Cooperative Multiple Binding of BisANS and Daunomycin to Tubulin†

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ABSTRACT: The binding of daunomycin and bisANS to tubulin was studied by direct equilibrium techniques. Both ligands generated abnormal Scatchard plots. Their concave-downward nature indicated positive cooperativity. The data conform to tubulin possessing ca. 35 daunomycin binding sites with a binding constant of 570–1430 M^{-1}. The binding of bisANS is characterized by 1 strong binding site (K_A = 4.5 × 10^5 M^{-1}) and 40–50 lower affinity sites. Hill plots of both showed low degrees of cooperativity (m = 1.8 for daunomycin and 2.3 for bisANS). A detailed analysis was carried out of the cooperativity of binding of daunomycin to tubulin. Concentration difference spectra and sedimentation velocity analysis of daunomycin showed that this molecule undergoes self-association in the drug concentration range used in the binding study. The low level of polymerization (~tetramer), however, indicated that this could not be the source of the observed cooperativity between 35 molecules. Both the shape and concentration dependence of the daunomycin concentration difference spectra were strikingly similar to those generated on the binding of daunomycin to tubulin, which indicates the stacking of daunomycin in both cases. The observed Scatchard plot of the binding was found to be consistent with a process that involves in part ligand–ligand interactions when complexed to tubulin. Examination of the binding of bisANS in the presence of daunomycin revealed a strong increase of bisANS binding to tubulin, which suggests a loosening of tubulin structure with the exposure of new sites as these ligands bind. The mutual interaction between the two ligands in dilute solution was demonstrated by difference spectroscopy. These observations have led to the conclusion that daunomycin and bisANS (with the exception of the high-affinity site) bind to probably the same flexible region on the surface of the tubulin molecule, that is large enough to accommodate 35–50 molecules of the ligands in mutual interactions and which can be enlarged by the binding. This region may be the same as that shown to multiply bind detergent molecules [Andreu & Munoz (1986) *Biochemistry 25,* 5220–5230].

Microtubules are widely distributed subcellular structures which are involved in cellular processes of such varied nature as mitosis (Dustin, 1984), the maintenance of cell shape (Hyams & Stebbings, 1979), cell movement (Warner & Mitchell, 1978; Bloodgood, 1982), transportation of structures within the cell (Hall, 1984), the ability to influence the distribution of membrane proteins (Helmreich & Elson, 1984), and cardiac cell contraction (Lampidis et al., 1992). The basic building block of microtubules is the protein tubulin which has the ability to assemble into a variety of structures modulated by the binding of ligands to the protein (Timasheff & Grisham, 1980; Ward et al., 1994). Although the detailed structure of tubulin is not known, a number of features have been identified. Both subunits of tubulin contain an acidic, carboxyl-rich peptide in the C-terminal region (Ponstingl et al., 1981; Krauhs et al., 1981). Both also contain large flexible hydrophobic patches where detergent molecules can bind cooperatively and reversibly and loosen the structure of the protein (Andreu & Munoz, 1986; Andreu et al., 1986). Our understanding on the molecular level of the mechanisms of ligand modulations of the various tubulin interactions should be enhanced if knowledge were available of the relative spacial distribution of the binding loci of these various ligands and of the cooperativity in their binding to the protein. In the pursuit of this aim, a detailed investigation of the binding of daunomycin and bis(8-anilino-1-naphthalenesulfonic acid) (bisANS)[1] to tubulin and of the interrelationship of their binding has been undertaken. The strong visible absorption characteristics of these two molecules and the extreme environmental sensitivity of bisANS fluorescence make both compounds potentially useful tools for probing conformational states of the tubulin dimer. The fluorescent probe bisANS has been reported to inhibit microtubule assembly substoichiometrically (Horowitz et al., 1984) and to possess one strong and six weaker binding sites on tubulin (Prasad et al., 1986a) as determined by spectroscopic means. Daunomycin is an anthracycline antibiotic used widely in cancer chemotherapy (Venditti et al., 1966; Whang-Peng et al., 1969; Aubel-Sadron & Londos-Gagliardi, 1984) due to its antimitotic activity which involves its ability to interact with nucleic acids (Calendi et al., 1965). Daunomycin has also been demonstrated to bind to tubulin and to inhibit the *in vitro* assembly of microtubules stoichiometrically by binding to tubulin dimers (Na & Timasheff, 1977). This interaction results in a weak GTPase activity in the tubulin dimer (David-Pfeuty et al., 1979), similar to that of tubulin–colchicine (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981). The induction of GTPase activity, which normally is associated with tubulin within microtubules, has been interpreted in terms of the ability of these ligands to induce conformations partially mimicking

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; DAPI, 4,6-diamidino-2-phenylindole; GTP, guanosine 5′-triphosphate; bisANS, bis(8-anilino-1-naphthalenesulfonic acid).

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those present within microtubules (Carlier, 1982). Furthermore, daunomycin has been shown to act competitively with vinblastine in stimulating heart beat at what appears to be a microtubule level (Lampidis et al., 1992). Anthracyclines have also been shown to affect the MAP-related microtubule organization (Fromes et al., 1993).

The use of these molecules as probes of protein structure requires accurate knowledge of their binding properties. The aim of this study is first to investigate the binding of bisANS and daunomycin to tubulin by conventional equilibrium binding techniques and second to investigate the interrelationship between their binding prior to their potential use in donor–acceptor pairs for the spatial mapping of ligand binding sites on tubulin by fluorescence energy transfer.

MATERIALS AND METHODS

Chemicals. GTP[1] (type 2), chlorpromazine, Blue Dextran, daunomycin, colchicine, 4,6-diamidino-2-phenylindole (DAPI),[1] and glycerol were Sigma products. 8-Anilino-1-naphthalenesulfonic acid (ANS)[1] and bisANS[1] were obtained from Molecular Probes and used without further purification. Ruthenium Red was purified from commercial Ruthenium Red from Fluka by recrystallization in 0.5 N ammonia (Fletcher et al., 1961). [^{3}H]Daunomycin was from New England Nuclear. Vinblastine was a gift from Dr. P. Potier of the Institut de Chimie des Substances Naturelles, CNRS, Gif-sur-Yvette, France. Ultrapure guanidine hydrochloride was obtained from Heico. Biogel P100 and Sephadex-G50 Fine were Bio-Rad and Pharmacia products, respectively. All other chemicals were of reagent grade.

