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Binding of Vinblastine to Stabilized Microtubules

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SUMMARY

Addition of 2–200 μM vinblastine to microtubules at steady state *in vitro* causes the microtubules to depolymerize, with the formation of protofilament spirals and other aggregated forms of microtubule protein. The presence of such spirals and protein aggregates, which are difficult to separate from microtubules, has complicated attempts to measure the binding of vinblastine to microtubules. We have found that stabilizing bovine brain microtubules *in vitro* with dimethyl sulfoxide, taxol, or a combination of dimethyl sulfoxide and taxol prevents or greatly retards the formation of protofilamentous spirals, thus permitting us to measure the binding of vinblastine to intact microtubules. Reciprocal plots of binding data indicate the presence of 1.4–1.7 vinblastine binding sites/mol of tubulin in the microtubule, with a K_a of approximately 3–4 × 10^3 M^{-1}. The K_a value obtained is within 1 order of magnitude of the apparent intrinsic binding constant for the binding of vinblastine to tubulin dimers. The results support the idea that depolymerization of microtubules by intermediate and high concentrations of vinblastine occurs by stoichiometric binding of vinblastine to tubulin along the microtubule surface.

The *Catharanthus roseus* dimeric alkaloids (*Vinca* alkaloids) are antimitotic drugs that exert their antiproliferative effects on mammalian cells, at least in part, by interfering with the organization and function of microtubules. The binding of the *Vinca* alkaloids to tubulin, the dimeric subunit of microtubules, has been well characterized (1–6) and recent work has helped explain major differences in the reported affinity constants (7–9). Much less is known about the binding of the alkaloids to microtubules. A major question is whether the alkaloid binds along the wall of the microtubule or just to the ends. In previous work with VLB at concentrations less than 1 μM (10, 11), we found that binding occurs at microtubule ends to a small number of sites with apparent high affinity. Data were also obtained that suggested that at higher concentrations (approximately 2 to 100 μM) binding occurs stoichiometrically to tubulin along the microtubule wall, with an apparent lower affinity than that observed at the ends of the microtubules. Our hypothesis has been that, at low concentrations, VLB interacts with sites located at the microtubule ends, and such interactions affect the dynamics of tubulin subunit addition and loss at the ends. At higher concentrations, VLB interacts with binding sites located along the microtubule, and these interactions result in the splaying and peeling of protofilaments (11). A problem encountered in the studies on the binding of the *Vinca* alkaloids to microtubules at the higher concentrations is that the rapid peeling into protofilament spirals made it difficult to exclude the possibilities that 1) the VLB binding to the small quantities of residual microtubules was due to binding to amorphous aggregates or spirals and 2) binding was to a very small subpopulation of stable microtubules.

In the work reported here, we have studied VLB binding to stabilized microtubules under conditions in which the formation of spirals was largely reduced or prevented entirely. The results demonstrate that stoichiometric binding of VLB to the walls of microtubules does occur and that the binding has a weak apparent association constant.

Materials and Methods

Purification of microtubule protein and tubulin. Bovine brain MTP was isolated by two cycles of disassembly and assembly, by the procedure of Shelanski *et al.* (12), and was stored as described by Nath *et al.* (13). Tubulin was purified from the MTP preparation by phosphocellulose chromatography, as described by Algaier and Himes (14).

VLB binding experiments. MTP was thawed and polymerized at 37° in the presence of PEM buffer (0.1 M PIPES, 1 mM $MgSO_4$, 1 mM EGTA, pH 6.9) and 0.5 mM GTP. The microtubules were pelleted at 100,000 × *g* for 20 min and resuspended in cold buffer. Suspensions were kept on ice for 15 min to depolymerize the microtubules and then were centrifuged at 27,000 × *g* for 10 min. The resultant supernatants were polymerized at a protein concentration of 2.5–3 mg/ml, at 37°, for 15 min in the presence of 0.5 mM GTP. In certain experiments, DMSO was included during polymerization or taxol was added after steady state was attained. In some cases, the microtubule suspension was transferred to a 25° bath after steady state was achieved.

