Identification of the Cysteine Residue of β-Tubulin Alkylated by the Antimitotic Agent 2,4-Dichlorobenzyl Thiocyanate, Facilitated by Separation of the Protein Subunits of Tubulin by Hydrophobic Column Chromatography

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ABSTRACT: The mechanism of action of the antimitotic drug 2,4-dichlorobenzyl thiocyanate (DCBT) has been examined in detail. Shown in previous studies to inhibit tubulin polymerization [Abraham, I., Dion, R. L., Duanmu, C., Gottesman, M. M., & Hamel, E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6839–6843] and to form a covalent bond preferentially with β-tubulin [Bai, R., Duanmu, C., & Hamel, E. (1989) *Biochim. Biophys. Acta 994*, 12–20], DCBT has now been documented to interact at low concentrations with a high degree of specificity at cysteine residue 239 of β-tubulin. These low DCBT concentrations also result in the partial inhibition of tubulin polymerization. Such findings strongly indicate that cysteine-239 of β-tubulin is essential for microtubule assembly. Although α-tubulin is alkylated almost as well as β-tubulin when the drug:tubulin ratio = 5:1 (Bai et al., 1989), β-tubulin is alkylated about 25 times as extensively as α-tubulin, almost exclusively at Cys-239, when the drug:tubulin ratio = 1:5. In addition, we find that low concentrations of DCBT do not affect the binding of colchicine to tubulin but that colchicine and related compounds do reduce the alkylation of tubulin by DCBT. This suggests that Cys-239 of β-tubulin is not involved in the binding of colchicine to tubulin but that this amino acid residue is at least partially masked by the drug when it is bound to the protein. We also describe a column chromatography procedure (hydrophobic chromatography on decylagarose) useful for the preparative resolution of unalkylated, although denatured, α- and β-tubulin.

Antimitotic drugs are of great interest both for potential practical uses, such as cancer chemotherapy, and as tools to understand the structure and function of microtubules. Our laboratory has been examining 2,4-dichlorobenzyl thiocyanate (DCBT;[1] NSC 145813; structure in Figure 1), a cytolytic agent with antineoplastic properties which causes cells to accumulate in metaphase arrest (Abraham et al., 1986; Bai et al., 1989).

Initial studies (Abraham et al., 1986) demonstrated that multi-drug-resistant cell lines retained their sensitivity to DCBT and that the interphase microtubule network of Chinese hamster ovary (CHO) cells disappeared following DCBT treatment, being replaced by spiral aggregates containing tubulin. Further, CHO cell lines with a mutant gene for β-tubulin were resistant to DCBT, roughly in proportion to their content of the mutant gene (i.e., multiple gene copies enhanced resistance). DCBT inhibited in vitro tubulin polymerization at substoichiometric concentrations, but this inhibition required a drug–protein preincubation which was time, temperature, and concentration dependent.

Subsequently (Bai et al., 1989), we observed that DCBT formed a covalent bond with tubulin. The data, including the ability of dithiothreitol to release covalently bound drug, were most consistent with a reaction in which the 2,4-dichlorobenzyl mercaptan moiety was transferred to a cysteine sulfhydryl(s), to form a mixed disulfide, with release of cyanate anion to the medium. At high drug concentrations, multiple alkylation reactions occurred, and both subunits reacted with DCBT. At stoichiometric and substoichiometric DCBT concentrations, however, the β-tubulin subunit was the primary target of the drug.

Since tubulin polymerization (Kuriyama & Sakai, 1974; Ikeda & Steiner, 1978; Deinum et al., 1981; Lee et al., 1981), but not the colchicine or GTP binding activities of tubulin (Kuriyama & Sakai, 1974; Nishida & Kobayashi, 1977), is highly sensitive to sulfhydryl reactive agents, we decided to determine whether at low DCBT concentrations a specific cysteine residue of β-tubulin was alkylated. In this report, we document that Cys-239 of β-tubulin is the drug's major target and correlate this finding with reduced activity of tubulin in polymerization, but not in the binding of colchicine. In addition, we describe here initial studies on the separation of unalkylated, but denatured, tubulin subunits by hydrophobic column chromatography on decylagarose.

Materials and Methods

Materials. Nonradiolabeled DCBT and maytansine were obtained, respectively, from the Drug Synthesis and Chemistry and Natural Products Branches of the National Cancer Institute; [*benzyl*-^{3}H]DCBT and [*nitrile*-^{14}C]DCBT from Moravek Biochemicals; [^{3}H]colchicine from Amersham; decylagarose from ICN Immunobiologicals; cyanogen bromide from Alfa; podophyllotoxin and nocodazole from Aldrich; GDP, GTP (both repurified by ion-exchange chromatography), colchicine, and vinblastine from Sigma. All reagents used in sequencing peptides were highly purified products of Sigma, Pierce, and Baker. Electrophoretically homogeneous bovine

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[1] Abbreviations: DCBT, 2,4-dichlorobenzyl thiocyanate; CHO, Chinese hamster ovary; NEM, *N*-ethylmaleimide; HPLC, high-performance liquid chromatography; EBI, *N*,*N*′-ethylenebis(iodoacetamide).

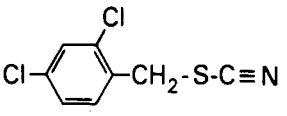

FIGURE 1: Molecular formula of DCBT.

brain tubulin was prepared as described previously (Hamel & Lin, 1984). DCBT was dissolved in either ethanol or dimethyl sulfoxide.

