Fasella, P., Hammes, G. G., & Schimmel, P. R. (1965) *Biochim. Biophys. Acta 103*, 708-710.
Frigon, R. P., & Lee, J. C. (1972) *Arch. Biochem. Biophys. 153*, 587-589.
Gellote, B. (1960) *J. Chromatogr. 3*, 330-342.
Hirose, M., & Kano, Y. (1971) *Biochim. Biophys. Acta 251*, 376-379.
Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530-532.
Janson, J.-C. (1967) *J. Chromatogr. 28*, 12-20.
Kegeles, G., & Cann, J. R. (1978) *Methods Enzymol. 48*, 248-270.
Lee, J. C., Frigon, R. P., & Timasheff, S. N. (1973) *J. Biol. Chem. 248*, 7253-7262.
Lee, J. C., Harrison, D., & Timasheff, S. N. (1975) *J. Biol. Chem. 250*, 9276-9282.
Malawista, S. F., & Sato, H. (1969) *J. Cell Biol. 42*, 596-599.
Na, G. C., & Timasheff, S. N. (1980a) *Biochemistry 19*, 1347-1354.
Na, G. C., & Timasheff, S. N. (1980b) *Biochemistry 19*, 1355-1365.
Na, G. C., & Timasheff, S. N. (1980c) American Chemical Society Meeting, Las Vegas, NV, Abstract Biol. 170.
Na, G. C., & Timasheff, S. N. (1981a) *J. Mol. Biol. 151*, 165-178.
Na, G. C., & Timasheff, S. N. (1981b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1548.
Na, G. C., & Timasheff, S. N. (1982) *Methods Enzymol. 85*, 393-408.
Na, G. C., & Timasheff, S. N. (1986) *Biochemistry* (following paper in this issue).
Nichol, L. W., & Winzor, D. J. (1976) *Biochemistry 15*, 3015-3019.
Pearlman, W. H., & Crêpy, O. (1967) *J. Biol. Chem. 242*, 182-189.
Weisenberg, R. C., & Timasheff, S. N. (1970) *Biochemistry 9*, 4110-4116.
Weisenberg, R. C., Borisy, G., & Taylor, E. (1968) *Biochemistry 7*, 4466-4479.
Wilson, L., Bryan, J., Ruby, A., & Mazia, D. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 807-814.
Wilson, L., Creswell, K. M., & Chin, D. (1975) *Biochemistry 14*, 5586-5592.
Wilson, L., Morse, A. N. C., & Bryan, J. (1978) *J. Mol. Biol. 121*, 255-268.

Interaction of Vinblastine with Calf Brain Tubulin: Effects of Magnesium Ions†

George C. Na‡ and Serge N. Timasheff*
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254
Received January 17, 1986; Revised Manuscript Received May 28, 1986

ABSTRACT: The effects of magnesium ions on the binding of the anticancer drug vinblastine to calf brain tubulin were investigated by a batch gel equilibration method. Magnesium ions at 1 mM strongly enhanced the binding of the first vinblastine molecule to each tubulin dimer without affecting either the drug affinity toward the rest of the binding site or the total stoichiometry of the vinblastine binding to tubulin. Sedimentation velocity studies indicated that magnesium ions can enhance strongly the vinblastine-induced tubulin self-association and suggested that the drug-induced self-association still proceeds through the isodesmic indefinite mechanism in the presence of the divalent cation. In PG buffer (0.01 M NaP_i, 10^{-4} M GTP, pH 7.0) containing more than 2.5 mM $MgCl_2$, vinblastine induced tubulin to form large amorphous aggregates. The aggregate formation was rapid and took place at a drug stoichiometry between 0.7 and 1.0 mol of vinblastine per mole of tubulin dimers. Increasing the solution ionic strength decreased the rate of aggregate formation. Between an ionic strength of 0.05 and 0.1, the self-association led to the formation of paracrystalline aggregates instead of the amorphous ones. The results indicated that the binding of only the first vinblastine molecule to each tubulin dimer is linked to the self-association of the protein. They also confirmed our previously proposed rationale for the disagreement among the vinblastine–tubulin binding constants reported in the literature in terms of the different magnesium ion concentrations and ionic strength of the buffers used in the various studies.

In the preceding paper (Na & Timasheff, 1986), we have examined the binding of vinblastine to tubulin as a function of protein concentration and probed the equilibrium linkages between the drug binding and the protein self-association reactions. In that study, in order to simplify the system, magnesium ions were not added to the solution. This rendered vinblastine the only strong ligand effector in the solution. Magnesium ions, however, are known to exert a strong influence on the vinblastine-induced tubulin self-association. Early studies by Weisenberg and Timasheff (1970) had shown that, in the presence of magnesium ions, vinblastine induced the formation of large tubulin aggregates that precipitated from the solution without centrifugation. More recently, we have shown that magnesium ions are required for the in vitro formation of vinblastine–tubulin paracrystals (Na & Timasheff, 1982).

