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Effect of Tubulin Binding and Self-Association on the Near-Ultraviolet Circular Dichroic Spectra of Colchicine and Analogues†

Renee M. Chabin, Francisco Feliciano, and Susan Bane Hastie*

Department of Chemistry, State University of New York, Binghamton, New York 13901

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ABSTRACT: Near-UV circular dichroic (CD) spectra of three colchicine analogues that differ at the C-10 position have been obtained in the presence and absence of tubulin. All three colchicine analogues show dramatic alterations in the low-energy near-UV CD band upon tubulin binding that cannot be mimicked by solvent, but in no event does the rotational strength of the CD band decrease to nearly zero as in the case of colchicine [Detrich, H. W., III, Williams, R. C., Jr., Macdonald, T. L., & Puett, D. (1981) *Biochemistry 20*, 5999-6005]. The effect of self-association of colchicine and one of the C-10 analogues, thiocolchicine, on the near-UV CD band was also investigated. A qualitative similarity was seen between the near-UV CD spectra of colchicine and thiocolchicine dimers and the spectra of these molecules bound to tubulin. These observations support the previous suggestion that ligands bound to the colchicine site on tubulin may be interacting with an aromatic amino acid in the colchicine binding site [Hastie, S. B., & Rava, R. P. (1989) *J. Am. Chem. Soc. 110*, 6993-7001].

Colchicine binding to tubulin, a 100 000-dalton protein which is the major protein component of the microtubule, is accompanied by ligand spectral alterations that are unique to tubulin binding and have resisted conventional explanation. [For reviews of the colchicine–tubulin association, see Brossi et al. (1988) and Luduena (1979).] The near-UV absorption spectrum of colchicine is a broad band that occurs at lower energy than tubulin absorption (λ_{max} = 353 nm in aqueous solution); the differences in energies of the tubulin and colchicine absorption spectra allow for examination of low-energy colchicine electronic spectra without interference by tubulin. Furthermore, the colchicine–tubulin complex, although noncovalent, dissociates so slowly that it is considered to be essentially irreversible, and thus spectra of the complex can be examined in the absence of unbound species.

The near-UV absorption band of colchicine is composed of two π–π^* transitions that are primarily located on the tropone C ring, and tubulin binding alters at least one of these transitions in a manner that has not yet been precisely duplicated in the absence of the protein (Hastie & Rava, 1989). The spectral changes in colchicine bound to tubulin are manifested in the absorption, fluorescence, and circular dichroic spectra of this low-energy absorption band. The overall absorption band of colchicine shifts to higher energy when the solvent dielectric constant is decreased; tubulin binding results in a small red shift in the absorption band (Bhattacharyya & Wolff, 1974). Tubulin binding also induces a dramatic enhancement of colchicine fluorescence (λ_{ex} = 350 nm, λ_{em} = 440 nm, ϕ = 0.03; Bhattacharyya & Wolff, 1974) that is not the result of simple hydrophobic effects, as the quantum yield of colchicine in aqueous solution and in low dielectric constant solvents is so low that the luminescence is difficult to detect. The sole condition that has been found so far to partially mimic tubulin-bound colchicine fluorescence is increased viscosity (Bhattacharyya & Wolff, 1984), leading to the hypothesis that tubulin binding serves to "immobilize" colchicine.

Perhaps the most dramatic change in colchicine's electronic spectra upon tubulin binding is observed by circular dichroism (CD).[1] The CD spectrum of colchicine in aqueous solution displays two negative bands in the 250–400-nm region of the spectrum (Hrbek et al., 1982). The band at 340 nm ($[\theta] \sim -34\,000$ deg·cm²/dmol; Detrich et al., 1981) has been shown to arise primarily from the interaction of the tropone C ring with the trimethoxyphenyl A ring (Yeh et al., 1988), which are oriented in an "S" biaryl configuration with a dihedral angle of about 53° (Lessinger & Margulis, 1978). When colchicine is bound to tubulin and free ligand removed, the rotational strength of the 340-nm band is reduced to nearly zero (Detrich et al., 1981).

At least two suggestions have been put forth to explain the loss of the 340-nm CD band in the colchicine–tubulin complex. One suggestion is that the colchicine binding site on tubulin

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[1] Abbreviations: CD, circular dichroism; Pipes, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMEG buffer, 0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, and 0.1 mM GTP, pH 6.90 at 23 °C.

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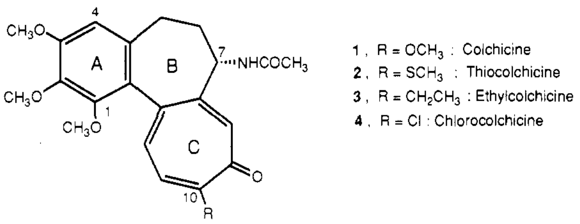

FIGURE 1: Structures of colchicine and derivatives. The conventional numbering system and ring designation (A, B, and C) are shown.

could cancel the inherent asymmetry of colchicine. Alternatively, a conformational change in the colchicine molecule could accompany tubulin binding and result in a colchicine conformation in which the relative orientations of the μ's and m's are modified in such a way that the rotational strength of the 340-nm band is reduced. One way by which colchicine could adopt such an altered conformation is through a boat–boat interconversion of the tropone C ring (Detrich et al., 1981).