Preparative Separation of the α- and β-Subunits of Tubulin. A variety of conditions have been used to evaluate the usefulness of decylagarose for the resolution of α- and β-tubulin. Specific details of successful experiments will be presented below, but no technique has given completely reproducible separation. Both guanidine hydrochloride and urea have been used as the denaturant, both unbuffered and buffered at pH values ranging from 6 to 9, and varying concentrations of dithiothreitol have been employed. Both reverse NaCl gradient and step elutions have been used. Although some separations have been highly successful, in other experiments little resolution was achieved. In still other experiments, the β-tubulin peak was not symmetrical, suggesting at least partial resolution of isoforms (George et al., 1981). Often the recovery of α-tubulin was significantly less than that of β-tubulin. The reasons for this variability are unknown.

Separation of α- and β-Subunits of Alkylated Tubulin. A reaction mixture containing 2.5 mg/mL tubulin, 5 μM [*benzyl*-^{3}H]DCBT or 5 μM nonradiolabeled DCBT, and 1.0 M monosodium glutamate was incubated at 37 °C for 2 h. The reaction was stopped with 2.5 mM *N*-ethylmaleimide (NEM), which precipitated the tubulin. The sample was left overnight at 4 °C. The supernatant was discarded after centrifugation, and the precipitate was dissolved in 4 M guanidine hydrochloride (pH 5.0)–2 M NaCl (solution A). The protein was applied to a decylagarose column (1.5 × 25 cm) equilibrated with solution A. Following sample application, the column was washed with solution A until protein elution ceased. The column was then washed with 4 M guanidine hydrochloride (pH 5.0). Protein was determined as described by Bradford (1976), and aliquots of protein-containing fractions were counted. The two protein peaks, representing β- and α-tubulin, respectively, were pooled separately, dialyzed against water, and lyophilized. Only preparations of the tubulin subunits estimated to be at least 95% pure by polyacrylamide gel electrophoresis (Stephens, 1975) were used in further studies.

Cyanogen Bromide Cleavage. One part α- or β-tubulin alkylated with [*benzyl*-^{3}H]DCBT and NEM was mixed with three parts α- or β-tubulin alkylated with nonradiolabeled DCBT and NEM. The protein (2.0 mg/mL) was digested in 70% formic acid containing 20 mg/mL cyanogen bromide for 24 h at 37 °C in the dark (Gross & Witkop, 1961). The reaction mixture was diluted 10-fold with distilled water and lyophilized.

Peptide Purification. Following lyophilization, cyanogen bromide peptides of α- and β-tubulin were dissolved in 4 M guanidine hydrochloride (pH 5.0). Initial separation of the peptides was by high-performance liquid chromatography (HPLC) on a Brownlee Laboratories C18 Spheri-5 RP-18 column (0.4 × 10 cm) in 0.1% trifluoroacetic acid with a 0–60% acetonitrile gradient (room temperature; 1.0 mL/min) (Waterfield, 1986). An aliquot of each fraction was counted, and the radioactive peak from the β-tubulin digest was collected and lyophilized.

The radiolabeled peptide was dissolved in 4 M guanidine hydrochloride (pH 5.0) and repurified by HPLC on a Brownlee Laboratories C8 Aquapore RP-300 CO3-GU column (0.46 × 3 cm) using a gradient of 0–35% 2-propanol in 0.1% trifluoroacetic acid (room temperature; 1.0 mL/min) (Mahoney, 1982). The radiolabeled peak was collected and lyophilized.

Amino Acid Sequence Analysis. Automated Edman degradation for determination of the amino acid sequence of the radiolabeled peptide was performed with an Applied Biosystems Model 470A gas-phase sequencer (Hewick et al., 1981). Identification of phenylthiohydantoin derivatives was carried out with an Applied Biosystems Model 120A PTH analyzer, essentially as described by Zimmerman et al. (1977).

Functional Assays. Tubulin polymerization was followed turbidimetrically (Gaskin et al., 1974) in Gilford spectrophotometers equipped with electronic temperature controllers as described previously (Abraham et al., 1986). The binding of radiolabeled DCBT or colchicine to tubulin was followed by the DEAE-cellulose filter technique (Borisy, 1972) (stacks of three filters were used). Release of radiolabeled cyanate anion from [*nitrile*-^{14}C]DCBT was measured by ethyl acetate extraction as described previously (Bai et al., 1989).

RESULTS

Chromatographic Separation of α- and β-Tubulin on Decylagarose. Preliminary experiments indicated that a single cysteine residue of β-tubulin was the primary target of DCBT at lower drug concentrations. To identify this highly sensitive amino acid specifically, we utilized a method previously developed in this laboratory to separate α- and β-tubulin in preparative amounts. We will first briefly describe this separation procedure, as it has not yet been reported.

Although the separation of alkylated subunits by hydroxyapatite column chromatography has been described (Lu & Elzinga, 1977; Little, 1979), we were unable to achieve any resolution of unalkylated tubulin subunits with this medium. Alternative materials were explored, and only hydrophobic chromatography on decylagarose was effective.[2] Figure 2 presents a separation achieved following denaturation of tubulin with 8 M urea. (Comparable results were also obtained following denaturation of unalkylated tubulin with 3 M guanidine hydrochloride.) Both decreasing NaCl step elutions and reverse NaCl gradient elutions have been effective in separating the subunits, although there has been considerable variation between nominally duplicate experiments. Figure 2 presents a successful reverse gradient elution, with β-tubulin (centered at 2.1 M NaCl) eluting before α-tubulin (centered at 1.2 M NaCl). The upper panel presents the conductivity and protein content of the individual fractions, while the lower panel presents their band patterns on polyacrylamide gel electrophoresis. Amino acid composition analysis of peak fractions confirmed the identification of the first peak as β-tubulin and the second as α-tubulin.[3] There were no significant differences between the amino acid compositions we obtained and those derived from the published sequences of porcine α- and β-tubulin (Ponstingl et al., 1981; Krauhs et al., 1981).