In the analysis of the vinblastine-induced tubulin self-association, we proposed that if magnesium ions can enhance the vinblastine-induced tubulin self-association, they should also enhance the vinblastine binding to the protein, resulting

† This work was supported in part by NIH Grants CA-16707 and GM-14603. This paper is Publication No. 1594 from the Graduate Department of Biochemistry, Brandeis University.
‡ Present address: U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118.

0006-2960/86/0425-6222$01.50/0

in a strong increase in the apparent binding constants (Na & Timasheff, 1980b). This proposal accounted for the differences between the vinblastine–tubulin binding constants reported in the literature, since their magnitudes appear to increase with the magnesium ion concentration used in the buffer (Lee et al., 1975; Wilson et al., 1975; Bhattacharyya & Wolff, 1976). To substantiate the above proposition and to further probe the effect of magnesium ions on the activity of the drug, a study was carried out on the effects of magnesium ions on both the binding of vinblastine to tubulin and the vinblastine-induced tubulin self-association, and the results are reported in this paper. Preliminary reports of this work have been presented earlier (Na & Timasheff, 1980c, 1981).

Materials and Methods

Measurement of Vinblastine Binding to Tubulin. The binding of vinblastine to calf brain tubulin was measured with the same batch gel equilibration method as described in the preceding paper (Na & Timasheff, 1986). The only exception was that the PG buffer[1] contained 1 mM $MgCl_2$. The correction for drug adsorption to the gel matrix was carried out with the same distribution curve as shown in the preceding paper, since it was found that the presence of 1 mM $MgCl_2$ did not alter the adsorption of the drug to the gel.

Sedimentation Velocity. Sedimentation velocity experiments were performed with a Beckman[2] Model E analytical ultracentrifuge. Kel-F centerpieces and quartz windows were used. Sedimentation boundary images were recorded on Kodak metallographic plates.

Turbidimetric Titrations. The formation of tubulin aggregates during titration with vinblastine was monitored from the solution turbidity by using a Cary 118 UV–visible spectrophotometer. One milliliter of tubulin solution was added to a semimicro cuvette with a fitted Teflon cap. The titration was performed at ambient temperature by the addition of small aliquots of 10^{-2} M vinblastine, followed by gentle shaking of the mixture for 5 min and measurement of its turbidity at 350 nm.

Results

Vinblastine–Tubulin Binding in the Presence of 1 mM $MgCl_2$. The binding of vinblastine to calf brain tubulin in the presence of 1 mM $MgCl_2$ was determined at a tubulin concentration of 1.6×10^{-5} M. The results are shown in Figure 1 as a Scatchard plot, together with the results obtained at the same protein concentration in the absence of magnesium ions (Na & Timasheff, 1986). It is evident that 1 mM $MgCl_2$ caused a striking strengthening of the binding of the first vinblastine molecule to each tubulin dimer. This is reflected by the increase of the intercept on the ordinate of the Scatchard plot by more than 1 order of magnitude, from ca. 1×10^5 M^{-1} to ca. 2×10^6 M^{-1}. On the other hand, the binding of vinblastine to the rest of the binding sites appeared to be unaffected by the presence of the magnesium ions. The total stoichiometry of the vinblastine–tubulin binding also remained unchanged in the presence of magnesium ions. Thus, by adding 1 mM $MgCl_2$ to the PG buffer, the vinblastine binding isotherm changed from one that is only slightly curvilinear to one that is strongly concave upward and which resembles that of two heterogeneous classes of binding sites,