A colchicine conformation deformation has also been suggested to rationalize the fluorescence enhancement of the bound ligand (Detrich et al., 1981), the slow kinetics of the association, and the association properties of certain colchicine analogues with tubulin (Bane et al., 1984; Hastie et al., 1989). In spite of the mechanistic importance of the CD behavior of colchicine, the effect of tubulin binding on the CD spectra of only two other colchicinoids has been investigated (Yeh et al., 1988). Moreover, recent work in our laboratory does not support a colchicine conformational change via a boat–boat interconversion of the tropone ring as accompanying tubulin binding (Hastie & Rava, 1989; Hastie, 1989).

In this work, we have studied the CD spectra of several colchicine derivatives that differ in substituents at the C-10 position (see Figure 1 for structures). The C-10 analogues were of interest as the C-10 substituent may be in direct conjugation with the tropone π system and may therefore affect the low-energy electronic transitions of the molecule. We previously suggested that the unusual resonance Raman, absorption, and excitation spectra observed when certain colchicinoids bind to tubulin may be due to an interaction of the ligand with an aromatic amino acid in the binding site (Rava et al., 1987; Hastie & Rava, 1989; Hastie, 1989). In this work, we show that changes in the colchicinoid near-UV CD spectra upon tubulin binding may be explained by the same mechanism.

EXPERIMENTAL PROCEDURES

Materials. Pipes, EGTA, and GTP (type II-S) were obtained from Sigma. Colchicine was obtained from Aldrich. Thiocolchicine was prepared from colchicine by using a published procedure (Shiau et al., 1975). The methods for preparing the two novel C-10 analogues of colchicine (chlorocolchicine and ethylcolchicine) were developed in this laboratory and will be reported elsewhere (M. E. Staretz and S. B. Hastie, unpublished results). All colchicinoids were characterized by infrared and proton NMR spectroscopy and mass spectrometry, and all compounds migrated as a single spot by thin-layer chromatographic analysis.

The near-UV absorption spectra of the colchicinoids in aqueous solution each displayed a broad band. The absorption maxima and extinction coefficients of the compounds in aqueous solution were as follows: colchicine ($\epsilon_{353} = 1.69 \times 10^4$ M^{-1} cm^{-1}), thiocolchicine ($\epsilon_{380} = 1.70 \times 10^4$ M^{-1} cm^{-1}), chlorocolchicine ($\epsilon_{354} = 1.10 \times 10^4$ M^{-1} cm^{-1}), and ethylcolchicine ($\epsilon_{342} = 7.75 \times 10^3$ M^{-1} cm^{-1}).

Tubulin Purification and Protein Determination. Bovine brain tubulin, free of microtubule-associated proteins, was prepared by two cycles of assembly/disassembly followed by phosphocellulose chromatography as described previously (Williams & Lee, 1982) and stored in liquid nitrogen. Tubulin obtained by this procedure is routinely >98% pure when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Prior to use, the frozen pellets were gently thawed, centrifuged at 5000g for 10 min at 4 °C, and then desalted into PMEG buffer (0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, and 0.1 mM GTP, pH 6.90 at 23 °C) on 1-mL Sephadex G-50 columns according to the method of Penefsky (1977). Tubulin concentrations were determined spectrophotometrically by the use of an extinction coefficient at 278.5 nm of 1.23 (mg/mL)$^{-1}$ cm^{-1} in PMEG buffer (Detrich & Williams, 1978).

Absorption Spectroscopy. Absorption difference spectra of colchicine derivatives in the presence and absence of tubulin were measured on a Perkin-Elmer 559A UV–visible spectrophotometer. Tandem cells (0.437-cm path length in each compartment) were used in the following manner: the front compartment of each cell was filled with an equal volume of 60 μM ligand in PMEG buffer and the rear compartment with the same volume of 10 μM tubulin in PMEG buffer. The temperature of the sample cell was held constant at 37 °C; the reference cell was at ambient temperature. A base line was obtained at half-full-scale using the automatic base-line feature (5 nm/min scan rate, 1-nm spectral bandwidth, 0.05 absorbance unit full-scale). To obtain the difference spectrum, the sample cell was removed, mixed by inversion, incubated at 37 °C for 30 min in the sample chamber, and scanned. Since the absorption of the ligand in the presence and absence of tubulin is equal at the isosbestic point of the difference spectrum, the extinction coefficient of the unbound ligand at the wavelength corresponding to the isosbestic point could be used to calculate the concentration of the tubulin-bound ligand in later experiments.

Thiocolchicine self-association was also analyzed by using absorption difference spectroscopy. Concentration difference spectra were obtained by measuring absorption spectra of thiocolchicine solutions from 250 to 500 nm on a Hewlett-Packard 8451A diode array absorption spectrometer using cells of different path lengths (1.0 cm, 1.0 mm, and 0.14 mm). The digitized data of dilute (<100 μM) solutions were subtracted from appropriate spectra of more concentrated solutions using an IBM PC to generate the concentration difference spectra. All concentration difference spectra showed an isosbestic point at approximately 410 nm. The wavelength at which the largest deviation from Beer–Lambert absorptivity was seen was 424 nm. The increase in absorption at 424 nm over the expected Beer–Lambert value (ΔA_{424}) was determined as a function of concentration up to a total thiocolchicine concentration of 7 mM, which was approximately the solubility limit of the compound. The solvent used was 90:10 water/methanol; thiocolchicine was soluble to only about 2.5 mM in water alone.