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ABBREVIATIONS: VLB, vinblastine; MTP, microtubule protein; DMSO, dimethylsulfoxide; PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethylether)-*N,N,N'N'*-tetraacetic acid.

Binding to microtubules was measured by a centrifugation assay. VLB (a gift of Eli Lilly & Co. or purchased from Sigma Chemical Co.), containing sufficient [^{3}H]VLB (Amersham or Moravek) to give specific activities ranging from 6×10^6 to 6×10^7 cpm/μmol, was added to a suspension of microtubules to achieve the desired final concentration. Polymerized material was collected by centrifuging 150 μl at 120,000 × *g* for 4 min in a Beckman airfuge or 300 μl at 200,000 × *g* for 5 min in a Beckman TL 100 ultracentrifuge. After removal of the supernatants, the walls of the tubes were rinsed quickly three times with warm PEM buffer and the pellets were dissolved in 200 μl of 0.1 M NaOH. These tubulin-VLB samples typically contained 4×10^4 to 2×10^5 cpm/ml. To control for label being trapped in the pellets, [^{3}H]sodium formate was added before centrifugation to samples that did not contain VLB. The amount of formate trapped was very small, usually about 1% of the amount added. From this determination, it was calculated that 9 to 16% of the VLB found in the pellet, depending on the initial concentration, was due to trapped VLB. Some centrifugations were done through sucrose cushions. In these cases, samples were layered on top of 4 ml of a solution of 40 or 60% sucrose in PEM buffer and were centrifuged in a Beckman 65 rotor at 130,000 × *g* for 90 min (40% sucrose) or 150 min (60% sucrose). After the supernatants were carefully removed, the pellets and sides of the centrifuge tubes were washed three times with warm PEM buffer. Pellets were dissolved in 200 μl of 0.1 M NaOH for protein and radioactivity determinations. In calculating the amount of VLB bound to tubulin, it was assumed that the tubulin represented 70% of the total protein. Protein determinations were done by the method of Bradford (15).

Binding to unpolymerized MTP and tubulin was measured by a column centrifugation assay, using Sephadex G-25 packed in 1-ml syringes, as described previously (9). Samples containing 200 μl of 2 μM tubulin and 5 μM [^{3}H]VLB in PEM buffer were centrifuged through 0.5 ml of Sephadex G-25 after incubation for 5 or 90 min at room temperature.

For electron microscopic examination of negatively stained products, aliquots were diluted 10- to 20-fold in PEM buffer containing 0.25% glutaraldehyde. The solutions were then applied to formvar- and carbon-coated grids. After 30 sec, excess fluid was drawn off using torn edges of filter paper. Grids were washed three times with H_2O, followed by a 30-sec exposure to 2% uranyl acetate. For thin-section examination, samples were pelleted in a Beckman TL 100 ultracentrifuge. Pellets were fixed with 2.5% glutaraldehyde/8% tannic acid in 50 mM phosphate buffer, pH 6.8, post-fixed with 1% osmium tetroxide, dehydrated in an acetone series, and embedded in Araldite. Sections were placed on uncoated copper grids and stained with 2% methanolic uranyl acetate and lead citrate. Grids were viewed in a Philips 300 electron microscope.

Results

Binding stoichiometry of VLB to unpolymerized MTP and tubulin. We first determined the amount of binding obtained to unpolymerized tubulin, using the gel filtration centrifugation technique under the solution conditions used in most of the experiments reported in this work. In PEM buffer, using 5 μM VLB and 2 μM tubulin, 0.4 mol of VLB/mol of tubulin was bound, an amount similar to that found in earlier work (9). The amount of binding increased little between 5 and 90 min. DMSO (10%) and 20 μM taxol, the two stabilizing compounds used in this work, had no effect on the VLB binding stoichiometry either alone or in combination. The lack of an effect of taxol is consistent with the view that this drug and active derivatives of it bind to microtubules but not to unpolymerized tubulin (16–18). Binding of VLB to unpolymerized MTP was also not affected by DMSO or taxol.