Preparation of Tubulin and of the Stable Tubulin–Colchicine Complex. The modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970; Lee et al., 1973) was used to isolate calf brain tubulin from freshly slaughtered animals, with the small procedural changes described by Prakash and Timasheff (1983). After removal from liquid nitrogen, tubulin was equilibrated in the desired buffer by using the procedure of Na and Timasheff (1982). The tubulin–colchicine complex was formed by incubating together millimolar concentrations of colchicine and tubulin (Andreu & Timasheff, 1982) before the usual equilibration procedure. The concentrations of tubulin and the stable tubulin–colchicine complex were calculated using absorptivity values of 1.03 mL·mg^{-1}·cm^{-1} (Na & Timasheff, 1982) and 1.16 mL·mg^{-1}·cm^{-1} (Andreu & Timasheff, 1982) at 275 nm in 6 M guanidine hydrochloride, respectively.

UV/Visible Spectroscopy. All spectra were performed on a Perkin Elmer Lambda 3B UV/visible spectrophotometer after base-line correction. The sample cuvette was maintained at a constant temperature of 20 °C by way of a thermoelectric cell holder, and the reference cuvette was thermostated by circulation of water from an external water bath. The visible difference spectra for the tubulin–daunomycin interaction were obtained using 1 cm cells, with tubulin and daunomycin in the sample cuvette and daunomycin in the reference compartment, after subtracting the low absorbance due to tubulin. Similarly, the influence of bisANS on the tubulin–daunomycin difference spectra was assessed with tubulin, daunomycin, and bisANS in the sample cell and daunomycin and bisANS in the reference cell. The base line was recorded by reading tubulin–bisANS relative to bisANS in the absence of protein to negate the contribution of these components to the difference spectra. The UV/visible tubulin–bisANS spectra and the bisANS–daunomycin spectra were recorded using 0.4 + 0.4 cm tandem cells.

Concentration Difference Spectra. The concentration difference spectra of daunomycin were recorded by using a 1 mm cell in the sample compartment held in place with a 9.5 mm spacer relative to a 1 cm cell in the reference compartment. The exact ratio of a sample path length to reference path length was calculated by recording concentration difference spectra of potassium chromate in KOH, a ligand known not to associate under these conditions (Strazza et al., 1985). Use of a potassium chromate concentration in the sample compartment that was 9.93 times that of the reference cuvette resulted in no recorded difference spectrum.

Fluorescence Spectroscopy. Fluorescence measurements were performed on a Perkin Elmer 650-40 spectrofluorometer using the ratio mode. Background was routinely subtracted for all spectral measurements, and any instrumental variation as a function of wavelength was corrected for by generating correction factors using Rhodamine B and a light diffuser.

Analytical Ultracentrifugation. Sedimentation velocity experiments were carried out at 20 °C in a Beckman Model E analytical ultracentrifuge fitted with electronic speed control and RTIC temperature control. The experiments were performed at 60 000 rpm in an AND rotor using Kel F centerpieces. The movement of the boundary was monitored using the photoelectric scanner at 590 nm. The weight average sedimentation coefficient was measured directly from the tracings of absorbance versus distance, as described by Schachman (1959).

Tubulin–Daunomycin Binding. The binding of daunomycin to tubulin was studied both by frontal gel chromatography (Nichol & Winzor, 1964) and by a gel partition method (Fasella et al., 1965; Pearlman & Crepy, 1967; Hirose & Kano, 1971; Na & Timasheff, 1985, 1986a). Use of the frontal chromatography method entailed application of a tubulin–daunomycin–[^{3}H]daunomycin mixture to an 11 cm × 1 cm column of Sephadex G-25 Fine. The trailing plateau of the elution profile was used to estimate the free daunomycin concentration of the equilibrium mixture (Nichol & Winzor, 1964). Experiments were performed at varying flow rates from 0.2 to 0.4 mL/min, maintained by an LKB peristaltic pump, and the concentration of daunomycin in the column effluent was monitored by taking small aliquots, adding to 10 mL of aqueous scintillation fluid (ACS Amersham), and counting in a Beckman L-100 liquid scintillation spectrometer. The procedure used to determine the binding of daunomycin to tubulin by the batch equilibrium partition method was essentially that used by Na and Timasheff (1985, 1986a) for monitoring the binding of vinblastine to tubulin. To 12 × 75 mm borosilicate culture tubes were added 40 mg of Biogel P100 and 0.7 mL of PG buffer, and swelling was allowed to occur at room temperature overnight. After the swelling period, aliquots of daunomycin, [^{3}H]daunomycin, and tubulin were added, and the tubes were placed in a water bath at 20 °C and subjected to intermittent gentle shaking for 1 h. The gel was then allowed to settle, and aliquots were taken for liquid scintillation counting and protein concentration determinations. As a control, incubation was also done in the absence of tubulin in order to account for the nonspecific absorption of daunomycin to the gel matrix. The external volume was determined by using Blue Dextran 2000. In agreement with Na and Timasheff (1986b), tubulin was completely excluded from the inner volume of the gel. The inner volume of the Biogel P100 was found to vary from batch to batch, but was consistent within gel batches.

Tubulin–BisANS Binding. The binding of bisANS to tubulin was measured with an Amicon micropartition system.

