, photoincorporation of NAPDAC exhibited saturability.

The best evidence for specificity between NAPDAC and tubulin is obtained from experiments with the two diastereomers of NAPDAC. DIAS1 accounted for about 3% of pho-

toincorporated analogue when the isomeric mixture was used, and there was no preferential incorporation into either tubulin subunit. DIAS2 accounted for about 97% of the photoincorporated analogue, and there was a reproducible, specific distribution of DIAS2 into α- and β-tubulin. This specificity of incorporation of the two stereoisomers must be due to the conformations of the stereoisomers and clearly shows that incorporation of NAPDAC DIAS2 is not due to nonspecific interaction with tubulin.

NAPDAC Diastereomers. Structural representations of NAPDAC can be, in part, taken from crystal structure data of colchicine analogues (Margulis, 1974; Koerntgen & Margulis, 1977). Examination of molecular models [MM2(85); Burkert & Allinger, 1982] and analogy to the determined crystal structures indicate that the primary difference between the colcemid/NAPDAC and colchicine/ANPAH-CLC structures is the orientation of the carbonyl into the shallow cavity of the A- and C-rings with the alkyl moiety pointed away from the cavity for the colchicine-like compounds compared to the orientation of the aromatic (or methyl) moiety into that same cavity for the colcemid-like compounds (Chart I). Although the A- and C-ring methoxyls can experience a large number of conformational orientations, the remainder of the colchicine molecule is relatively rigid with the two nearly planar moieties (aryl and tropolone) twisted by approximately 50–60° from each other. All known derivatives (e.g., even isocolchicine) may be described in this manner (Margulis, 1974; Koerntgen & Margulis, 1977).

Consequently, the long spacer chain of ANPAH-CLC points to an entirely different region of the tubulin molecule than does NAPDAC. In fact, the geometric orientation of the azide of ANPAH-CLC is essentially orthogonal to the azide of NAPDAC. Therefore, they label different peptides; yet the α-subunit is clearly labeled by both ANPAH-CLC and NAPDAC. This argues strongly against the primary site on the β-subunit with the longer ANPAH-CLC reaching to α-subunit. Because of the rotational barrier exhibited by the NAPDAC diastereoisomers,[2] it is clear that the photoactivated nitrene is severely conformationally restricted and can only interact with a limited number of tubulin residues.

Molecular model representations of colchicine, NAPDAC, and the flattened conformation of colchicine according to Bane et al. (1984) and Detrich et al. (1981) show, as expected, that the internal rotation of both NAPDAC and colchicine is restricted by the B-ring. Since only four accessible conformations are available to the colchicine molecule (Margulis & Wilson, 1981), DIAS1 of NAPDAC must prevent the development of a productive conformation leading to binding. The planar positioning of the nitroaryl azido moiety relative to the trimethoxyaryl moiety probably prevents the dihedral angle between the biaryl rings from decreasing as observed when colchicine binds to tubulin (Bane et al., 1984). The existence of atropisomerism resulting from the rigid biaryl conformation of the phenyl–tropolone system in colchicine has been recognized for several years (Carfield et al., 1984). Recently, it has been shown that the required axial chirality of the biaryl ring system is the a*S* configuration (Yeh et al., 1988). The NAPDAC diastereomer designated DIAS2 (Chart I) is also the a*S*,7*S* configuration.[2] Consequently, the differential binding observed between NAPDAC DIAS1 and DIAS2 is consistent with and directly confirms the observation by Brossi (Yeh et al., 1988) that the structural and spatial requirements for productive binding of the colchicinoids include a critical dependence on the axial chirality of the biaryl ring system. Therefore, the stereochemical requirements of the colchicine binding domains are well established. However, we are not suggesting that colchicine and NAPDAC interact with tubulin in exactly the same way and to the same extent. The observations that B-ring analogues do not show fluorescence increases on binding to tubulin and that the kinetics of binding are more rapid and reversible (Bhattacharyya et al., 1986; Ray et al., 1981; Bane et al., 1984) clearly indicate that conformational changes in both the ligand and tubulin are part of the explanation of the binding phenomenon.