In our initial work with tubulin alkylated by radiolabeled DCBT (Bai et al., 1989), we observed that the covalent bond(s) formed between drug and protein was (were) relatively labile. Its stability was considerably enhanced at acid pH values and

[2] We also examined octylagarose and dodecylagarose for their potential in resolving α- and β-tubulin. We obtained no separation of the subunits on octylagarose, while tubulin bound so tightly to dodecylagarose that we were unable to elute either subunit from the resin.

[3] We are indebted to the late Dr. Erhard Gross for performing the initial amino acid composition analysis for us.

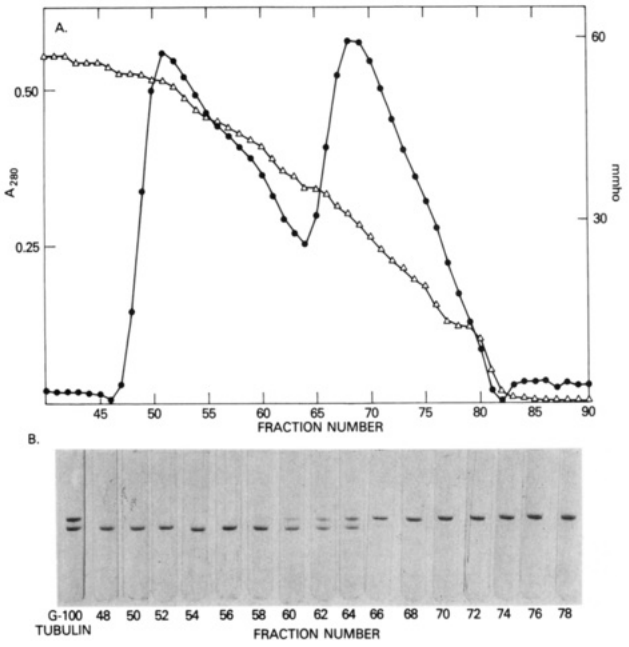

FIGURE 2: Separation of unalkylated α- and β-tubulin by reverse NaCl gradient chromatography on decylagarose. All operations were at room temperature. Solid urea and dithiothreitol (from a 2 M stock solution) were added to 180 mg of purified tubulin in 4 mL of 1.0 M monosodium glutamate (pH 6.6 with HCl) for final concentrations of urea and dithiothreitol of 8 M and 0.5 mM, respectively. The protein solution was applied to a 2.5 × 45 cm column of Sephadex G-100 (fine) equilibrated and developed with 8 M urea containing 0.5 mM dithiothreitol (solution B). Protein-containing fractions were pooled ("G-100 tubulin") (volume approximately 100 mL), and NaCl was added to a final concentration of 2.5 M. A decylagarose column (2.5 × 30 cm) was prepared and equilibrated with a solution containing 8 M urea, 2.5 M NaCl, and 0.5 mM dithiothreitol (solution C), the protein solution was applied, and the column was washed with 200 mL of solution C. During this phase of the procedure, 14-mL fractions were collected. A 400-mL reverse NaCl gradient was applied to the column, with the mixing chamber containing solution C (2.5 M NaCl) and the reservoir solution B (no NaCl). When the gradient was completed, the column was washed with 200 mL of solution B. Fraction size during the reverse gradient and the salt-free wash was 7 mL. (A) Protein (●, A_{280}) and conductivity (Δ) profiles of the column fractions. Only protein-containing fractions are displayed in the figure. (B) Polyacrylamide electrophoresis gel patterns of column fractions. Each gel contained 5 μg of the indicated fraction, except that the left-hand gel contained 10 μg of G-100 tubulin.

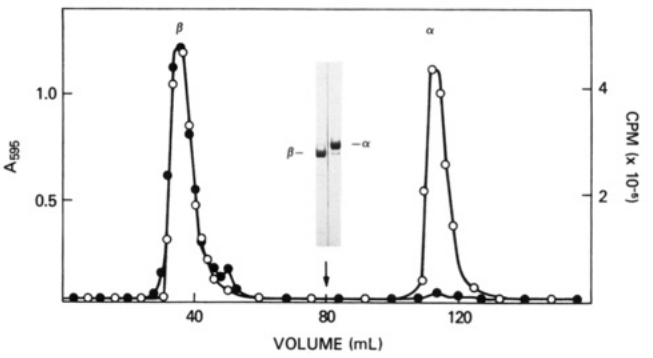

FIGURE 3: Separation of the α- and β-subunits of tubulin alkylated with [*benzyl*-^{3}H]DCBT by reverse NaCl step elution on decylagarose. Sample preparation and chromatography were described in detail in the text. The sample was applied and the column washed with a solution containing 4 M guanidine hydrochloride (pH 5.0) and 2 M NaCl. At the point indicated by the arrow, the column was further washed with a solution containing 4 M guanidine hydrochloride (pH 5.0) only. Open symbols represent protein; solid symbols represent radioactivity. Polyacrylamide gels (Stephens, 1977) of 10 μg of material from each of the two peaks are also presented in the figure.