Binding of VLB to microtubules under stabilizing and nonstabilizing conditions. Binding of VLB to microtubules that had previously been assembled to steady state was measured, under a variety of conditions, as a function of time of incubation. The data are presented in Fig. 1. These experiments were done under conditions in which unstabilized microtubules were rapidly converted into spiral structures upon addition of VLB and under a variety of conditions designed to retard the rate of spiral formation. When 40 μM VLB was added, there was a very rapid binding of VLB to the polymerized MTP, which varied between 0.1 and 0.4 mol of VLB/mol tubulin, depending on whether a stabilizing agent was present. At 37°, unstabilizied microtubule-associated protein-rich microtubules initially bound about 0.4 mol of VLB/mol of tubulin. This was followed by an increase in binding, which appeared to approach a plateau value of approximately 0.6 mol of VLB/mol of tubulin after about 30 min (Fig. 1, *curve 1*). When 10% DMSO was present, the initial binding was less than that obtained with unstabilized microtubules, but binding still increased with time to a plateau at approximately 40 min (Fig. 1, *curve 2*). In both cases, the formation of spiral structures was evident (Fig. 2, A and B) and it is probable that the observed time-dependent increase in binding was due to the exposure of more sites as the microtubules were converted into protofilament arrays (see Discussion).

Because of the relatively rapid rate of spiral formation, it was not possible to determine how much VLB binding was to spirals and how much was to intact microtubules. In an attempt to resolve the problem created by the formation of spirals, we sought ways of preventing or slowing the disruption of the microtubules into spiral structures. We found that taxol, a known stabilizer of microtubules (19), significantly retarded the rate of spiral formation. Spirals were not detected by electron microscopy until more than 30 min of incubation in

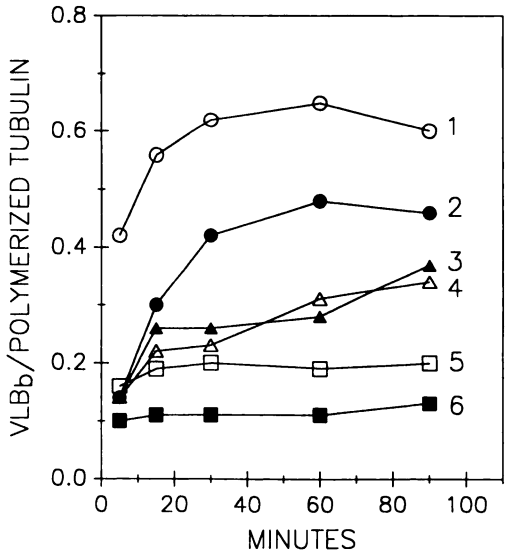

Fig. 1. Binding of VLB to preformed microtubules. MTP (2.5 mg/ml) was assembled for 15 min to steady state as described in Materials and Methods. In the experiments represented by *curves 2*, *3*, and *6*, 10% DMSO was included in the reaction mixture. In the experiment represented by *curve 4*, 20 μM taxol was added before incubation at 37°. In the experiments represented by *curves 5* and *6*, 20 μM taxol was added after the 37° incubation. In the experiments represented by *Curves 3*, *5*, and *6*, the microtubule suspension was shifted to 25° for 10 min after the initial assembly at 37°. [^{3}H]VLB (40 μM) was added to each sample and aliquots were centrifuged in the absence of sucrose in a Beckman TL 100 centrifuge, as described in Materials and Methods.