The dimerization constant for the association was calculated in the same manner as the equilibrium constant for colchicine self-association (Engelborghs, 1981) using the equation

$$K = (1 - \alpha)/2\alpha^2 C_t \qquad (1)$$

where K is the equilibrium constant for self-association, α is the concentration of thiocolchicine in monomeric form (C_m) divided by the total nominal thiocolchicine concentration, and C_t is the total nominal thiocolchicine concentration.

The value for α was determined at various thiocolchicine concentrations by the relationship

$$\alpha = C_m/C_t = (C_t\Delta\epsilon - \Delta A_{424})/C_t\Delta\epsilon \qquad (2)$$

Assuming a value for the change in extinction coefficient upon dimerization, values for α and K were calculated at each concentration. The calculations were repeated for several values of $\Delta\epsilon$ until a value for K was found which was independent of concentration.

The concentration difference spectra were also analyzed by the isodesmic model previously used for colchicine dimerization (Engelborghs, 1981). Essentially the same results were obtained by both methods.

CD Spectroscopy. CD spectra were obtained on a Jasco Model J-20 ORD/UV-5 spectropolarimeter equipped with a Sproul Scientific SS15-2CD modification. All spectra were taken at ambient temperature (~23 °C). Two quartz cells were used depending on the optical density of the sample (optical path = 1.0 cm and 0.1 mm). The base line was always recorded in the same cell as the sample, and the slit width and photomultiplier voltage were continuously monitored to ensure proper instrument performance. Full-scale sensitivities of 20 $\times 10^{-3}$ deg, 40 $\times 10^{-3}$ deg, or 10 $\times 10^{-2}$ deg, and a time constant of 1 s were used. At least three scans were made of all samples. The data were manually digitized at 2.5-nm intervals and input into the PC for subsequent manipulation.

Ligand–tubulin complexes for CD were prepared in the following manner. Tubulin (20 μM) was incubated with the colchicinoid (20 μM) in PMEG buffer for 45 min at 37 °C. The complex was separated from bound ligand by rapid gel filtration by the method of Penefsky (1977). The concentration of tubulin after gel filtration was assessed by treating a tubulin solution without added ligand to the same procedure and measuring the absorbance of the effluent. The concentration of the tubulin after gel filtration was routinely 20 μM. The concentration of the tubulin-bound ligand after gel filtration was determined by measuring the absorption intensity of the sample at the wavelength corresponding to the isosbestic point of the absorption difference spectrum.

The CD spectra of dimerized colchicine and thiocolchicine in the absence of monomeric species were determined by digitally subtracting the contribution of the monomer in the following manner. CD spectra of high-concentration ligand solutions were converted to molar ellipticity assuming no self-association. The percentage of the total ligand in monomeric form was calculated for each solution by using the self-association constant for each compound. A CD spectrum of a dilute solution of the ligand (at a concentration where no self-association was evident) in molar ellipticity was multiplied by the appropriate factor to match the percent monomer in the concentrated solutions and digitally subtracted from the appropriate spectrum. The resulting spectrum was then multiplied by 0.5 to account for the concentration difference due to dimerization. This analysis was repeated for three different dimer-containing solutions of thiocolchicine varying in total ligand concentration from 2 to 7 mM. The spectra from each analysis were virtually identical within the noise level of the spectra. The resulting dimer CD spectra were digitally averaged, and the averaged spectrum is shown. The CD spectrum of dimerized colchicine was calculated in a similar fashion using duplicate measurements of two solutions in which the total nominal concentration of colchicine was 10 and 12.5 mM. The averaged spectrum is shown.

NMR Spectroscopy. Colchicine self-association was assessed by proton NMR spectroscopy on a Bruker AM-360 NMR spectrometer. Colchicine solutions (1–40 mg/mL) were made up by weight in D_2O containing 0.75% sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d_4* as the internal standard. The chemical shifts of the A and C ring protons at 23 °C as a function of colchicine concentration were analyzed by the equation (Horman & Dreux, 1984)

$$\delta_i = \delta_0 - \chi_i(\delta_0 - \delta_\infty) \qquad (3)$$

where δ_i is the observed chemical shift at a particular concentration of colchicine, δ_0 and δ_∞ are the intrinsic chemical shifts of the uncomplexed and the complexed species, respectively, and χ_i is the fraction of the total nominal concentration of colchicine (C_t) present in dimerized form (C_d), i.e.

$$\chi_i = 2C_d/C_t \qquad (4)$$

Values of χ_i were calculated from a equation based on the law of mass action for dimerization:

$$K = C_d/(C_t - 2C_d)^2 \qquad (5)$$

where K is the self-association constant.