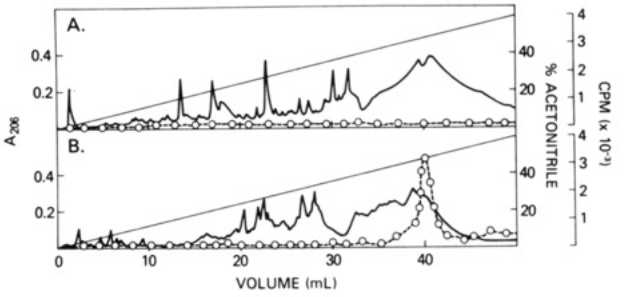

FIGURE 4: HPLC profile by C18 reverse-phase chromatography of cyanogen bromide peptides drived from α-tubulin (panel A) and from β-tubulin (panel B) alkylated with [*benzyl*-^{3}H]DCBT. Absorbance is indicated by the heavy solid lines, the acetonitrile gradients by the thin solid lines, and radioactivity by the open circles. Further details are described in the text.

if the tubulin was further alkylated with NEM, whereas its lability was increased in the presence of dithiothreitol. In the studies described here, the reaction of DCBT with tubulin was therefore stopped by the addition of NEM to the drug–tubulin complex (this precipitated the tubulin). Thereafter, all experimental manipulations were performed at acid pH values. In addition, to minimize reaction of DCBT with less reactive cysteine (or possibly other amino acid) residues, the alkylation reactions performed in the experiments described here were at a protein to drug ratio of 5:1.

Figure 3 demonstrates the resolution obtained on decylagarose with step elution of tubulin alkylated with [*benzyl*-^{3}H]DCBT and NEM (similar separations have been obtained with unalkylated tubulin). Densitometric scans of the gels shown in the figure indicated that the two subunits were each about 95% pure. There was about 25 times as much radioactivity derived from the [*benzyl*-^{3}H]DCBT in the β-tubulin peak as in the α-tubulin peak. Stoichiometry of the [*benzyl*-^{3}H]mercaptan moiety in the two subunits was determined from the total protein and radioactivity recovered: there was 0.076 pmol of drug/pmol of β-tubulin and 0.003 pmol of drug/pmol of α-tubulin. Prior to the decylagarose chromatography, there was 0.145 pmol of drug covalently bound per picomole of tubulin. Thus, some loss of bound drug still occurred despite the precautions described above. Since the total input of DCBT relative to tubulin was 0.2, about 75% of the drug reacted with the protein, assuming no initial losses. The reaction was almost entirely with β-tubulin, assuming no differences between retained and lost drug moieties.[4]

Identification of the Amino Acid Residue Alkylated by DCBT. Both trypsin and cyanogen bromide were examined as agents for generating peptides suitable for sequence analysis, and we found the chemical agent much more satisfactory for obtaining reproducible peptide patterns on HPLC analysis. Figure 4 presents the patterns obtained with cyanogen bromide digests of α- (panel A) and β-tubulin (panel B) alkylated with [*benzyl*-^{3}H]DCBT and NEM. These separations were performed on a C18 reverse-phase column. Both the absorbance and radioactivity profiles are presented in Figure 4. No distinct radioactive peak was obtained from the α-tubulin peptide digest. With β-tubulin, almost all the radioactivity appeared to be associated with a single peak, but this peak was not adequately pure for sequence analysis. Attempts to purify this material further by chromatography on the C18 column were unsuccessful.

[4] In earlier studies (Bai et al., 1989), we observed little variation in the relative amounts of radiolabeled drug retained by β- and α-tubulin, although absolute stoichiometry was highly variable depending on how the drug-protein complex was processed.

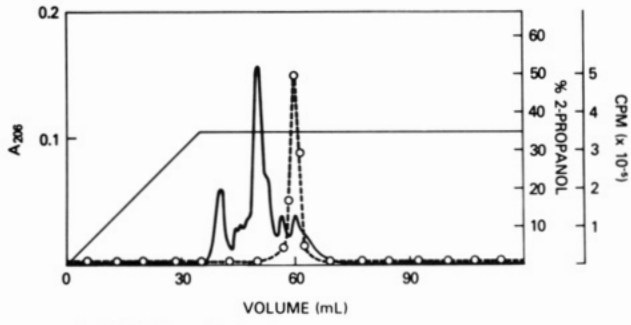

FIGURE 5: HPLC profile by C8 reverse-phase chromatography of the radiolabeled peptide peak from β-tubulin alkylated with [*benzyl*-^{3}H]DCBT, isolated by C18 reverse-phase HPLC (as described in Figure 4). Absorbance is indicated by the heavy solid line, the 2-propanol gradient by the thin solid line, and radioactivity by the open circles. Further details are described in the text.

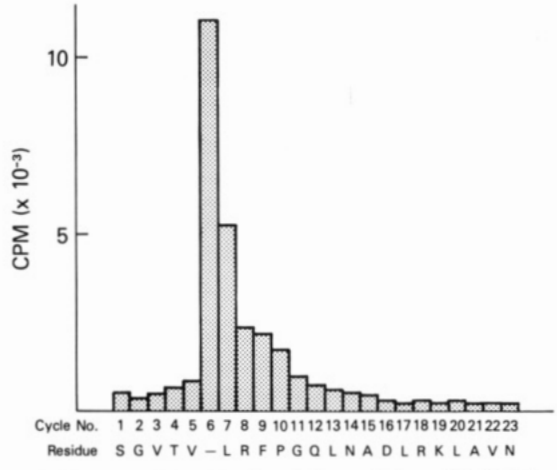

FIGURE 6: Sequence determination of the purified, radiolabeled peptide derived from β-tubulin alkylated with [*benzyl*-^{3}H]DCBT (as described in Figure 5). The entire sequence of the peptide was determined as described in the text. The cycle number and amino acid (indicated by the standard single-letter code) recovered are shown at the bottom of the figure, with radioactivity recovered in each cycle shown in the body of the figure. No amino acid was recovered in the sixth cycle, consistent with a cysteine residue at this position.