Photolabeling of Tubulin by NAPDAC. Values for the stoichiometry of NAPDAC in the α-subunit by photoincorporation and binding are reasonable in light of the data for colchicine binding and information on photolability with aryl azides and NAPDAC (Table I). The stoichiometry of binding for colchicine has historically been reported as varying between 0.1 and 1.0 depending upon the filter assay conditions, whether the tubulin–colchicine complex was isolated, and the source of tubulin (Zweig & Chignell, 1973; Patzett et al., 1975; Raybin & Flavin, 1977; Bhattacharyya & Wolff, 1974; Bryan, 1972; Cortese et al., 1977; Owellen et al., 1972). For colchicine, a stoichiometry of 0.46 was obtained by a Scatchard analysis and 0.29 by a computer fit to a one-site model (Table I). The stoichiometry of less than 1 may be due to several factors including the decay of the colchicine binding capacity of renal tubulin (Barnes & Roberson, 1979), the nature of the filter assay technique (Barnes et al., 1983), and tubulin microheterogeneity.

Colchicine has been shown to cause a conformational change in tubulin upon binding (Andreu & Timasheff, 1982; Garland, 1978; Lambeir & Engelborghs, 1981a,b; Detrich et al., 1981), and colchicine itself has been suggested to undergo a conformational change upon binding (Bane et al., 1984; Detrich et al., 1981). The lack of reversibility of colchicine binding at the high-affinity site has been attributed to the observed conformational changes (Andreu & Timasheff, 1982; Garland, 1978; Bane et al., 1984; Detrich et al., 1981). Furthermore, B-ring analogues of colchicine have been shown to bind more rapidly and reversibily than colchicine (Bhattacharyya et al., 1986). If NAPDAC prevents a conformational change in tubulin or if itself does not undergo the same conformational change as colchicine, then its rapid, reversible binding will produce the low stoichiometry of binding because of the inherent problems associated with the filter assay technique. Vinblastine binds to two sites on tubulin; however, the kinetic filter disk assay technique yields an observed stoichiometry of 0.45, which must be explained by invoking a rapid and reversible binding site (Wilson et al., 1975).

A secondary factor that can contribute to low stoichiometry for the photolabeled high-affinity site on the α-subunit is dependent upon lability of the bound NAPDAC. If the loss is selectively from the α-subunit due to the nature of the chemical linkage between tubulin and NAPDAC, the apparent stoichiometry in the α-subunit will be low. Partial loss of photoincorporated aryl azides has been reported (Okamoto & Yount, 1985). The nature of the covalent link between the NAPDAC and the protein has not yet been established. Recent studies have shown that 3- and 4-nitrophenyl azides produce four classes of reactive intermediates: singlet nitrene, dehydroazepine, triplet azide, and triplet nitrene (Liang & Schuster, 1986, 1987). The triplet nitro-substituted nitrenes are formed more efficiently and account for the majority of products when the nitro azides are irradiated. Liang & Schuster (1986, 1987) have demonstrated that 3- and 4-nitro-substituted azides will only be effective when there is a nucleophilic functional group at the targeted site. Even then,

covalent bond formation is inefficient because of the short lifetime of the singlet nitrene and dehydroazepine intermediates. NAPDAC may be effectively trapped, whereas another 3-nitroaryl azide might not, due to two factors. The first is the possible presence of a suitable nucleophilic group in close proximity to the photoactivated nitrene (e.g., singlet nitrene or dehydroazepine), and the second is partial stabilization of these intermediates since NAPDAC is an aminonitroaryl azide. Nevertheless, the low stoichiometries may result from inefficient covalent bond formation. Since the geometric constraints on NAPDAC are severe, the low stoichiometries observed may arise from triplet nitrene (Liang & Schuster, 1987) and its subsequent reaction to produce a nitroaniline that inhibits NAPDAC binding. This product can no longer photoincorporate, and consequently it would be lost in the dialysis/workup of the photolabeled material. Ongoing attempts to sequence NAPDAC-labeled peptide fragments, although exhibiting loss of label in the purification steps, will provide information in the nature of the covalent attachment to tubulin.