Table 1: Binding of Ligands to Tubulin[a]

daunomycin		bisANS			
		in buffer		in presence of daunomycin[d]	
[S]$_f$[b]	r[c]	[S]$_f$[b]	r[c]	[S]$_f$[b]	r[c]
7.35×10^{-6}	0.5	6.88×10^{-7}	0.22	4.2×10^{-6}	10
1.15×10^{-5}	0.9	6.5×10^{-7}	0.26	1×10^{-5}	12.8
1.29×10^{-5}	1.1	1.8×10^{-6}	0.36	1.8×10^{-5}	18
2.1×10^{-5}	1.9	1.43×10^{-5}	1	3.41×10^{-5}	25.6
1.82×10^{-5}	2	1.63×10^{-5}	1.3	4.7×10^{-5}	33
2.16×10^{-5}	2.5	2.4×10^{-5}	5.4	7×10^{-5}	39.4
3.15×10^{-5}	4	2.6×10^{-5}	1.8	1×10^{-4}	44
4.52×10^{-5}	5.2	2.84×10^{-5}	2.7		
4.91×10^{-5}	6	2.96×10^{-5}	8.6		
6.50×10^{-5}	8	3.16×10^{-5}	3		
6.59×10^{-5}	11	3.6×10^{-5}	4		
9.07×10^{-5}	16.5	4×10^{-5}	13.2		
1.15×10^{-4}	19.5	4.66×10^{-5}	17		
1.11×10^{-4}	23.3	5.56×10^{-5}	20.6		
1.26×10^{-4}	24	6.76×10^{-5}	28.4		
1.37×10^{-4}	27.4	9.26×10^{-5}	33.8		
3.23×10^{-4}	30.4	1.27×10^{-4}	38.2		
3.38×10^{-4}	30.8				
2.1×10^{-4}	31.2				
1.26×10^{-4}	31.8				
2.8×10^{-4}	34.6				

[a] The tubulin concentration was 1×10^{-5} M. [b] Concentration of free ligand in moles per liter. [c] Number of ligand molecules bound to tubulin. [d] The daunomycin concentration was 1.5×10^{-4} M.

YMT membranes and bromobutyl O rings were routinely used. A 1.2 mL solution of protein and ligand was equilibrated for 5 min at 20 °C before application to the micropartition system. The mixture was spun at 4000 rpm for 1 min, the effluent was collected, and the concentration of free or unbound bisANS was measured. The spin time was designed to give 0.2 mL of effluent so as to change minimally the equilibration mixture. The concentration was calculated from the intrinsic fluorescence of free bisANS or its absorbance at 385 nm. Fluorescence measurements were performed with an excitation wavelength of 385 nm and an emission wavelength of 530 nm with 15 nm excitation and emission slits. The fluorescence of the assay solutions was compared to a standard curve of free bisANS calculated under the same conditions. Controls were performed in the absence of tubulin to correct for any binding of free bisANS to the membrane.

RESULTS

Binding of Daunomycin to Tubulin. The binding of daunomycin to tubulin was measured by a combination of frontal gel chromatography (Nichol & Winzor, 1964) and gel partition experiments. The data are presented in Table 1, and the results expressed in Scatchard format are given in Figure 1. It is quite obvious from the abnormal Scatchard plot obtained that the binding of daunomycin to tubulin is not described by a simple rectangular hyperbolic relationship. The concave-downward nature of the Scatchard plot is indicative of positive cooperativity (Nichol & Winzor, 1981). These data indicate that tubulin possesses multiple sites for the binding of daunomycin, which by inspection total approximately 35. The ordinate intercept of a Scatchard plot can be equated to nK_A where n is the total number of ligand binding sites and K_A is the association constant. From these data, the binding constant for the tubulin–daunomycin interaction was estimated to lie between 570 and 1430 M^{-1}, the exact value being difficult to evaluate because of the steep slope of the Scatchard plot in this region.

In order to gauge the degree of cooperativity of the interaction, the data were plotted according to the Hill equation

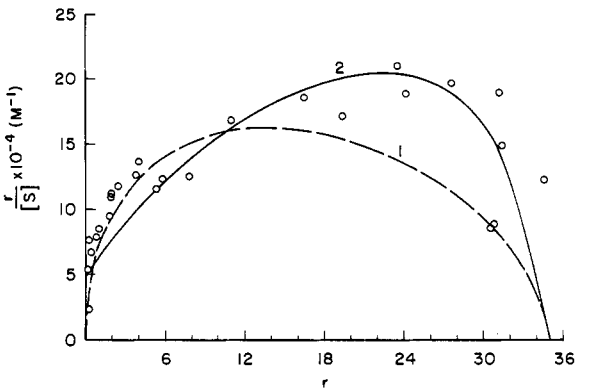

FIGURE 1: Scatchard plot of the equilibrium binding of daunomycin to tubulin in 10 mM phosphate/0.1 mM GTP, pH 7.0, buffer at 20 °C. The tubulin concentration was 1×10^{-5} M. Curve 1 (– –) was generated by fitting the data to the Hill equation (eq 1) using values of $K = 1.04 \times 10^4$ M^{-1}, $n = 35$, and $m = 1.8$; these values have been calculated from a nonlinear regression analysis of the data plotted in the Hill format (Figure 2). Curve 2 (—) was generated according to the relation of McGhee and von Hippel (1974), described by eq 2, using values of $n = 35$, $w = 8$, $K' = 1400$, and $q = 1$.

(Hill, 1910) in logarithmic form:

$$\log \frac{n-r}{r} = -m \log K_A - m \log [S] \qquad (1)$$

where [S] is the free ligand concentration, K_A is an apparent binding constant, n is the total number of sites, r is the average number of sites occupied, and m is the Hill coefficient, which is a measure of the degree of cooperativity of the interaction. The Hill plot is presented in Figure 2. The data fit quite well a linear relation, although a deviation is apparent at the higher free daunomycin concentrations. The line drawn through the data is the result of linear regression analysis. It has a slope of 1.8, which corresponds to the Hill coefficient. This indicates relatively weak cooperativity. The calculated value of K was 1.04×10^4 M^{-1}. The simulated Scatchard plot utilizing these values is shown in Figure 1, curve 1. As expected from the Hill plot, the data fit well to the relation until the degree of saturation becomes greater than 0.5.