For a given value of K, a set of χ_i values corresponding to different C_t values were calculated by using eq 4 and 5, and the resulting data were plotted against δ_i by using eq 3. The optimum K gives the best straight line fit for δ_i vs χ_i (Horman & Dreux, 1984). The optimum value for K from this analysis (60 M^{-1}) was essentially identical with the value determined for colchicine self-association using concentration difference spectroscopy (Engelborghs, 1981).

The proton NMR data for $C_t \leq 15$ mM were also analyzed according to Dimicoli and Hélène (1973) to determine if higher order aggregation occurs in this concentration range. Only dimerization was observed by this method.

The effect of colchicine self-association on the ^{13}C NMR spectrum was also investigated. The ^{13}C NMR spectra of colchicine in D_2O (5–20 mg/mL) were measured on the Brucker AM-360 NMR spectrometer.

RESULTS

Effect of Tubulin Binding on the Near-UV CD Band. We have previously shown that thiocolchicine (**2**) binds with high affinity to the colchicine site on tubulin ($K = 1.1 \times 10^6$ M^{-1} at 23 °C) and that the thiocolchicine–tubulin complex is only very slowly reversible (Chabin & Hastie, 1989). When thiocolchicine was bound to tubulin and unbound ligand removed, the CD spectrum in Figure 2A resulted. Unlike colchicine, tubulin binding does not eliminate the lower energy CD band of thiocolchicine. Rather, the near-UV CD band of the bound species appears to have split into two bands: a band with reduced rotational strength is found slightly red shifted relative to unbound ligand, and a new negative band centering at 410 nm emerges. Altering solvent polarity had little effect on the lower energy band of the CD spectrum, although a shoulder at ~260 nm of the higher energy band became more intense in dioxane (data not shown).

The dramatic difference in the tubulin-bound CD spectra of thiocolchicine and colchicine (Detrich et al., 1981) led us to investigate the effect of tubulin binding on the near-UV CD spectra of other C-10 analogues of colchicine. Chlorocolchicine (**3**) and ethylcolchicine (**4**), two novel colchicine analogues in which the C-10 substituent is a chlorine atom and an ethyl moiety, respectively, have recently been prepared in our laboratory. These compounds are competitive inhibitors of colchicine binding to tubulin (M. E. Staretz and S. B. Hastie, unpublished results). The CD spectra of both molecules exhibit two negative bands at approximately the same energies as colchicine, and changing solvent has little effect on the wavelength or rotational strength of the two bands (data not

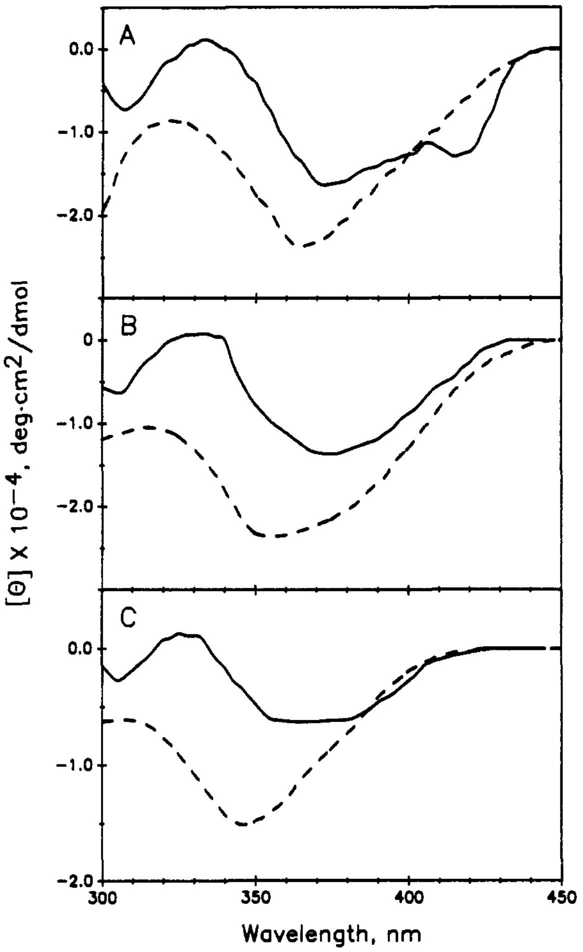

FIGURE 2: Near-UV CD spectra of thiocolchicine (A), chlorocolchicine (B), and ethylcolchicine (C) in the presence and absence of tubulin. The dashed line of each panel is the spectrum of the colchicinoid (20 μM) in PMEG buffer; the solid line is the spectrum of each ligand bound to tubulin after removal of unbound ligand. The colchicinoid (20 μM) was incubated with 20 μM tubulin for 45 min at 37 °C to form the complex. Unbound ligand was removed by rapid gel filtration, and the concentration of bound ligand was determined as described under Experimental Procedures. The spectra are presented as molar ellipticity.

shown). The effect of tubulin binding on the near-UV CD band of the two colchicinoids is shown in Figure 2B,C. Like thiocolchicine, the CD spectra of the molecules are greatly altered when complexed to tubulin, but significant optical activity is still observed in the protein-bound ligands.

Effect of Self-Association on the Near-UV CD Band. Colchicine undergoes self-association in aqueous solution (Engelborghs, 1981), and concentration difference absorption spectra of the dimerized species resemble the absorption difference spectrum of the molecule bound to tubulin (Bhattacharyya & Wolff, 1974; Andreu & Timasheff, 1986). It was therefore of interest to determine whether a similar process occurs with other colchicinoids and the effect that dimerization may have on the optical activity of these molecules.