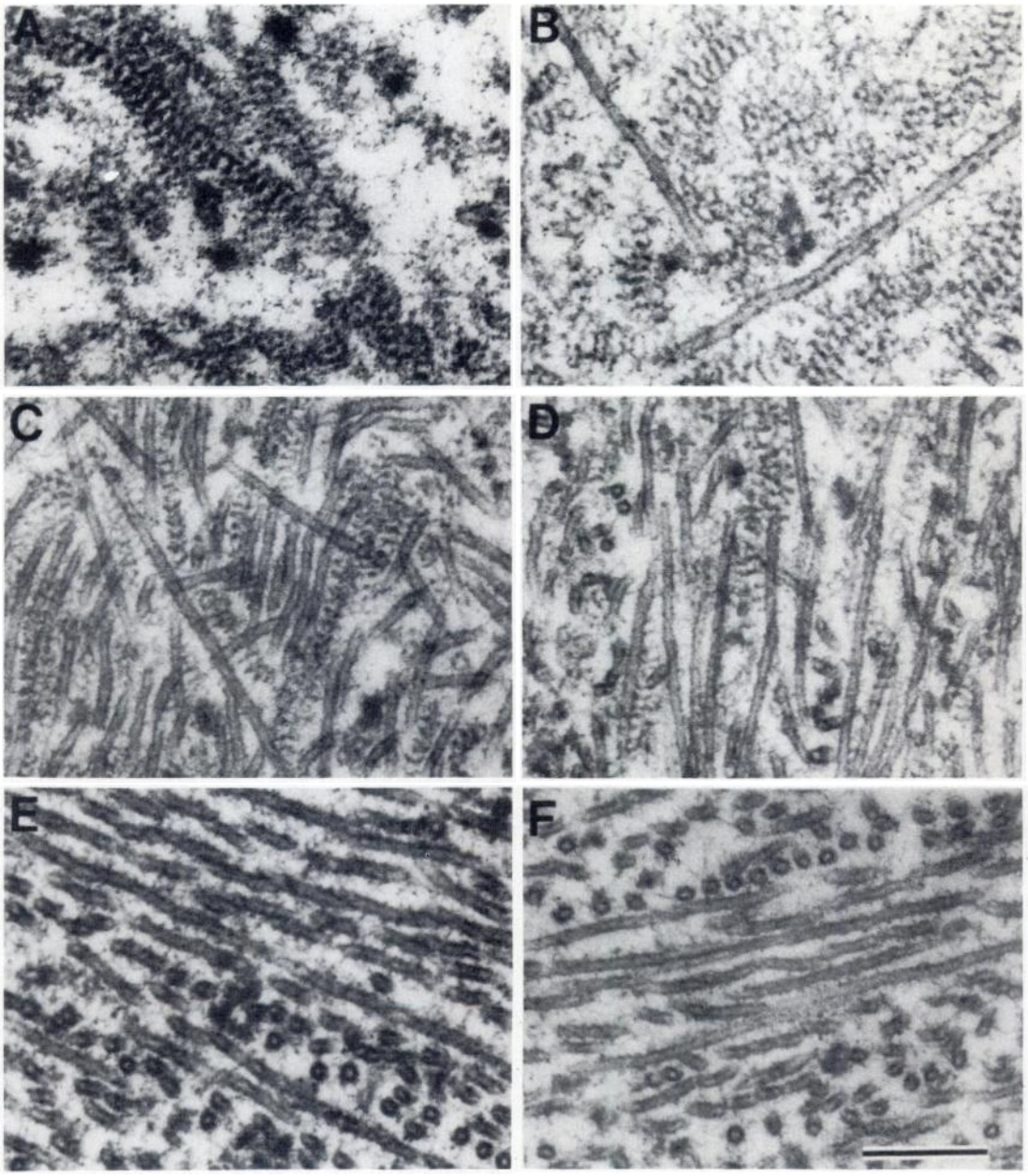

Fig. 2. Electron micrographs of samples treated with VLB. An experiment, similar to the one described in Fig. 1, was done and samples of pellets representing 60 min of incubation with VLB were sectioned and stained. A–F represent *curves 1–6*, respectively, in Fig. 1. *Magnification bar*, 228 nm.