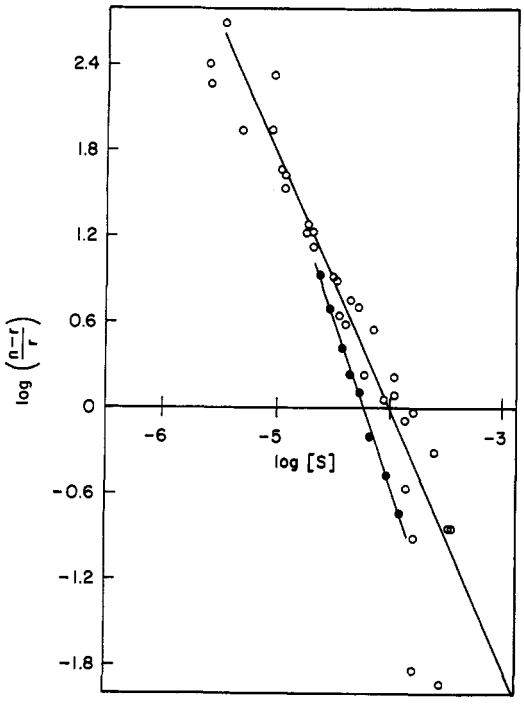

FIGURE 2: Hill plots of the equilibrium binding of daunomycin (○) and bisANS (●) to tubulin in 10 mM phosphate/0.1 mM GTP, pH 7.0, buffer at 20 °C. The tubulin concentrtaion was 1×10^{-5} M. For bisANS, this describes only the binding to low-affinity sites, the contribution of the high-affinity bisANS site having been subtracted at each concentration. The hand-fitted curves were described by the binding parameters $K_A = 1.04 \times 10^4$ M^{-1}, $n = 35$, and $m = 1.8$ for daunomycin and $K_A = 1.6 \times 10^4$ M^{-1}, $n = 45$, and $m = 2.3$ for bisANS. These values were obtained by linear regression analysis of the data plotted in Hill format. The values for n were taken as the points toward which the data tended by inspection of Figures 1 and 3.

Equilibrium Binding of BisANS[1] to Tubulin. The equilibrium binding of bisANS[1] to tubulin–colchicine was measured by ultrafiltration, a method that had been shown to be thermodynamically equivalent to equilibrium dialysis (Sophianopoulos et al., 1978). Tubulin–colchicine was used in preference to tubulin because of the known stabilizing effect of colchicine against the "aging" of tubulin (Ventilla et al., 1972; Prakash & Timasheff, 1992) and the reported increase in the binding of bisANS to aging tubulin (Prasad et al., 1986a). It has also been observed by Prasad et al. (1986a) that the fluorescence signal generated on the binding of bisANS to tubulin increased with time, even when the stock tubulin solution was kept on ice. The binding of colchicine did not interfere with the interaction of bisANS with tubulin, since the biphasic dependence of the fluorescence increase on the binding of bisANS to pure tubulin reported by Prasad et al. (1986a) was also observed with tubulin–colchicine. The binding data are presented in Table 1 and expressed in Scatchard format in Figure 3.

The binding response is unusual. It is characterized by 1 high-affinity binding site (Figure 3B) and 40–50 lower affinity sites. The high-affinity site has an association constant of the order of 4.5×10^5 M^{-1}, which is in agreement with the value of 5×10^5 M^{-1} calculated from fluorescence measurements (Horowitz et al., 1984). As with daunomycin, the concave nature of the Scatchard plot of the binding of the lower affinity sites indicates that they are related in a positively cooperative manner. The degree of cooperativity was assessed by plotting the data in Hill format (Figure 2), after subtracting the contribution of the high-affinity site $[K_1[S]/(1 + K_1[S])$ where $K_1 = 4.5 \times 10^5$ $M^{-1}]$ at each free ligand concentration. The

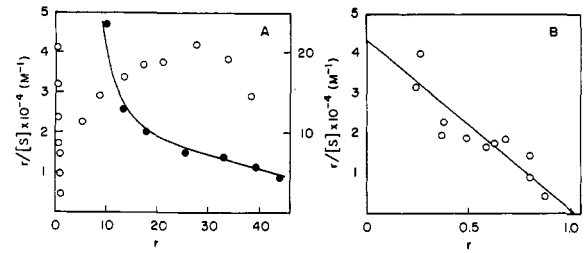

FIGURE 3: (A) Scatchard plot of the equilibrium binding of bisANS to tubulin–colchicine in 10 mM phosphate/0.1 mM GTP, pH 7.0, buffer at 20 °C in the presence of 1.50×10^{-4} M daunomycin (●) and in the absence of daunomycin (○). (B) Scatchard plot in the region of the high-affinity binding site; the line drawn through the data corresponds to $K_A = 4.5 \pm 0.4$ nM^{-1} and $n = 1.01$. The tubulin concentration was 1×10^{-5} M.

degree of cooperativity of the binding of the lower affinity sites is moderate, since it is described by a Hill coefficient of 2.3. Thus, bisANS and daunomycin bind to tubulin in similar manner. Both possess a large number of binding sites (>35) related in a positively cooperative manner.

The binding of ANS[1] to tubulin was also measured by this technique. The observed data could be described in terms of a single binding site with an association constant of 1×10^4 M^{-1}. No low-affinity positively cooperative sites were observed, which is in agreement with the fluorescence titration studies of Lee et al. (1975) and Ward and Timasheff (1988).

Influence of Daunomycin on BisANS Binding. Because of the similar binding responses of daunomycin and bisANS, the binding of the latter was measured in the presence of daunomycin to probe whether any linkage (competition or cooperativity) exists between these two groups of sites. A Scatchard plot that describes the binding of bisANS to tubulin–colchicine in the presence of 1.50×10^{-4} M daunomycin is presented in Figure 3A. The actual data are listed in Table 1. At this concentration, 10 daunomycin sites are occupied when this ligand is used alone, so that binding competition would be clearly detectable. In fact, the binding of bisANS was found to be enhanced in the presence of daunomycin relative to that in its absence, as the number of sites increased to approximately 70. At this concentration of daunomycin, no positive cooperativity was observed, but the curve is indicative of heterogeneous classes of binding sites.