Of the C-10 analogues, thiocolchicine was chosen for detailed study. Thiocolchicine self-association was first noted by the observation of a concentration difference absorption spectrum (Figure 3A). The absorption of thiocolchicine in aqueous solution deviated from Beer–Lambert law beginning at ~750 μM, and the concentration difference spectra consistently displayed a positive band at ~425 nm and a negative band centering around 385 nm. The concentration difference spectra of thiocolchicine in aqueous solution were also seen to qualitatively resemble the difference spectrum observed between thiocolchicine in the presence and absence of tubulin (Figure 3B).

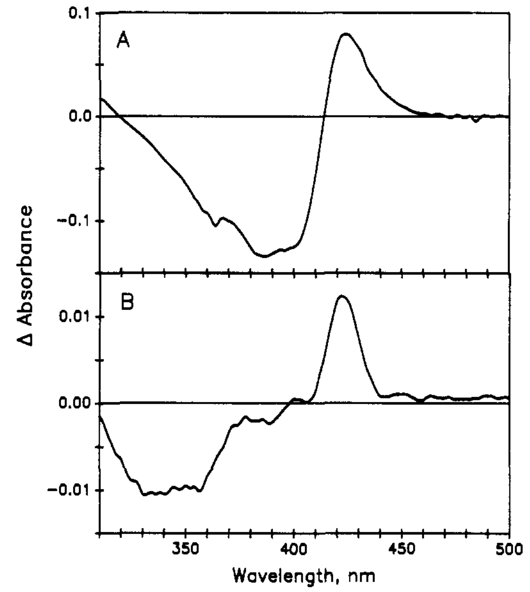

FIGURE 3: (A) Concentration difference spectrum of thiocolchicine. The absorbance spectrum of a low concentration of thiocolchicine in 90:10 water/methanol obtained in a 1-cm cell was digitally subtracted from the absorbance spectrum of thiocolchicine in a 1-mm cell as described under Experimental Procedures. The average of three separate determinations is shown. (B) Absorption difference spectrum of thiocolchicine in the presence and absence of tubulin. The difference spectrum was obtained by using tandem cells, and the concentrations of thiocolchicine and tubulin before the sample cell was mixed were 60 and 10 μM, respectively. The spectrum was recorded at ambient temperature (~23 °C) as described under Experimental Procedures.

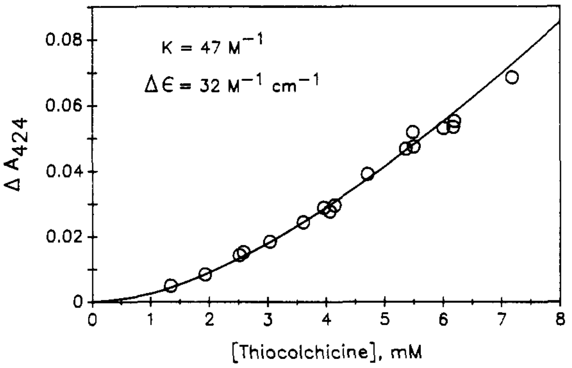

FIGURE 4: Concentration dependence of the absorption change of thiocolchicine at 424 nm. The deviation of thiocolchicine absorbance at 424 nm (ΔA_{424}) from Beer–Lambert absorptivity was determined from concentration difference spectra. The curve was calculated with an extinction coefficient change at 424 nm ($\Delta\epsilon_{424}$) of 32 M^{-1} cm^{-1} as described under Experimental Procedures.

The absorption at 424 nm was monitored as a function of thiocolchicine concentration as described under Experimental Procedures to yield a dimerization constant of 47 M^{-1} at room temperature in 90:10 water/methanol (Figure 4). This value is slightly less than the self-association constant for colchicine in aqueous solution (60 M^{-1}; Engelborghs, 1981).

The effect of increasing concentration on the CD spectrum of thiocolchicine is shown in Figure 5A. The spectrum, in which about 30% of the total nominal concentration of thiocolchicine is in dimeric form (curve 2 of Figure 5A), shows a remarkable resemblance to the CD spectrum of thiocolchicine bound to tubulin (Figure 2A). The CD spectrum of the dimer in the absence of monomer could be calculated by using the dimerization constant, and the CD spectrum of