The partially purified material from the C18 column was resolved into several peaks on a large-pore reverse-phase C8 column by gradient chromatography (0–35% 2-propanol in 0.1% trifluoroacetic acid), as shown in Figure 5. Again, a single radiolabeled peak was obtained. Radiolabeled material was rechromatographed under the same conditions, and an apparently homogeneous peptide with coincident radioactivity and absorbance was obtained.

This peptide was sequenced, with the radioactivity of each residue determined. The results are presented in Figure 6. The peptide was 23 residues long, corresponding to that predicted for the cyanogen bromide fragment from β-tubulin residues 234–256 (Krauhs et al., 1981; Little & Luduena, 1985), with the exception of position 5 (see Discussion). The sixth residue in this peptide bore the radioactive moiety derived from [*benzyl*-^{3}H]DCBT, and no amino acid was identified at this position. This result is expected for a cysteine residue, and position 239 in most isoforms of β-tubulin is a cysteine (Krauhs et al., 1981; Little & Luduena, 1985; Sullivan & Cleveland, 1986).[5] These findings therefore strongly indicate that the primary target for DCBT is Cys-239 of β-tubulin.

Functional Correlates. In previous studies (Abraham et al., 1986; Bai et al., 1989), we examined the effects on tubulin

[5] The alternative amino acid at this position is serine.

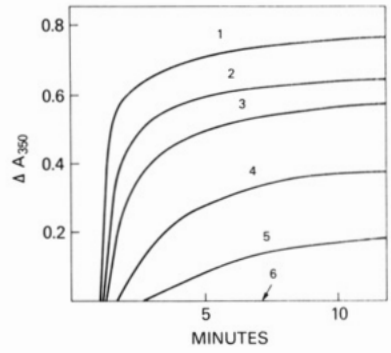

FIGURE 7: Inhibition of tubulin polymerization by substoichiometric concentrations of DCBT. Each 0.5-mL reaction mixture contained 2.5 mg/mL (25 μM) tubulin, 1.0 M monosodium glutamate (pH 6.6 with HCl), 1.5% (v/v) ethanol, and the following concentrations of DCBT: curve 1, none; curve 2, 5 μM; curve 3, 10 μM; curve 4, 15 μM; curve 5, 20 μM; curve 6, 25 μM. They were incubated at 37 °C for 2 h and then chilled on ice. GTP from a 0.18 M stock solution was added to a final concentration of 2 mM. Polymerization was then followed spectrophotometrically. At zero time, the electronic temperature controller was set at 30 °C, and the temperature rose at about 0.5 °C/s.

primarily of stoichiometric and superstoichiometric concentrations of DCBT. As demonstrated above, at low DCBT concentrations, Cys-239 of β-tubulin is by far the most reactive residue of the protein, while at higher concentrations significant alkylation of α-tubulin also occurs (Bai et al., 1989; no information is currently available on potential alkylation of additional cysteine residues of β-tubulin at high DCBT concentrations). It was therefore of importance to examine effects of substoichiometric DCBT concentrations on tubulin polymerization.

Figure 7 presents a study in which tubulin was treated with increasing substoichiometric amounts of DCBT under the same reaction condition used to alkylate Cys-239 of β-tubulin with [*benzyl*-^{3}H]DCBT. The higher the drug concentration in the preincubation without GTP, the greater the subsequent inhibition of tubulin polymerization, as described earlier (Abraham et al., 1986), until total inhibition occurred at a stoichiometric concentration of DCBT (curve 6). It is important to note that even at the lowest DCBT concentration (curve 2; 5 μM, one-fifth the tubulin concentration, i.e., the precise condition used to generate virtually specific alkylation of β-tubulin Cys-239), significant inhibition relative to the drug-free control occurred. The turbidity reading at 10 min in this experiment of the 5 μM DCBT sample was at 84% of the control value (compare to the 15% alkylation of tubulin noted above), but a still more dramatic inhibition occurred when the maximal rate of turbidity increase was evaluated (56% of the rate of the control reaction mixture).[6]

Another aspect of the effect of DCBT on tubulin which was evaluated earlier at stoichiometric and superstoichiometric concentrations was reversibility of the alkylation reaction. Formation of the covalent bond(s) between tubulin and the 2,4-dichlorobenzyl mercaptan moiety of DCBT appeared to be reversible at all DCBT concentrations by dithiothreitol treatment, but polymerization could only be partially restored with dithiothreitol following treatment with stoichiometric amounts of DCBT, and minimally or not at all following treatment of the protein at still higher drug concentrations (Bai et al., 1989). In comparable experiments with substoichiom-

[6] With 10 μM DCBT (curve 3), the maximum rate was 35% of that of the control reaction, and the 10-min turbidity reading was 74% of the control reaction. With 15 μM DCBT (curve 4), the comparable values were 11% and 49%, and with 20 μM DCBT (curve 5), 4% and 23%.