Effects of Other Ligands on the Binding of Daunomycin to Tubulin. The possible overlap of the daunomycin binding site with those of other ligands and the ability of other ligands to influence the binding of daunomycin to tubulin were assessed by their ability to perturb the tubulin–daunomycin difference spectra. Vinblastine (1×10^{-4} M), chlorpromazine (3×10^{-4} M), ANS (1×10^{-4} M), and DAPI (3.3×10^{-5} M) all had no effect on the difference spectra over a daunomycin concentration range of $(2–7) \times 10^{-5}$ M at a tubulin concentration of 1×10^{-5} M. On the basis of the association constants that have been reported for these ligands (Na & Timasheff, 1980a,b; Hinman & Cann, 1976; Lee et al., 1975; Bonne et al., 1985), the inclusion of these ligands at the listed concentrations would be expected to influence daunomycin binding had there been any linkage between daunomycin and the above ligands. In addition, daunomycin bound identically to the tubulin–colchicine and the tubulin–Ruthenium Red complexes. The latter is characterized in the following paper (Ward et al., 1994).

Ligand–Ligand Interactions. Although the binding of daunomycin and bisANS to tubulin can both be described by a positive cooperative binding model, the question remains as

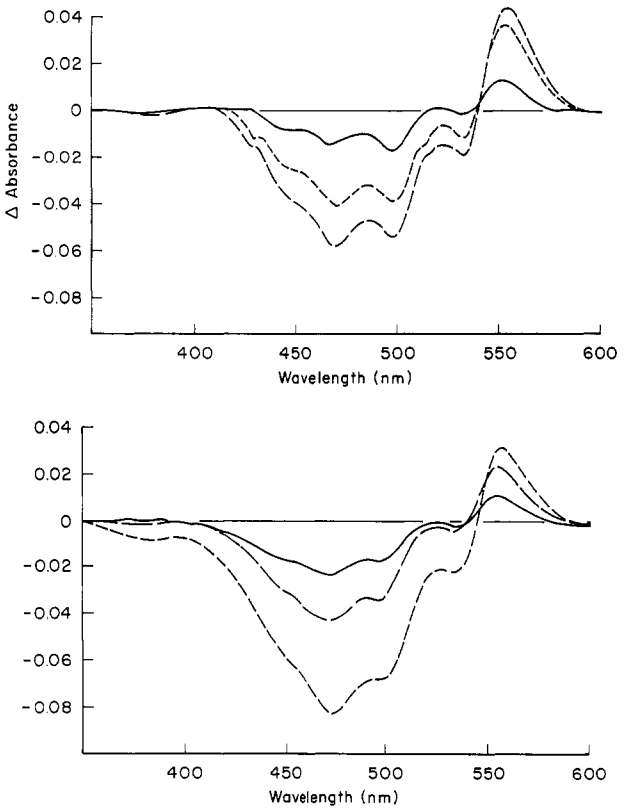

FIGURE 4: (A) Concentration difference spectra of daunomycin in 10 mM phosphate/0.1 mM GTP, pH 7.0, buffer at 20 °C. All spectra were recorded using a 1 mm cell in the sample compartment with a 9.5 mm spacer, relative to a 1 cm cell in the reference compartment. Daunomycin solutions were prepared such that the concentration in the sample cuvette was 9.93 times that in the reference cuvette. The concentration of daunomycin in the sample cuvette was 1.93×10^{-4} M (—), 5.93×10^{-4} M (- - -), and 7.9×10^{-4} M (– –). (B) Visible difference absorption spectra obtained on the binding of daunomycin to tubulin in 10 mM phosphate/0.1 mM GTP, pH 7.0, buffer at 20 °C. The curves correspond to daunomycin concentrations of (—) 2.2×10^{-5} M, (– –) 4.9×10^{-5} M, and (- - -) 7.4×10^{-5} M. The concentration of tubulin was 1×10^{-5} M in all the assays.

to how such an unusual binding response, i.e., the positive cooperativity between such an extremely large number of binding sites on a soluble protein, can be explained at the molecular level. Positive cooperativity has been reported as resulting from the preferential binding of the ligand to altered conformational or polymeric forms of the acceptor (Nichol & Winzor, 1981), from the binding of polymeric forms of the ligand (Hinman & Cann, 1976), and from the contribution to the binding response of interactions between neighboring ligand molecules (Blake & Peacocke, 1968).

Self-Association of Daunomycin. The appreciable interaction between dye molecules required by the last two mechanisms seems possible, since daunomycin is known to self-associate in aqueous solution (Barthelemy-Clavey et al., 1974; Schuetz et al., 1979; Chaires et al., 1982; Martin, 1980; Eksborg, 1978). To probe this possibility, the self-association of daunomycin in the current buffer (10 mM phosphate/0.1 mM GTP, pH 7.0) was investigated by concentration difference spectroscopy and sedimentation velocity analysis. Figure 4A shows the concentration difference spectra of daunomycin. These clearly indicate that the visible absorbance characteristics of the daunomycin chromophore are indeed dependent on concentration. The shapes of the spectra are very similar to the visible difference spectra of daunomycin when bound to tubulin (Figure 4B), with a peak centered at 553 nm, a trough with minima at 497 and 470 nm, and an