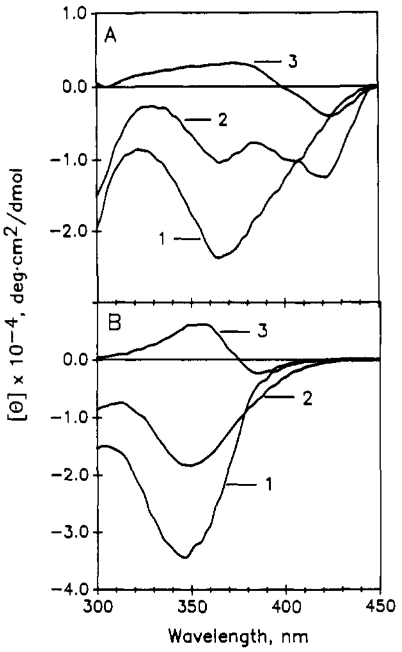

FIGURE 5: Concentration dependence of the near-UV CD spectrum of thiocolchicine (A) and colchicine (B). (A) Curve 1 is the CD spectrum of 70 μM thiocolchicine in which no self-association of thiocolchicine is observed. Curve 2 is the spectrum of a 7.2 mM solution of thiocolchicine in which approximately 30% of the total nominal concentration of thiocolchicine is in dimeric form. Note the similarities between curve 2 and the CD spectrum of thiocolchicine bound to tubulin (solid line, Figure 2A). Both spectra are expressed in molar ellipticity based on the total nominal concentration of thiocolchicine. Curve 3 is the CD spectrum of dimerized thiocolchicine, found by subtracting the contribution of the monomer from spectra containing monomers and dimers as described under Experimental Procedures. The average of three separate analyses is shown. The solvent for all spectra was 90:10 water/methanol. (B) Curve 1 is the CD spectrum of 70 μM colchicine in which no self-association of colchicine is observed. Curve 2 is the spectrum of a 12.3 mM solution of colchicine in which approximately 55% of the total nominal concentration of colchicine is in dimeric form. The spectra are expressed in molar ellipticity based on the total nominal concentration of colchicine. Curve 3 is the CD spectrum of dimerized colchicine. The CD spectrum of the dimer was found by subtracting the contribution of the monomer from spectra containing monomers and dimers as described under Experimetnal Procedures. The average of two separate analyses is shown. The solvent for all spectra was water.

the dimer is shown by curve 3 of Figure 5A. In the pure dimer, the lower energy negative band is still present, but the higher energy band appears as a positive peak in the dimer spectrum.

The effect of dimerization on the CD spectrum of colchicine was also investigated. Figure 5B shows a concentration-dependent reduction of ellipticity at 340 nm that is accompanied by an increase in ellipticity at lower energy (curve 2). A CD spectrum of dimerized colchicine (curve 3), calculated by subtracting the contribution of the monomer to the CD spectrum, resembles the thiocolchicine dimer spectrum.

It is clear that self-association alters the optical properties of the tropone ring, but the structure of the dimerized species is not apparent from the absorption (Hastie & Rava, 1989) and CD spectra. The effect of concentration on the proton NMR spectrum of colchicine was therefore examined. At relatively low ($\leq$15 mM) concentrations of colchicine, the A and C ring protons shifted upfield with increasing concentration (Figure 6A), indicating that both conjugated rings are involved in the dimerization process. There was no evidence of a colchicine conformational change, such as atropisomerism, with increasing concentration. The dimerization constant obtained by analysis of these data (60 M^{-1}) was identical with the value obtained when absorption spectroscopy is used (Engelborghs, 1981). At higher concentrations, the change in chemical shift deviates from values expected for a simple dimerization, and some protons begin to show downfield shifts (Figure 6B), implying that higher order aggregation may be occurring at the higher concentrations.

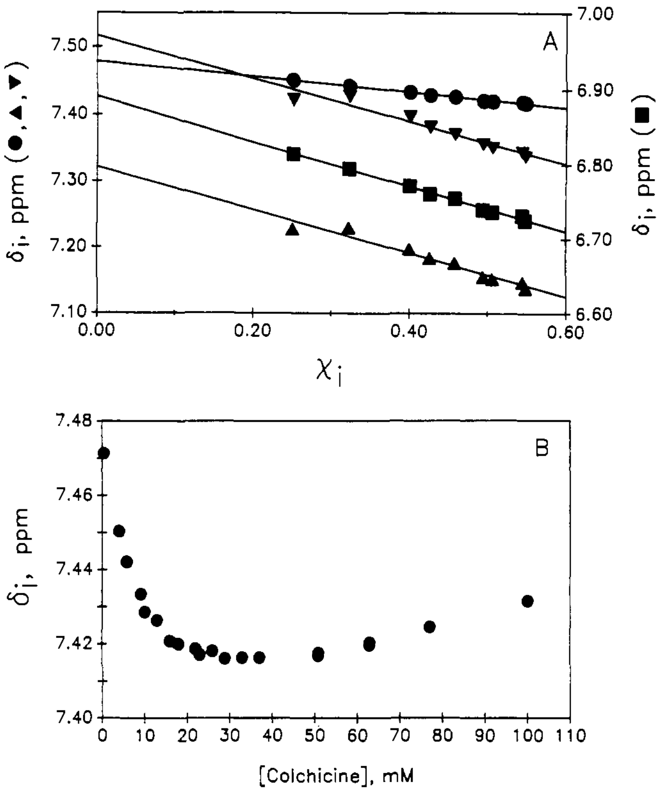

FIGURE 6: Colchicine self-association monitored by proton NMR spectroscopy. (A) The chemical shifts of the A and C ring protons (positions 4, 8, 11, and 12 from Figure 1) of colchicine were determined as a function of colchicine concentration in D_2O at 23 °C. The symbols used are as follows: (●) C-8 proton; (■) C-11 proton; (▲) C-12 proton; (▼) C-4 proton. The observed chemical shift (δ_i) is plotted as a function of χ_i, the fraction of the total nominal concentration of colchicine present in dimeric form, as outlined under Experimental Procedures. Values for χ_i were calculated by assuming a self-association constant of 60 M^{-1}. All four ring protons experience upfield shifts with increasing colchicine concentration up to about 15 mM. (B) At colchicine concentrations >15 mM, the chemical shifts of the molecule do not behave as described by a simple dimerization process. The effect of higher colchicine concentrations on the chemical shift of the C-8 proton is illustrated.