Preblocking or Prephotolysis Followed by NAPDAC Binding. Results summarized in Tables II and III clearly show significant reduction of NAPDAC incorporation when either a prephotolysis with NAPDAC or ANPAH-CLC or a preincubation with ANPAH or colchicine was performed. These data are consistent with and strongly support specificity of the NAPDAC interaction at the colchicine and ANPAH-CLC binding domains.

Further analysis of the tubulin subunit patterns was undertaken to start to acquire a more coherent picture of colchicine–tubulin interactions. Garland (1978) and Lambeir and Engelborghs (1981b) proposed the currently accepted two-step binding mechanism in which the stable tubulin–colchicine complex exists as a conformationally altered tubulin that is produced slowly and irreversibly. Detrich et al. (1981) have shown by circular dichroism that colchicine is conformationally altered upon interaction with tubulin due to a putative change in angle between the biaryl A- and C-rings. This leads to both a loss of the 340-nm circular dichroism band and promotion of fluorescence as shown by Bane et al. (1984). All studies have focused on the final stable tubulin–colchicine complex. Our results with preincubation and/or prephotolysis of ligands followed by photolysis of [^{3}H]NAPDAC does not probe the tubulin in that final complex form; rather, they provide the first direct evidence of intermediate tubulin conformations that are not identical with the stable tubulin–colchicine complex. This argument is based upon several observations: (1) [^{3}H]NAPDAC preincubation is not necessary since photoincorporation into tubulin is the same for no preincubation as observed for a 3-hour incubation, thus indicating a rapid and reversible interaction between NAPDAC and tubulin; (2) this same observation was made for the ANPAH-CLC interaction with tubulin (Williams et al., 1985); (3) NAPDAC inhibits polymerization of tubulin into normal microtubules and initiates aggregation of the protein; (4) NAPDAC DIAS1 does not show any significant incorporation into the α- or β-subunits of tubulin; and (5) preincubation and prephotolysis with different ligands alters the subunit distribution pattern when compared to the typical distribution of [^{3}H]NAPDAC (Tables II and III).

Preincubation with ANPAH-CLC or colchicine at either 4 or 25 °C or preincubation with NAPDAC at 4 °C shows essentially the same 1:1 subunit distribution of tubulin-incorporated NAPDAC. The incorporation into the α-subunit for all of these cases is slightly higher than observed for NAPDAC incorporation at 25 °C with a 3-h NAPDAC preincubation. Thus preincubation does not cause any substantial differential effect in either the α- or β-subunits with regard to NAPDAC photolysis. Therefore, the tubulin must be controlling the outcome of the binding interaction. This is consistent with the reduced binding that occurs with colchicine at 4 °C (Barnes et al., 1983). The tubulin–colchicine complex that is normally observed at 25 °C does not form under these conditions, and NAPDAC can successfully compete with colchicine. These results show that the conformation of tubulin cannot be the same at 4 and 25 °C. The slightly higher incorporation into the α-subunit may be due to several factors including a stabilization of the protein to decay. However, we suggest that the results indicate an intermediate conformation of tubulin that allows for a more favorable interaction with NAPDAC at the binding site on the α-subunit. Comparing the 25 °C data for NAPDAC to the 4 °C data (Table II), there is a decrease of approximately 33 mol % in the amount of total incorporation at 4 °C. The most significant reduction is the 48 mol % reduction of binding in the β-subunit. Taking into account that the α-subunit increased by 9 mol %, we can account for 42 mol % of the 48 mol % reduction in β-subunit incorporation. These data support the argument that we are looking at different conformations of the protein at the two temperatures, and their subunit labeling patterns reflect this reduction primarily in the β-subunit caused by a conformational alteration.