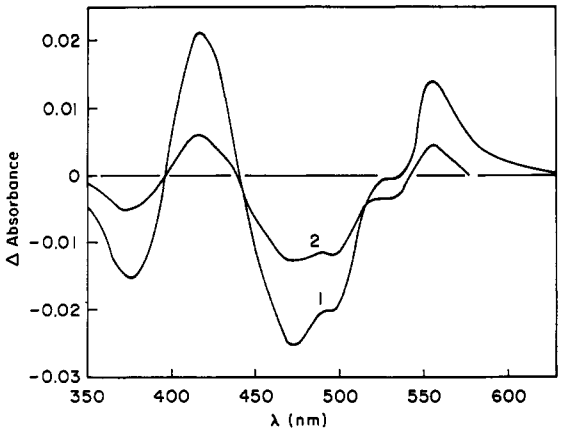

FIGURE 5: Visible daunomycin–bisANS difference spectra generated on the interaction of bisANS and daunomycin in 10 mM phosphate, pH 7.0, buffer at 20 °C. The spectra were obtained on mixing 1.82×10^{-5} M bisANS with 5×10^{-5} M daunomycin (curve 1) and 2.5×10^{-5} M daunomycin (curve 2) and reading relative to identical concentrations of the two reactants in the different compartments of (0.4 + 0.4) cm tandem cells.

isosbestic point at 540 nm. For both the tubulin–daunomycin difference spectra and the concentration difference spectra, the individual regions of the spectra do not display identical concentration dependences. This nonidentical dependence on concentration is paralleled by the increasing prominence of a shoulder at 524 nm. Such a varying concentration dependence of the different regions of the spectrum indicates that daunomycin is self-associating beyond the dimer and that the higher polymers have different absorption characteristics from those of the daunomycin dimer. In order to gain insight into how large the daunomycin polymers are, it was subjected to a sedimentation velocity analysis. The sedimentation coefficient (s_{20}) of daunomycin was found to increase from 0.25 S at 4×10^{-4} M to 0.65 S at 1.2×10^{-3} M and to plateau at that value at higher concentrations. This indicates that the degree of association is low, not proceeding much beyond tetramer. These results eliminate the preferential binding of a large polymeric form (35mer) to tubulin as being the source of the positive cooperativity, as had been found to be the case in the binding of chlorpromazine to tubulin (Cann et al., 1981).

Daunomycin–BisANS Interaction. An alternative mechanism for explaining the positively cooperative nature of the binding is contributions to the binding response of interactions between neighboring ligand molecules (Blake & Peacocke, 1968). In such a case, the binding of daunomycin (Figure 1) and of bisANS (Figure 3) could be explained in terms of the self-association of daunomycin and of bisANS molecules on the surface of the tubulin molecule as well as loosening of the tubulin structure by binding, thus exposing additional sites. The daunomycin enhancement of bisANS binding to tubulin could be explained then by the ability of bound daunomycin molecules to interact with neighboring bisANS molecules on tubulin as well as by binding to newly exposed sites in the partially unfolded protein. Such an interpretation would require bisANS to be able to interact with daunomycin. Figure 5 shows visible difference spectra obtained when free bisANS was mixed with free daunomycin. Interactions between the two molecules do obviously take place, as judged from the large spectroscopic changes with peaks at 555 and 415 nm and troughs at 470 and 375 nm. The perturbations of the daunomycin chromophore on interaction with bisANS, which result in the appearance of the peak and trough at 555 and 470 nm, respectively, are similar to those observed both on the binding of daunomycin to tubulin (Figure 4B) and on the

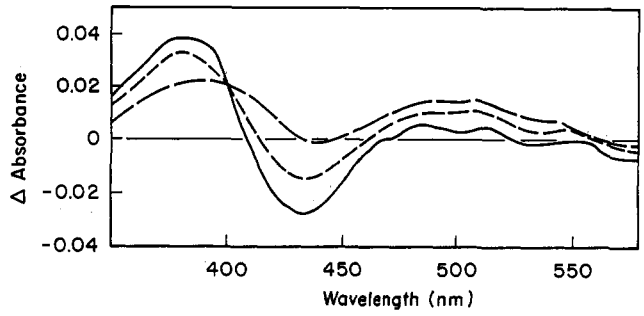

FIGURE 6: Tubulin–daunomycin difference spectra in the presence of bisANS. The concentrations of tubulin and bisANS were 1×10^{-5} M and 2×10^{-5} M in all cases. The daunomycin concentrations were 2.1×10^{-5} M (– –), 3.07×10^{-5} M (- - -), and 4.51×10^{-5} M (—). All assays were performed in 1 mL cuvettes and read relative to the identical concentrations of bisANS and daunomycin in the absence of tubulin in the reference compartment. The base line was recorded by reading tubulin–bisANS in the sample compartment relative to bisANS in the reference compartment to negate the contributions of these components to the difference spectra.

self-association of daunomycin (Figure 4A).

The difference spectra obtained when daunomycin and bisANS are mixed simultaneously with tubulin are presented in Figure 6. Such spectra are difficult to interpret as they actually represent the difference between the difference spectra observed on the binding of bisANS–daunomycin to tubulin and on the binding of bisANS to daunomycin. Nevertheless, these spectra testify to the mutual interaction between the two ligands when bound to tubulin. The net positive absorbance change from 470 to 530 nm indicates that the decrease in absorbance in this region on the binding of daunomycin to bisANS is greater than that on the binding of daunomycin to tubulin in the presence of bisANS. The net positive difference spectrum in this region decreases in magnitude with increasing daunomycin concentration, which indicates that the individual daunomycin molecules bound do not encounter the same environment, something that was observed in the tubulin–daunomycin difference spectrum performed in the absence of bisANS (Figure 4B). The magnitudes of the peak and trough at 381 and 433 nm, which are attributable predominantly to the bisANS chromophore [see following paper (Ward et al., 1994)], increase with increasing daunomycin concentration. These spectra are actually opposite in sign to what is observed on the binding of bisANS to tubulin or on the binding of bisANS to daunomycin (Figure 5). The difference spectra obtained on the binding of bisANS to tubulin [see Ward et al. (1994)] are characterized by peaks at 290 and 415 nm and troughs at 260, 348, and 370 nm. Thus, the net peak at 380–370 nm in Figure 6 actually results from subtraction of a larger negative absorption change in this region on the binding of daunomycin to bisANS than what occurs when daunomycin perturbs tubulin-bound bisANS. Similarly, the trough at 433 nm results from subtraction of a larger positive absorbance in this region on the binding of bisANS to daunomycin than what occurs as a result of perturbation by daunomycin of tubulin-bound bisANS.