In a ^{13}C NMR study of colchicine binding to tubulin, the C-10 methoxy resonance of colchicine was broadened but did not shift upon tubulin binding (Ringel & Sternlicht, 1984). This observation led to the suggestion that colchicine is not bound to an aromatic pocket on tubulin. Since ring current effects on ^{13}C NMR chemical shifts are generally quite small ($\leq$1 ppm; Becker, 1980), it is possible that the presence of an aromatic ring in the colchicine binding site would not be detectable by ^{13}C NMR. The ^{13}C NMR spectrum of colchicine in D_2O as a function of colchicine concentration should therefore provide insight to the magnitude of the ring current effect on the C-10 methoxy resonance. The chemical shift of the C-10 methoxy resonance was found at 56 ppm in D_2O, and was invariant with colchicine concentrations up to 50 mM (data not shown), well above colchicine concentrations at which significant dimerization is observed by proton NMR spectroscopy.

DISCUSSION

Modification of the C-10 substituent of colchicine alters the near-UV absorption spectrum in predictable fashions: substitution of the C-10 oxygen with a sulfur atom results in a red shift in the absorption maximum, while a chlorine or ethyl substituent produces only minimal change in the absorption maximum. The near-UV CD band of the colchicinoids in the absence of tubulin mirrors the absorption spectra changes, and solvent properties have little effect on either the shape or the intensity of this band. The near-UV CD band of the colchicinoids bound to tubulin, however, is quite sensitive to the nature of the C-10 substituent. In all four cases examined, the ellipticity at the maximum wavelength of the negative CD band is greatly diminished, but only in colchicine itself is the rotational strength of this band reduced to nearly zero.

The dramatic differences between the tubulin-bound colchicinoids and solution CD spectra indicate that the colchicine binding site is interacting with the colchicinoids in a manner that is not describable by simple solvent effects. We have found previously that the near-UV absorption spectrum of colchicine bound to tubulin could be partially modeled by colchicine dimerization, implying that the optical changes that occur upon tubulin binding may be the result of the interaction of colchicine and an aromatic amino acid residue in the binding site (Hastie & Rava, 1989). We therefore set out to determine if colchicinoid self-association could also mimic the CD behavior of the tubulin-bound ligand.

We have shown in this work that, like colchicine, thiocolchicine undergoes self-association in aqueous solution. The dimerization constant (47 M^{-1}) for thiocolchicine at ambient temperature is very similar to the value previously found for colchicine (60 M^{-1}; Engelborghs, 1981). Thiocolchicine concentration difference spectra were qualitatively similar to the difference spectrum of thiocolchicine in the presence and absence of tubulin, implying that dimerization of thiocolchicine also results in changes in the absorption spectrum of the molecule that resemble tubulin binding.

The CD spectrum of thiocolchicine at concentrations where significant dimerization is present was remarkably similar to the tubulin-bound spectrum. When the contribution due to monomer was subtracted, the resemblance was less striking, but the general features of the spectrum remained. The CD spectrum of a colchicine solution in which a significant portion of the molecules were in dimers showed a decrease in ellipticity near 350 nm in a manner similar to the tubulin-bound ligand, but like thiocolchicine the pure dimer showed a positive band at higher energy near the wavelength maximum and a negative peak at lower energy. Although the dimer spectra did not precisely duplicate the tubulin-bound spectra, they show for the first time experimental conditions in which the major CD band of colchicinoids complexed to tubulin can be partially simulated in the absence of tubulin and provide more evidence for our hypothesis that colchicine is associating with an aromatic amino acid residue of the protein.

Whether the interaction of the A or the C ring (or both) with an aromatic amino acid in the binding site is responsible for the alterations in the CD spectra with tubulin binding cannot be precisely defined from this study. The NMR analysis of colchicine as a function of concentration showed that both rings are involved in the dimerization process; thus, the electronic effects observed by dimerization probably reflect contributions from both rings. Since the CD band of colchicine near 350 nm arises from the interaction of the A and C rings (Yeh et al., 1988), it is possible that perturbation of either ring by another aromatic system could lead to the changes in the CD spectrum observed upon ligand binding. On the basis of previous analysis of colchicine absorption spectra (Engelborghs, 1981; Hastie & Rava, 1989), it is reasonable to suggest that the C ring is the portion of the colchicine molecule that is in close proximity to an aromatic amino acid.

The NMR studies also did not show any indication of conformational changes in colchicine accompanying the self-association process, implying that the spectral changes observed with self-association are not due to conformational readjustments of the molecule upon dimerization.