20 μM taxol at 37°, and many microtubules were still present after 60 min of incubation (Fig. 2D). The binding of VLB in the presence of 20 μM taxol at 37° is shown in Fig. 1, *curve 4*. Initial binding was reduced to somewhat below 0.2 mol of VLB/mol of tubulin, and binding increased slowly to approximately 0.3 mol of VLB/mol of tubulin at 90 min, a value approximately half of that obtained with unstabilized microtubules.

To increase stability further, the temperature of the microtubule suspensions formed at 37° in the absence or presence of DMSO was reduced to 25°, followed in some cases by the addition of taxol, before the addition of VLB (Fig. 1, *curves 3, 5,* and *6*). When taxol was present with or without DMSO, there was little increase in the amount of VLB bound following the initial binding (Fig. 1, *curves 5* and *6*). Electron microscopic examination showed that, at early time points, spirals were not present and only very few had formed after 60 min of incubation (Fig. 2, E and F).

Effect of taxol on VLB binding. As shown in Fig. 1, the amount of binding that occurred within the first few minutes after addition of VLB in the presence of 20 μM taxol was lower than the initial binding to unstabilized microtubules (Fig. 1, *curves 1* and *4–6*). We found that taxol reduced the amount of the initial binding in a concentration-dependent manner. The data in Fig. 3 demonstrate this effect of taxol on VLB binding under conditions of an 11-fold excess of VLB over tubulin.

Effect of VLB concentration on initial binding. By using microtubules stabilized at 25° with either taxol or DMSO or with both taxol and DMSO, it was possible to measure the

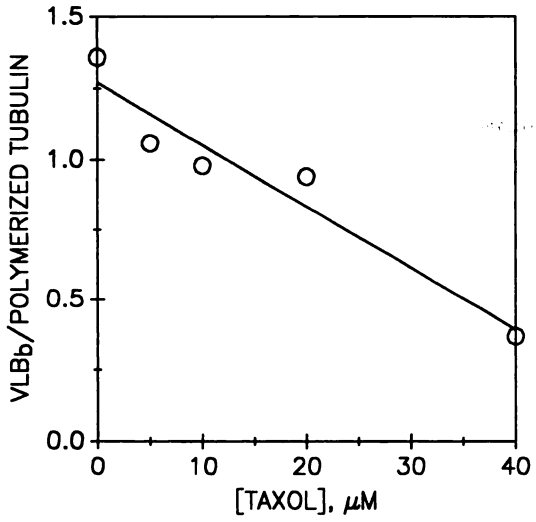

Fig. 3. Effect of taxol concentration on the initial binding of VLB. MTP (2.5 mg/ml) was polymerized at 37° for 15 min and then transferred to 25°. Taxol at the concentrations shown was added, followed 10 min later by 200 μM [^{3}H]VLB. After several minutes, samples were centrifuged in the absence of sucrose.

initial binding (within 5 min) of VLB to microtubules as a function of VLB concentration. Under these conditions, no spirals were observed. Data are presented in reciprocal plot form in Fig. 4. Reciprocals of the ordinate intercepts gave values of 1.4 to 1.7 VLB binding sites/mol of tubulin in microtubules. K_a values obtained from the abscissa intercepts were 3.9×10^3 M^{-1} in DMSO alone, 3.8×10^3 M^{-1} in 20 μM taxol alone, and 2.9×10^3 M^{-1} in DMSO and 20 μM taxol. These values are good estimates, but, because the highest VLB concentration used (150 μM) was less than the estimated K_d values, the extrapolations are quite long and, as a result, there is uncertainty in the values. It was not practical to use higher concentrations of VLB because it would have caused the protofilaments to splay apart and the protein to precipitate. Binding was unaffected by a 50-fold reduction in the Mg^{2+} concentration or by the addition of 3 mM Ca^{2+} in the absence of EGTA. However, the K_a increased approximately 2-fold when the PIPES concentration in the buffer was reduced from 0.1 M to 0.02 M by diluting a concentrated microtubule suspension into a 20 μM taxol/10% DMSO solution.