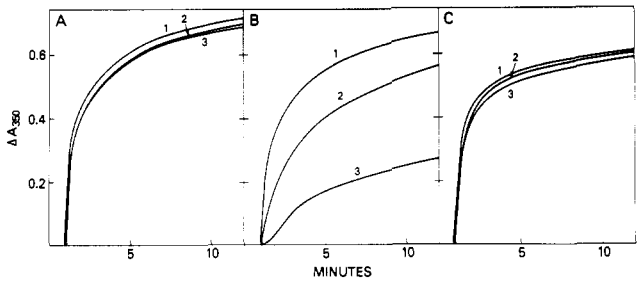

FIGURE 8: Reversibility of DCBT inhibition of tubulin polymerization by dialysis against dithiothreitol. Reaction mixtures (2.0 mL) containing the following components were prepared: 1.0 M monosodium glutamate (pH 6.6 with HCl), 0.1 M glucose 1-phosphate, 1.0 mM $MgCl_2$, 0.1 mM GDP, 0.2% (v/v) dimethyl sulfoxide, 2.0 mg/mL (20 μM) tubulin, and DCBT (curves 1, none; curves 2, 5μM; curves 3, 10 μM). For panel A, 0.25 mL of each reaction mixture was removed, GTP was added to a final concentration of 2 mM from a 0.18 M stock solution, and polymerization was followed spectrophotometrically at 30 °C as described for Figure 7. The remainder of the reaction mixtures were incubated at 37 °C for 2 h and then chilled on ice. For panel B, 0.25 mL of each reaction mixture was removed, GTP was added to a final concentration of 2 mM, and polymerization was followed as described above. Dithiothreitol was added to the remainder of the reaction mixtures to a final concentration of 50 mM from a 2.5 M stock solution. They were incubated for 5 min at 37 °C, chilled on ice, and then dialyzed separately for 4 h at room temperature (about 22 °C) against a solution containing the following components: 50 mM dithiothreitol, 1.0 M monosodium glutamate (pH 6.6 with HCl), 0.1 M glucose 1-phosphate, 1.0 mM $MgCl_2$, and 0.1 mM GDP. The dialyzed samples were chilled on ice. For panel C, 0.25-mL aliquots were taken, GTP was added to a final concentration of 2 mM, and polymerization was followed spectrophotometrically as described above.

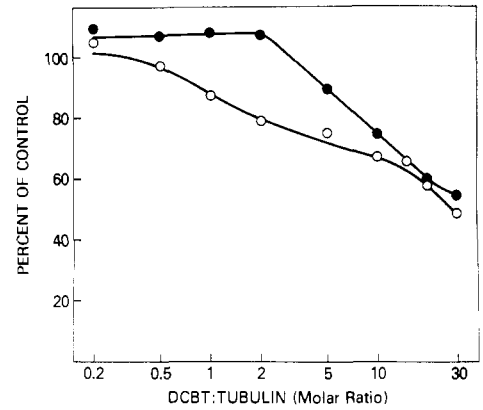

FIGURE 9: Effect of DCBT treatment on the binding of colchicine to tubulin. In the experiment represented by the closed symbols, reaction mixtures contained 0.1 mg/mL (1.0 μM) tubulin, 1.0 M monosodium glutamate (pH 6.6 with HCl), 10% (v/v) dimethyl sulfoxide, and the indicated concentrations of DCBT in 0.5 mL. Samples were incubated for 2 h at 37 °C and then chilled on ice. Triplicate 90-μL aliquots were mixed with 500 pmol of radiolabeled colchicine in 10 μL and incubated for 10 min at 37 °C. Triplicate values were within 10% of the average values presented in the figure. Data are expressed in terms of control samples without DCBT. In the experiment represented by the open symbols, reaction mixtures contained 2.5 mg/mL (25 μM) tubulin, 1.0 M monosodium glutamate (pH 6.6 with HCl), 5% (v/v) dimethyl sulfoxide, and the indicated concentrations of DCBT in 0.1 mL. Samples were incubated for 2 h at 37 °C and then chilled on ice. Triplicate 25-μL aliquots were mixed into final volumes of 0.3 mL containing 1.0 M monosodium glutamate (pH 6.6 with HCl), 0.1 M glucose 1-phosphate, 1.0 mM $MgCl_2$, 1.0 mM GTP, 0.5 mg/mL albumin, and 5 μM radiolabeled colchicine (final tubulin concentration 2.1 μM). Reaction mixtures were incubated for 10 min at 37 °C. Triplicate values were within 10% of the average values presented in the figure. Data are expressed in terms of control samples without DCBT.

etric DCBT concentrations, we still found only partial reversal of the inhibition of polymerization by dithiothreitol treatment (data not presented). These experiments were all performed in 1.0 M monosodium glutamate, as were most of the experiments presented here. We noticed, however, a steady deterioration in the control (i.e., drug-free) polymerization reaction during the many hours required for the drug–tubulin preincubation and for the dialysis against dithiothreitol.

We therefore explored whether the activity of tubulin in polymerization, like in colchicine binding (Hamel & Lin, 1981b; Huang et al., 1985), could be further protected by supplementation of the glutamate with glucose 1-phosphate, Mg^{2+}, and GDP. As the control reactions presented in Figure 8 demonstrate, little change occurred either following a 2-h drug-free preincubation at 37 °C or after the preincubation and a 4-h room temperature dialysis against dithiothreitol. Figure 8A,B demonstrates that in the supplemented glutamate reaction condition substoichiometric DCBT concentrations had little effect on tubulin polymerization unless drug and tubulin were preincubated at 37 °C [cf. Abraham et al. (1986)]. Without a preincubation (Figure 8A), using 20 μM tubulin (2.0 mg/mL), 5 μM DCBT had negligible effects on both the rate and extent of polymerization while 10 μM DCBT slightly affected the rate (89% of control) but not the extent of polymerization. Following preincubation for 2 h at 37 °C (Figure 8B), both DCBT concentrations resulted in significant inhibition of polymerization: with 5 μM DCBT, the rate was 55% of the control reaction, and the extent was 82% of the control; with 10 μM drug, the comparable values were 13% and 42% of control values (average of duplicate determinations in both cases). Following a 4-h dialysis at room temperature against 50 mM dithiothreitol (in the supplemented glutamate solution), polymerization of both drug-treated samples was again almost identical with the control reaction mixture (Figure 8C). Thus, in this reaction condition, the inhibitory effects of substoichiometric amounts of DCBT on tubulin polymerization appear to be fully reversible.