Preincubation of ANPAH-CLC at 25 °C causes a 21 mol % reduction in total photoincorporation of NAPDAC but an increase of 18 mol % in the α-subunit. This corresponds to a reduction of 39 mol % in the β-subunit. Again the data show a redirection of labeling pattern that can best be explained by a tubulin conformation that is somewhat different than that finally obtained by either colchicine or NAPDAC. Whether binding to the α-site induces a conformational change on the β-subunit or binding to the β-subunit induces the conformational change cannot be conclusively established from the present results; however, the former seems most consistent with the available data.

The prephotolysis experiments further support the proposed conformational changes in tubulin (Table III). When tubulin is prephotolyzed with NAPDAC prior to photolysis with [^{3}H]ANPAH-CLC, there is a clear reduction of about 20% in both the total mol % of ANPAH-CLC incorporated into tubulin and the amount of ANPAH-CLC label in the α-subunit. ANPAH-CLC has been shown to specifically label the α-subunit (Williams et al., 1985), and the decrease in the amount of ANPAH-CLC label corresponds to the value obtained for NAPDAC labeling of the α-subunit in the absence of ANPAH-CLC.

Prephotolysis with ANPAH-CLC at either 4 or 25 °C would be expected to block the α-subunit exclusively. However, this is not observed. As shown in Table III, significant decreases of total incorporation of [^{3}H]NAPDAC are observed at both 4 and 25 °C, yet the β-subunit incorporation is most strongly affected. The α-subunit incorporation is at the same level as observed previously for the preincubation experiments. These observations most strongly argue for conformationally altered tubulin that affects subunit distributions. After ANPAH-CLC is photoincorporated, the tubulin establishes a conformation that is different from the initial tubulin state, yet it is not the same as the colchicine–tubulin complex that is normally isolated. Due to the long spacer an ANPAH-CLC the tropolone moiety can dissociate from the α-site according to its equilibrium characteristics. This would allow NAPDAC

to bind and be photoincorporated into the α-subunit as if ANPAH-CLC was not present. The kinetic photolysis would compete successfully with the equilibrium of ANPAH-CLC binding at the α-site. Consequently, it is expected and observed that the NAPDAC labeling would be similar to the preincubation results since the ANPAH-CLC has produced a tubulin conformation that provides for better incorporation into the α-subunit. Clearly the conformational change alters the distribution pattern to prevent β-subunit incorporation. The 4 °C prephotolysis of ANPAH-CLC simply shows a decrease in the α-subunit labeling because the conformational mobility of the tubulin has been further restricted by the temperature reduction.

Limited Proteolysis of Labeled Tubulin. Only one peptide from the α-subunit was labeled for both the NAPDAC and ANPAH-CLC treated tubulin, and the labeled peptides were different as predicted from the different geometric orientation of the azides. Five labeled peptides were detected in the β-subunit of NAPDAC-labeled tubulin. This result is compatible with the binding and photolabeling data that suggest the presence of two low-affinity sites and the known reactivity of photoactivated aryl azido compounds (Chowdry & Westheimer, 1979).

Colchicine Binding Sites on Tubulin. Coupling the available information on ANPAH-CLC and other B-ring-modified colchicine analogues with the stereochemical information obtained from NAPDAC diastereomers allows a simplified representation of the high-affinity site for colchicine on the α-subunit to be approximated. Upon initial interaction of colchicine, ANPAH-CLC, or NAPDAC-DIAS2 with tubulin, the conformation of tubulin is altered to optimize binding. NAPDAC-DIAS1 cannot effectively bind to the tubulin because of unfavorable steric interactions.