In previous studies (10, 11), it was found that microtubules, but not spirals, were effectively pelleted by centrifugation through 60% sucrose cushions but that both microtubules and large spirals were sedimented through 40% sucrose. As further evidence that spirals were not produced under the most effective stabilizing conditions, we found that, after 130 min in the presence of 40 μM VLB, 20 μM taxol, and 10% DMSO, 76% of the protein was recovered in the pellet after centrifugation through 60% sucrose, as described in Materials and Methods. This compares with 85% that was recovered in a pellet by direct centrifugation. In contrast, in the absence of taxol and DMSO, no pellet was obtained after centrifugation through 60% sucrose. It was also found that about 50% of the VLB that was bound to stabilized microtubules was released during centrifugation through the sucrose solution. Spirals, which sediment through 40% sucrose, retained all of the bound VLB after such a centrifugation. This result suggests that VLB that is bound to the walls of stabilized microtubules is more easily released than that bound to spiral structures.

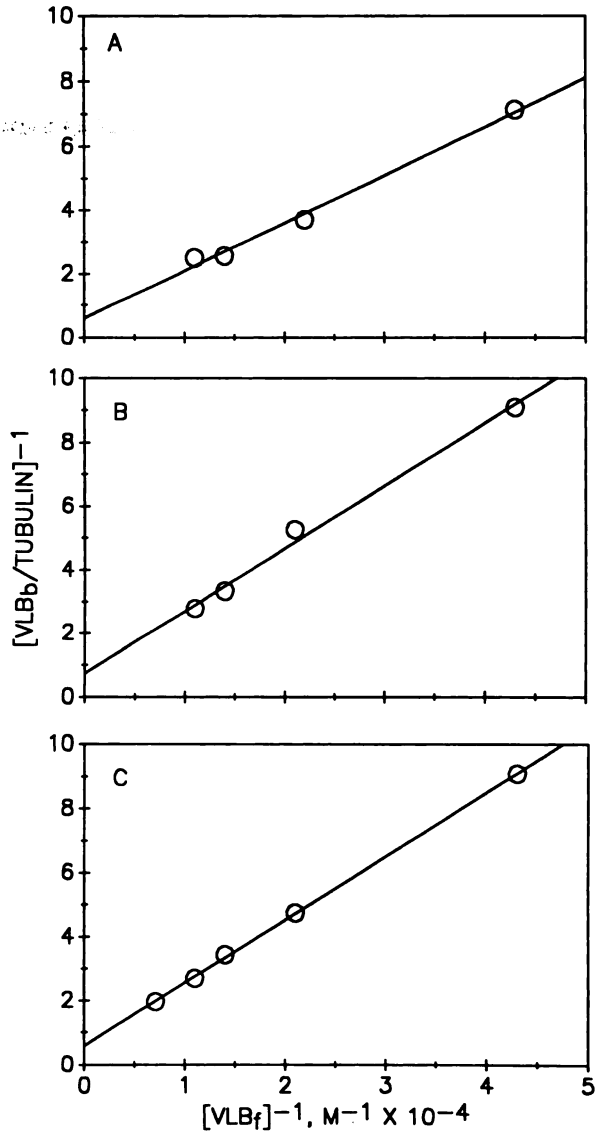

Fig. 4. Concentration dependence of the initial VLB binding. MTP (2.5 mg/ml) was polymerized in the absence or presence of 10% DMSO at 37° for 15 min and the samples were then transferred to 25°. Taxol (20 μM) was added and after 10 min was followed by different concentrations of [^{3}H]VLB. After 5 min, samples were centrifuged in the absence of sucrose. A, DMSO; B, taxol; C, DMSO and taxol. *VLB_f*, the calculated free VLB concentration.