The effects of sulfhydryl alkylation on the binding of colchicine to tubulin are more complex than on polymerization. On the one hand, tubulin must be much more heavily alkylated before its ability to bind colchicine is lost relative to its loss of activity in polymerization (Kuriyama & Sakai, 1974; Ikeda & Steiner, 1978). On the other hand, however, preincubation of tubulin with colchicine, or with other drugs which bind at the colchicine site, inhibits subsequent alkylation of tubulin by sulfhydryl reactive agents, including formation of a cross-link between Cys-239 and Cys-354 of β-tubulin (Luduena & Roach, 1981b; Luduena et al., 1982; Roach & Luduena, 1984; Roach et al., 1987).

Identical results have been obtained with DCBT. Figure 9 documents the relative resistance of the colchicine binding activity of tubulin under two different reaction conditions following a preincubation of the protein with varying concentrations of DCBT. Most importantly, after treatment of tubulin with a stoichiometric amount of DCBT, which consistently results in total loss of polymerization, the ability of the protein to bind colchicine was essentially intact. In contrast, Table I documents that colchicine and other agents (podophyllotoxin, nocodazole) binding at the colchicine site reduce both binding of DCBT to tubulin and the release of cyanate anion which occurs when cysteine residues are alkylated (Bai et al., 1989). Although we have not studied these phenomena in detail, it seems probable that the colchicine site drugs inhibit the reaction of DCBT with Cys-239 (perhaps with a shift of reactivity to other cysteine residues) just as they inhibit formation of the Cys-239/Cys-354 cross-link.

Data with two additional drugs, maytansine and vinblastine, are presented in Table I. Maytansine had little effect on either

Table I: Effect of Antimitotic Drugs on Interactions of Radiolabeled DCBT with Tubulin

drug added	binding of [*benzyl*-^{3}H]DCBT[a] (% of control)	release of ^{14}CN[b] (% of control)
colchicine	62	59
podophyllotoxin	43	50
nocodazole	60	77
vinblastine	96	36
maytansine	93	90

[a] Each 100-μL reaction mixture contained 0.5 mg/mL (5 μM) tubulin, 5 μM [*benzyl*-^{3}H]DCBT, 1.0 M monosodium glutamate (pH 6.6 with HCl), 1% (v/v) dimethyl sulfoxide, 0.5% (v/v) ethanol, and the indicated second antimitotic drug at 100 μM. Incubation was at 37 °C for 2 h. Samples were processed by filtration through DEAE-cellulose filters as described in the text. Data are expressed relative to control reaction mixtures which contained [*benzyl*-^{3}H]DCBT but no second antimitotic drug. [b] Each 200-μL reaction mixture contained 0.5 mg/mL (5 μM) tubulin, 5 μM [*nitrile*-^{14}C]DCBT, 1.0 M monosodium glutamate (pH 6.6 with HCl), 1% (v/v) dimethyl sulfoxide, 0.5% (v/v) ethanol, and the indicated second antimitotic drug at 100 μM. Incubation was at 37 °C for 2 h. Samples were processed by ethyl acetate extraction of unreacted drug as described previously (Bai et al., 1989). Data are expressed relative to control reaction mixtures which contained [*nitrile*-^{14}C]DCBT but no second antimitotic drug.

the binding or the cleavage of DCBT by tubulin [a comparable result has been obtained in formation of the Cys-239/Cys-354 cross-link; see Roach and Luduena (1984)]. Vinblastine did not greatly affect binding of radiolabeled DCBT to tubulin, but this agent substantially inhibited release of cyanate anion by the protein. This differs from the results obtained for formation of the Cys-239/Cys-354 cross-link (enhanced by vinblastine; Luduena & Roach, 1981b, Roach & Luduena, 1984)[7] and suggests that binding of DCBT to tubulin and its alkylation of cysteine residues are distinct events.

Discussion

Studies on the effects of sulhydryl reactive agents on microtubule assembly have demonstrated that alkylation of as few as two cysteine residues results in complete inhibition of polymerization, but partial inhibition was observed at even the lowest concentrations of these agents added to reaction mixtures (Kuriyama & Sakai, 1974; Ikeda & Steiner, 1977; Deinum et al., 1981).[8] This suggests that while a single key cysteine was required for assembly, several cysteines were being simultaneously alkylated. In addition, sulfhydryl reactive agents disrupt mitosis in cells (Ramel, 1969; Nath & Rebhun, 1976; Oliver et al., 1976).

Experiments with radiolabeled sulfhydryl reactive agents have thus far yielded somewhat contradictory results in efforts to localize cysteine(s) required for polymerization. With [^{14}C]NEM, α-tubulin was labeled more heavily than β-tubulin at all NEM concentrations examined (Deinum et al., 1981), and with iodo[^{14}C]acetamide, both subunits were equally labeled (Luduena & Roach, 1981a). With 1-fluoro-2,4-dinitro[^{14}C]benzene, only β-tubulin was alkylated at low concentrations, but both subunits reacted with the agent at higher concentrations (Lee et al., 1981), similar to the results obtained with radiolabeled DCBT (Bai et al., 1989).