DISCUSSION

The results of the present studies indicate that daunomycin and bisANS interact with tubulin in a highly unusual fashion both with respect to the high number of binding sites and with respect to the positively cooperative nature of the binding process. In the case of bisANS, the existence of two classes of binding sites is in agreement with previous spectroscopic studies that showed (i) a biphasic dependence of the observed fluorescence of the bisANS fluorophore on binding to tubulin and (ii) quenching of the intrinsic tubulin protein fluorescence by bound bisANS (Prasad et al., 1986a,b). In these spectroscopic studies (Prasad et al., 1986a), however, only six lower affinity sites were reported. This may reflect the difficulty in evaluating stoichiometries from spectroscopic measurements when the binding isotherm (Figures 1 and 3) is not described by a simple rectangular hyperbolic relationship (Ward, 1985), and the observed fluorescence change on binding of ligand is not directly proportional to the degree of saturation. In addition, the present study was performed over a wider bisANS concentration range [(0–4) $\times 10^{-4}$ M] than that a Prasad et al. (1986a) [(0–1) $\times 10^{-4}$ M], as one-third of the points were above 1×10^{-4} M. Nevertheless, it is difficult to reconcile the fluorescence enhancement data of Prasad et al. (1986a) with six low-affinity binding sites. First, at 1×10^{-4} M bisANS, there is no sign of the fluorescence signal leveling out and, therefore, saturating the lower affinity sites. At this concentration, the fluorescence intensity is already 15 times higher than the intensity when the first site is apparently saturated (Prasad et al., 1986a). Assuming a quantum yield of 0.45 for the high-affinity site and 0.38 for the low-affinity sites (Prasad et al., 1986a), this means that there are already 16–17 bisANS molecules bound at 1×10^{-4} M.

The binding of daunomycin to tubulin had been detected previously on the basis of the ability of daunomycin to quench the intrainsic protein fluorescence of tubulin (Na & Timasheff, 1977). This quenching is probably due, at least in part, to energy transfer between protein and bound daunomycin (Förster, 1948), since daunomycin absorbs strongly in the region of the protein fluorescence. Using this approach, two daunomycin binding sites with $K_A = 3 \times 10^3$ M^{-1} were reported. Inherent to this analysis is the assumption that the different bound ligand molecules possess nonoverlapping quenching volumes and, thus, the fluorescence change is directly proportional to fractional occupancy (Holbrook 1972; Ward, 1985). Such a situation would be expected for a mechanism that involved two binding sites for the ligand on tubulin. Knowledge of the results of the present study would not lead to the expectation that some 35 daunomycin molecules possess nonoverlapping quenching volumes. This rationalizes the different conclusions drawn from the two approaches. It is also not surprising that a deviation from a rectangular hyperbolic relation was not observed in the fluorescence quenching study of Na and Timasheff (1977), as the considerable nonspecific absorption of light by daunomycin, both at the excitation and at the emission wavelengths, prevents data analysis over a wide range of ligand concentrations. Although the inner filter effects can be corrected for over a limited concentration range (Ward, 1985), both the small range of daunomycin concentrations that could be used in the binding analysis and the correction procedure would shield the unusual behavior revealed in the equilibrium binding studies. Similar considerations apply to the failure of Prasad et al. (1986a) to detect the large number of low-affinity binding sites for bisANS.

It is of interest to speculate as to how one obtains a positively cooperative relation for such a large number of binding sites for both bisANS and daunomycin. One possible mechanism could involve conformational changes induced by ligand binding that lead to the enhanced binding of subsequent ligand molecules. In this regard, detergents such as octyl glucoside (60 binding sites) and deoxycholate (95 bindings sites) have

been shown to bind multiply and in a positively cooperative manner to tubulin (Andreu & Munoz, 1986). Associated with the detergent binding is a loosening of the normal compact three-dimensional structure of tubulin (Andreu & Munoz, 1986; Andreu et al., 1986) that results in the increased exposure of hydrophobic and, thus, detergent binding sites. A similar mechanism could be postulated to explain the binding of daunomycin and bisANS to tubulin, particularly in view of the reported increase in bisANS binding on the "aging" of tubulin (Prasad et al., 1986a). The ability of daunomycin to increase the binding of bisANS (Figure 3) could also be accommodated by such a scheme, if daunomycin induced a similar loosening of the tubulin structure that favors the association of other hydrophobic ligands, such as bisANS.

Other mechanisms that could be used to describe this unusual binding phenomenon involve interactions between the ligand molecules themselves. If ligands self-associated over the concentration range used in the binding study and the polymeric forms of the ligand bound preferentially to the protein, a positive cooperative binding response would be obtained. Chlorpromazine binds to tubulin with a stoichiometry of ca. 9 in a positively cooperative manner (Hinman & Cann, 1976). This has been shown to be consistent with a model where a micellar form of chlorpromazine binds preferentially to tubulin (Cann et al., 1981). Sedimentation velocity experiments, however, have demonstrated that daunomycin does not self-associate much beyond trimer of tetramer and that the higher polymers become significant only at concentrations above 6×10^{-4} M. The fact that daunomycin is already binding multiply to tubulin at concentrations less than 5×10^{-5} M renders this mechanism unlikely.