Discussion

The results presented here corroborate, by an independent and more direct method, our previous results indicating that VLB binds to sites on the microtubule surface along the entire length of polymer. This class of sites appears to have a relatively low affinity, with one to two sites/molecule of tubulin in the microtubule. These sites have been hypothesized to be responsible for inducing peeling of protofilaments at microtubule ends (11), an action that occurs at concentrations of 2 μM VLB and higher (10, 11, 20–23). This concentration range could be attained initially in the blood of patients given the drug. Thus, microtubule disintegration through interaction of VLB with the surface sites described in this study may play a role in the mechanism of action of the drug.

Our previous studies designed to measure the binding of VLB

to microtubules at these concentrations had been problematic, due to 1) protofilament peeling during incubation with the drug, 2) the inherent lability of the microtubules, and 3) difficulties encountered in separating the microtubules that remain from the protofilament spirals and other forms of aggregated tubulin that are generated during drug incubation. To circumvent these difficulties, we stabilized microtubules with taxol and DMSO before addition of VLB. In this state, microtubules bind VLB rapidly, with an apparent K_a value of approximately $3–4 \times 10^3$ M^{-1} in the presence of taxol, DMSO, or both DMSO and taxol. This value may be lower than the true value in the absence of microtubule stabilizers, because the stabilizing agents decrease the stoichiometry of VLB binding (Figs. 1 and 3).

There is substantial evidence that these apparently low affinity sites belong to one of at least two and possibly three classes of VLB binding sites present on microtubules. One class of sites with apparent high affinity (5.3×10^5 M^{-1}) has been identified and appears to be located at microtubule ends (10). The binding of *Vinca* drugs to this class of sites is thought to be responsible for the ability of *Vinca* drugs to poison tubulin addition and loss reactions at microtubule ends at low concentrations. The second class of sites of apparent lower affinity is described here and in previous work (11) and exists along the surfaces of microtubules. A third class of sites, which are considered to be entirely nonspecific and to exhibit extremely low affinity, has also been postulated to exist. This idea is based in part upon data obtained with sea urchin egg tubulin paracrystals, indicating that seven VLB binding sites/molecule of tubulin can be titrated at very high concentrations of the drug (>1 mM) and that, at very high concentrations, the drug, like high concentrations of calcium ions, causes tubulin to precipitate from solution. This apparently completely nonspecific action may be due to an ionic interaction of the drug with negatively charged residues exposed on the surface of the tubulin molecule (24). High concentrations of calcium (e.g., 5 mM) do not affect the binding of VLB to the two relatively high affinity VLB binding sites of tubulin in paracrystals (24).

The difference in apparent affinity of VLB for sites at the microtubule ends and those in the core of the microtubule could be explained by one of the following: 1) there is a real difference in the intrinsic affinity of the sites due to a conformational difference between tubulin in the core and tubulin at the ends or 2) binding sites in the core may have reduced affinity as compared with tubulin at the ends as a result of occlusion (concealment) by neighboring dimers. The first mechanism may be related to the differences in affinity observed in the binding of VLB to soluble tubulin, due to tubulin self-association (aggregation) as modeled by Na and Timasheff (7).

From an analysis of the multiple equilibria that exist in a tubulin-VLB solution (without microtubules present), Na and Timasheff (7) concluded that the intrinsic association constant for VLB binding to tubulin is 4×10^4 M^{-1}. According to their model, VLB binding to this site is specifically linked to the ability of VLB to induce tubulin self-association, the binding constant to polymerized tubulin being $\geq 4 \times 10^6$ M^{-1}. Additional nonspecific sites (not involved in VLB-induced tubulin self-association), having a K_a of $\leq 5 \times 10^3$ M^{-1}, were also modeled. It is interesting that the K_a we observed for the binding of VLB to DMSO- and/or taxol-stabilized microtubule-associated protein-containing microtubules is in the range for the additional sites and is about 5–10-fold lower than their reported intrinsic binding constant. It remains unclear whether the VLB binding activity we measured along the surface of intact microtubules is due to the binding of VLB to the specific site or to the additional sites [using the terminology of Na and Timasheff (7)].

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