We hope to narrow down the possible orientations of an external π system which could give rise to the tubulin-bound CD spectrum through continued molecular orbital calculations and molecular modeling. We have found that the near-UV band of colchicine is composed of two π–π^* transitions that differ greatly in the nature of the excited state (Hastie & Rava, 1989) and are in the process of performing a similar analysis from thiocolchicine. It is noted that the positive band in the colchicine dimer CD spectrum is at around the same energy as the higher energy transition found in the colchicine absorption spectrum (~340 nm), while the negative band is at approximately the same energy as the lower energy transition (~380 nm; Hastie & Rava, 1989). Preliminary analysis of the electronic transitions of thiocolchicine leads to a similar assignment. Although our understanding of the colchicinoid CD spectra is currently hampered by the complications introduced by the two transitions, the sharply different characteristics of the transitions should later assist us in defining the orientation of the π system in the binding site.

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Fluorescence Studies on Calmodulin Binding to Erythrocyte Ca^{2+}-ATPase in Different Oligomerization States[†]

Danuta Kosk-Kosicka,*[‡] Tomasz Bzdega,[‡] and J. David Johnson[§]

Department of Biological Chemistry, University of Maryland, School of Medicine, 660 West Redwood Street, Baltimore, Maryland 21201, and Department of Physiological Chemistry, The Ohio State University, Medical Center, Columbus, Ohio 43210

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ABSTRACT: The fluorescent spinach calmodulin derivative 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid-calmodulin (MIANS–CaM) was used to investigate calmodulin interaction with the purified, detergent-solubilized erythrocyte Ca^{2+}-ATPase. Previous studies have shown that the Ca^{2+}-ATPase exists in equilibria between monomeric and oligomeric forms. We report here that MIANS–CaM binds to both enzyme forms in a Ca^{2+}-dependent manner, with a ~50% fluorescence enhancement. These findings confirm our previous observation that enzyme oligomers retain their ability to bind calmodulin, even though they are fully activated in the absence of calmodulin. The Ca^{2+} dependence of MIANS–CaM binding to monomeric Ca^{2+}-ATPase is of higher affinity ($K_{1/2} = 0.09\ \mu M\ Ca^{2+}$) and less cooperative ($n_H = 1.1$) than the Ca^{2+} dependence of enzyme activation by MIANS–CaM ($K_{1/2} = 0.26\ \mu M\ Ca^{2+}$, $n_H = 2.8$). These Ca^{2+} dependences and the order of events, in which calmodulin binding precedes enzyme activation, demonstrate that calmodulin indeed could be a physiological activator of the monomeric enzyme. The calcium dependence of calmodulin binding to oligomeric Ca^{2+}-ATPase occurs at even lower levels of Ca^{2+} ($K_{1/2} = 0.04\ \mu M\ Ca^{2+}$), in a highly cooperative fashion ($n_H = 2.3$), and essentially in parallel with enzyme activation ($K_{1/2} = 0.05\ \mu M\ Ca^{2+}$, $n_H = 2.9$). The observed differences between monomers and oligomers suggest that the oligomerized Ca^{2+}-ATPase is in a conformation neccessary for efficient, cooperative calcium binding at nanomolar Ca^{2+}, which the monomeric enzyme acquires only upon interaction with calmodulin. Fluorescence titrations of Ca^{2+}-ATPase oligomers and monomers with MIANS–CaM reveal significant difference, indicating that the oligomer is saturated near 0.5 mol of calmodulin/mol of Ca^{2+}-ATPase while the monomer is saturated near 1 mol of calmodulin/mol of Ca^{2+}-ATPase. These results suggest the possibility that oligomerization of the Ca^{2+}-ATPase monomers results in the elimination of one calmodulin binding site per every two associated enzyme molecules.

Calmodulin has been shown to modulate the activity of a broad variety of enzymes, including the Ca^{2+}-ATPase of plasma membranes (Manalan & Klee, 1984; Schatzmann, 1982; Johnson & Mills, 1986). Despite extensive studies, the exact mechanism of calmodulin action on many enzymes has not been resolved. We have approached this problem by means of comparative studies of calmodulin's interaction with two forms of the same enzyme, the purified erythrocyte Ca^{2+}-ATPase, that differ in their oligomerization state. Previously we have studied the binding of calmodulin to the Ca^{2+}-ATPase by means of calmodulin affinity chromatography (Kosk-Kosicka & Bzdega, 1988). We have shown that both enzyme forms bind calmodulin whereupon only the interaction between calmodulin and the monomeric enzyme results in Ca^{2+}-ATPase activation. From the calmodulin dependence of the Ca^{2+}-ATPase activity of the monomeric form, we have derived a molar stoichiometry of 0.73:1 of calmodulin to enzyme, which implies that one calmodulin molecule binds to one enzyme monomer for activation. The stoichiometry of calmodulin binding to the oligomeric enzyme could not be measured by activity because the oligomers are fully active in the absence of calmodulin, and binding of calmodulin does not affect their Ca^{2+}-ATPase activity.

In the present study, we use spinach calmodulin, labeled at cysteine-26 with a sulfhydryl-selective fluorescent probe which undergoes a fluorescence increase with target protein binding (Mills et al., 1988). This 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid–calmodulin (MIANS–CaM)[1] was

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* To whom correspondence should be addressed.

[‡] University of Maryland.

[§] The Ohio State University.

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