Another mechanism which allows ligand–ligand interactions to promote ligand binding occurs when the ligand binding sites are in close enough proximity to permit interactions between neighboring bound ligand molecules. Such a mechanism has been associated with the binding of aminoacridines and their derivatives to nucleic acids (Blake & Peacocke, 1968) and to polyglutamic acid (Schwarz et al., 1970) at high dye: acceptor ratios. These dyes bind to the acceptor by an electrostatic or hydrogen bonding mechanism. Stacking interactions between neighboring dye molecules result in increased affinities at high degrees of saturation. Daunomycin has been reported to bind to DNA in this manner at high antibiotic to phosphate ratios (Barthelemy-Clavey et al., 1973), the circular dichroism spectrum of these complexes resembling those of self-associated daunomycin (Barthelemy-Clavey et al., 1974). Furthermore, it has been reported (Roche et al., 1994) that some daunomycin analogues bind in a positively cooperative manner to oligonucleotides. This type of mechanism could account for the binding patterns of daunomycin and bisANS to tubulin. The concentration difference spectra of daunomycin (Figure 4A) are very similar to those observed for the tubulin–daunomycin interaction (Figure 4B). Although this may be just a reflection of the involvement of similar functional groups in the two interactions, the hydrogen bonding of daunomycin hydroxyls having been proposed as the mechanism of its binding to tubulin (Na & Timasheff, 1977), the dipole–dipole interactions between stacked neighboring daunomycin molecules would be predicted to influence the spectral properties in the stacked form (Cantor & Timasheff, 1982). In fact, both daunomycin difference spectra show nonidentical concentration dependences at different wavelengths, a shoulder in the region of 530 nm becoming increasingly prominent at high degrees of saturation in both cases.

To test this mechanism, the data on Figure 1 were fitted to the theory of McGhee and von Hippel (1974), which describes the binding of ligands to acceptors with ligand–ligand interactions taken into account. This approach assumes that ligand can bind initially to any of the available binding sites. Cooperativity is introduced only when a ligand molecule binds to a site adjacent to another ligand. The binding equation, expressed in Scatchard format, is

$$\frac{\nu}{S} = K'(1 - q\nu)\left[\frac{(2w + 1)(1 - q\nu) + \nu - R}{2(w - 1)(1 - q\nu)}\right]\left[\frac{1 - (q - 1)\nu + R^2}{2(1 - q\nu)}\right] \quad (2)$$

$$R = \{[1 - (q + 1)\nu]^2 + 4w\nu(1 - 1\nu)\}^{1/2}$$

where S is the free ligand concentration, K' is the association binding constant that describes the interaction between ligand and acceptor, q is the number of lattice sites covered on the binding of each ligand molecule, w is the cooperativity parameter expressed in the form of a unitless equilibrium constant which describes moving a bound ligand from an isolated site to one neighboring another ligand molecule, and ν is the fractional occupancy of ligand, or $r/35$ in this case. In Figure 1, curve 2 shows a Scatchard plot simulated on the basis of this mechanism with the assumption of 35 daunomycin binding sites, with $q = 1$ (i.e., each daunomycin does not overlap more than 1 binding site), $K' = 1400$ M^{-1}, and $w = 8$. It is evident that data at $r > 8$ are fitted reasonably well by this mechanism. Below $r = 6$, however, the ordinate values are systematically lower than the experimental points. Therefore, while this mechanism may be partly involved in the cooperative binding, the situation is obviously more complicated.

Comparison of Figures 1 and 2 shows that, while the stacking cooperativity mechanism may describe the results at high ligand concentration, the low concentration region is described better by simple Hill cooperativity. This points to the complexity of the cooperativity in this system, which at present cannot be resolved. The increase in bisANS binding induced by daunomycin, however, strongly suggests that the process is complicated by loosening of the protein with the generation of new binding sites. In light of this, the binding constants, K_A of eq 1 and K' of eq 2, when applied to the current data, should be regarded simply as data describing parameters. The greater magnitude of K_A than K' reflects the fact that K_A contains the cooperativity, while in eq 2, linkage is expressed explicitly through the parameter w.

A mechanism that involves ligand–ligand interactions requires that the protein possess a domain that allows the multiple binding of ligand molecules in the vicinity of each other, thus permitting stacking interactions between neighboring ligand molecules to contribute to the free energy of the binding process. Daunomycin does possess the ability to bind to bisANS as judged from the changes in the chromophoric properties of both the bisANS and daunomycin molecules on mixing (Figure 5). Fluorescence polarization studies of the interaction of bisANS with tubulin have shown that tubulin-bound bisANS was characterized by a rapid rotational relaxation time, which was also time and concentration dependent (Prasad et al., 1986b). Such rapid rotational relaxation times could reflect the binding of bisANS to a flexible region of the protein. The fact that the total signal is characterized by this rapid relaxation time permits us to speculate that the majority of the bisANS sites lie in this flexible domain. The time dependence is consistent with a

loosening of protein structure. The depolarization of a fluorescence signal can also occur by mechanisms other than rotation of the fluorophore. As pointed out by Prasad et al. (1986b), one such alternate mechanism is fluorescence energy transfer. A mechanism of binding where ligand–ligand interactions introduce cooperativity obviously requires that ligands bind adjacent to one another, which would allow efficient energy transfer to occur. Such a mechanism would also be consistent with the concentration dependence of the rotational relaxation time.

As a final remark, the results of this study emphasize that care must be taken in equating indirect binding data, such as spectroscopic, with the true equilibrium binding, as measured by direct techniques. Tubulin appears to have the ability to bind hydrophobic ligands strongly, multiply, and in a positively cooperative manner accompanied by loosening of the protein structure. The mechanism of this process is not clear. This process is obviously not highly specific with regard to ligand structure, since similar results were obtained with daunomycin and bisANS. When studying linkages between such ligands, the possible implications of direct ligand–ligand interactions should be considered, since hydrophobic ligands, such as daunomycin and bisANS, can interact strongly with each other. The multiple binding of the two ligands makes them unsuitable for energy transfer experiments at high ligand concentrations. BisANS, however, at low concentration, 10^{-5} M, binds predominantly to a single high-affinity site and is suitable both as an energy donor and as an energy acceptor in fluorescence energy transfer studies. Such studies are reported in the following paper (Ward et al., 1994).

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