The sulfhydryl cross-linker *N*,*N*′-[^{14}C]ethylenebis(iodoacetamide) (EBI) reacted predominantly with β-tubulin (Luduena et al., 1982; Roach & Luduena, 1984). Little and Luduena (1985, 1987) described in detail two intra-β cross-links formed with this agent. One of these (between Cys-12 and either Cys-201 or Cys-211) was formed only when nucleotide-depleted tubulin was reacted with EBI (Roach & Luduena, 1984; Little & Luduena, 1987). [Our tubulin preparations contain a full complement of GDP bound in the exchangeable site; see Hamel and Lin (1981b, 1984) and Hamel et al. (1984).] The second cross-link formed with EBI was not affected by the exchangeable site nucleotide and is of special interest here, for it was between Cys-239 and Cys-354 (Little & Luduena, 1985). The formation of this intra-β cross-link results in loss of polymerizability of the tubulin (Luduena et al., 1982). In addition, drugs binding at the colchicine site inhibit formation of the Cys-239/Cys-354 cross-link (Luduena & Roach, 1981b; Luduena et al., 1982; Roach & Luduena, 1984; Roach et al., 1987).

In this study, we have examined the mechanism of action by which the antimitotic agent DCBT inhibits tubulin polymerization. Previous studies indicated an interaction of β-tubulin with the drug, for resistance is conferred by mutation of a β-tubulin gene (Abraham et al., 1986) and in vitro radiolabeled DCBT preferentially forms a covalent bond with β-tubulin (Bai et al., 1989), with specificity increasing as the drug to tubulin ratio falls. Here we have demonstrated almost total interaction of the drug with β-tubulin when the drug:tubulin ratio = 0.2. Furthermore, under this reaction condition, virtually all the covalently bound drug appears to react at a single amino acid residue of the protein, Cys-239. Moreover, this low DCBT concentration appropriately inhibits both the rate and extent of tubulin polymerization. This strongly indicates that Cys-239 must be in a reduced form if tubulin molecules are to participate in the microtubule assembly reaction and that Cys-239 probably represents the critical sulfhydryl group identified by many workers previously.

This, of course, does not exclude the possibility that other cysteine residues of tubulin are equally important in the assembly reaction. The reversibility of the DCBT–tubulin covalent bond by dithiothreitol may permit an approach to this question, for DCBT could be used to protect Cys-239 while additional cysteines are alkylated irreversibly with other agents.

The effects of DCBT on the binding of colchicine to tubulin and of colchicine site drugs on the interaction of DCBT with the protein are in complete agreement with previously published results with other sulfhydryl reactive agents. Drug concentrations which result in complete inhibition of polymerization do not have a significant effect on the binding of colchicine [cf. Kuryiyama and Sakai (1974) and Ikeda and Steiner (1978)]. At the same time, however, colchicine and other drugs which bind at the colchicine site inhibit the interaction of stoichiometric concentrations of DCBT with tubulin [cf. Luduena and Roach (1981b), Luduena et al. (1982), Roach and Luduena (1984), and Roach et al. (1987)]. This suggests that Cys-239 is not involved in the binding of colchicine to tubulin but that occupancy of the colchicine site at least partially masks this amino acid residue of β-tubulin.

In our determination of the sequence of the peptide bearing the radiolabeled DCBT moiety, we obtained an amino acid sequence that established the peptide as that corresponding to positions 234–256 (Krauhs et al., 1981; Little & Luduena, 1985). The only discrepancy was at the fifth position, where we obtained valine instead of threonine. The sequence analysis was therefore repeated a second time, and an identical result was obtained. Although it is possible that we have a variant tubulin at position 238, this seems highly unlikely. Direct

[7] It should be noted that high vinblastine concentrations more effectively induce tubulin aggregation in 1.0 M glutamate (used in our studies) than in the low ionic strength solutions used in the cross-linking studies (Luduena & Roach, 1981; Roach & Luduena, 1984).

[8] This occurs with DCBT, too (Figures 7 and 8).

sequence determination (Krauhs et al., 1981; Little & Luduena, 1985) and the sequence of tubulin genes [summarized in Sullivan and Cleveland (1986)] from many organisms (including bovine brain tubulin) have without exception yielded threonine at position 238. It seems most reasonable that our finding of valine at position 238 is an artifact resulting from the modification of its neighbor Cys-239 by DCBT, although we have no mechanistic explanation for this at present.

Since the separation of α- and β-tubulin by decylagarose chromatography has not been previously reported, a few brief remarks are warranted here. The two tubulin subunits have a high degree of homology both in their amino acid compositions and in their sequences (Ponstingl et al., 1981; Krauhs et al., 1981). Stephens (1975) reported their preparative separation following alkylation by large-scale polyacrylamide gel electrophoresis, and Lu and Elzinga (1977) and Little (1979) described the resolution of alkylated subunits on hydroxyapatite columns. We were not able to achieve separation of unalkylated subunits on hydroxyapatite and explored a number of other chromatographic media. The only success we have had is with hydrophobic chromatography on decylagarose, as described here. Like previous workers, thus far we have only worked with denatured tubulin. The amino acid compositions of α- and β-tubulin are very similar (Ponstingl et al., 1981; Krauhs et al., 1981), with most amino acids present in nearly identical amounts in the two subunits. Differences of five or more residues in the two polypeptide chains occur with only six amino acids. The α-subunit is richer in the hydrophobic amino acids alanine and isoleucine while the β-subunit is richer in the hydrophilic amino acids asparagine, glutamine, and serine. Although β-tubulin has eight more hydrophobic methionine residues than α-tubulin, the differences in content of the five other amino acids probably explain the significantly longer retention of α-tubulin by the hydrophobic column. Since the α-β dimer appears to reversibly dissociate under native reaction conditions (Detrich & Williams, 1978), decylagarose may also be useful in separating undenatured tubulin subunits under appropriate chromatographic conditions.

Registry No. DCBT, 7534-61-4; Cys, 52-90-4; colchicine, 64-86-8; podophyllotoxin, 518-28-5; nocodazole, 31430-18-9; vinblastine, 865-21-4; maytansine, 35846-53-8.

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