The stable tubulin–colchicine complex, normally observed, is apparently caused by a further conformational change in tubulin and probably the ligand. This secondary conformational change may be assisted by a carboxyl group at the B-ring amide through a hydrogen bond or other dipolar interaction. In the absence of such a secondary conformational change, the binding of a NAPDAC-DIAS2 or other ligand at the α-tubulin binding domain is rapid and reversible, yet the interaction still causes strong inhibition of microtubule formation. Binding of ligands to the β-site is diminished when ligands interact at the α-site. The converse is also true and reflects a high degree of conformational mobility that is likely to be connected to tubulin function.

ACKNOWLEDGMENTS

We thank Dr. Stuart Birnbaum for assistance with electron microscopy measurements, Connie L. Anderson for preliminary work with binding assays and NAPDAC separation procedures, and Ron Risinger for expertise with tubulin polymerization experiments.

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cDNA Cloning and Predicted Amino Acid Sequence of *Glycera dibranchiata* Monomer Hemoglobin IV[†,‡]

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Received April 14, 1989; Revised Manuscript Received June 16, 1989

ABSTRACT: The three major monomer hemoglobins from *Glycera dibranchiata* erythrocytes isolated in this laboratory were sequenced from their N-termini. A stretch of amino acid sequence identity was used to determine the sequence of a mixed oligodeoxynucleotide that would be complementary to all 12 possible mRNA sequences coding for the amino acids. A cDNA library was constructed by using poly(A+) RNA from *G. dibranchiata* erythrocytes, the library was probed with the oligonucleotide, and the longest positive inserts found were subcloned into a sequencing plasmid and then sequenced. The first one was 745 bases long, containing 85 bases of 5′-untranslated RNA, an open reading frame of 444 bases coding for 148 amino acids, and a 3′-untranslated region of 216 bases. The predicted amino acid sequence matches the first 25 amino acids of *G. dibranchiata* monomer globin component IV. The sequence contains an N-terminal methionine plus 18 other mostly conservative sequence changes compared to the published sequence of Imamura et al. (1972), which appears from our partial sequencing to be monomer globin component II. We confirm the presence of leucine in the E7 position, which is histidine in most myoglobins and hemoglobins.

The marine bloodworm *Glycera dibranchiata* has nucleated erythrocytes that contain hemoglobins separable on Sephadex chromatography into a polymeric fraction and a monomeric fraction (Vinogradov et al., 1970). Crystals obtained from this monomeric fraction led to partial (Li & Riggs, 1971) and complete amino acid sequences (Imamura et al., 1972) and a 2.8-Å crystal structure (Padlan & Love, 1974) that showed an unusual substitution at position E7 in the ligand binding pocket. The almost universally found histidine was replaced by leucine. Subsequently, we used CM-cellulose chromatography to reproducibly separate the monomer hemoglobin fraction into two minor components (Ia and Ib) and three major components (II–IV), all with molecular weights of approximately 16 kDa (Kandler & Satterlee, 1983; Kandler et al., 1984; Constantinidis & Satterlee, 1987; Constantinidis et al., 1989). Isoelectric focusing confirms the high purity of our preparations of these proteins (Constantinidis & Satterlee, 1987; Constantinidis et al., 1989).

Recent progress in characterizing the three major monomeric hemoglobins has focused on ligand binding kinetics since that is the function of these proteins. With available instrumentation, we have found that each of the monomer methemoglobins binds cyanide at a rate several orders of magnitude slower than those of metmyoglobin and other monomeric ferriheme proteins (Mintorovitch & Satterlee, 1985, 1987, 1988; Mintorovitch et al., 1989). Such slow rates have led to significantly revised estimates of the equilibrium constant for cyanide binding. These anomalous dynamics, in conjunction with limited structural and spectroscopic data (Satterlee, 1984; Cooke & Wright, 1985, Cooke et al., 1987), imply that all three major monomer hemoglobin components

[†] This work was supported by National Institutes of Health Grants AM30912 and HL01758 (Research Career Development Award) to J.D.S.

[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02873.

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0006-2960/89/0428-8525$01.50/0