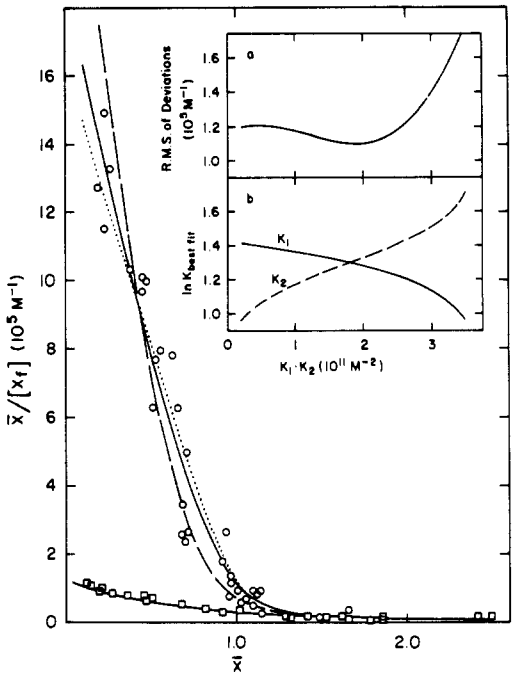

FIGURE 1: Scatchard plot of vinblastine binding to tubulin in PG buffer at 20 °C with (○) and without (□) 1 mM $MgCl_2$. The protein concentration was 1.6×10^{-5} M. The solid, dotted, and dashed curves at the top are theoretical binding isotherms calculated according to the ligand-mediated plus -facilitated self-association mechanism described in the preceding paper. They are the best fit isotherms obtained at three different values of K_1K_2: 1.95×10^{11} M^{-2}, 1.0×10^{11} M^{-2}, and 3.5×10^{11} M^{-2}, respectively. The intrinsic association constants of these theoretical isotherms are as follows: $K_1 = 3.76 \times 10^5$ M^{-1}, $K_2 = 5.18 \times 10^5$ M^{-1}, $K_3 = 4.42 \times 10^9$ M^{-1}, and $K_4 = 44.2$ M^{-1} (—); $K_1 = 8.17 \times 10^5$ M^{-1}, $K_2 = 1.23 \times 10^5$ M^{-1}, $K_3 = 3.16 \times 10^9$ M^{-1}, and $K_4 = 31.6$ M^{-1} (···); $K_1 = 1.44 \times 10^4$ M^{-1}, $K_2 = 2.44 \times 10^7$ M^{-1}, $K_3 = 5.92 \times 10^9$ M^{-1}, and $K_4 = 59.2$ M^{-1} (– –). Two self-association-independent binding sites with a ligand binding constant of 5×10^3 M^{-1} were included in all calculations. Inset b shows the best fit values of K_1 and K_2 as a function of K_1K_2 whereas inset a shows the root mean squares of deviations between the experimental data and the best fit theoretical isotherm as a function of K_1K_2.

with one site having a much stronger ligand affinity than the others.

The binding data were analyzed in a manner similar to that described in the preceding paper (Na & Timasheff, 1986). Since the shape of the Scatchard plot of the vinblastine binding in the presence of 1 mM $MgCl_2$ resembles the one obtained in the absence of magnesium ions, the same one-ligand–mediated plus -facilitated mechanism described in the preceding paper was used (Na & Timasheff, 1986). However, an analysis of the intercept on the ordinate of the Scatchard plot as a function of protein concentration could not be performed, since the binding in the presence of magnesium ion was determined at only one protein concentration. Consequently, all the information had to be derived from fitting the binding data to a single isotherm. Since tubulin did not show any measurable self-association in PG–1 mM $MgCl_2$ buffer in the absence of vinblastine (see the results of Figure 3), K_4 must have a very small value. By assigning $K_4 < 10^3$ M^{-1}, the resulting theoretical isotherms become independent of the values of K_3 and K_4, and only the values of K_1 and K_2 need to be treated. Theoretical ligand binding isotherms were calculated by using various values of the product K_1K_2. At any given value of K_1K_2, the values of K_1 and K_2 that generate the best fit isotherm were obtained. Inset b of Figure 1 shows the best fit values of K_1 and K_2 as a function of the product K_1K_2. At increasing values of K_1K_2, the best fit value of K_1 decreased whereas that of K_2 increased, and the resulting best fit isotherm

[1] Abbreviations: PG, 0.01 M NaP_i and 10^{-4} M GTP, pH 7.0; GTP, guanosine 5′-triphosphate.

[2] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

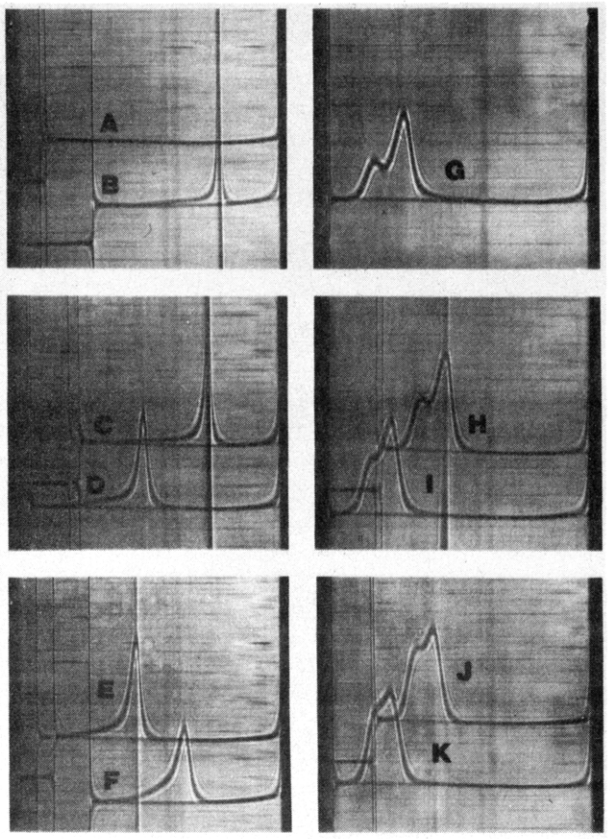

FIGURE 2: Effects of magnesium ions on the sedimentation velocity boundary of tubulin in the presence of vinblastine. Tubulin was equilibrated in either 5×10^{-5} M vinblastine–PG buffer (A–F) or 1×10^{-5} M vinblastine–PG buffer (G–K). Various concentrations of $MgCl_2$ were then added: (A) 5, (B and G) 2.5, (C and H) 1.0, (D and I) 0.5, (E and J) 0.1, or (F and K) 0 mM. The rotor speed was 60 000 rpm, and pitcures were taken at 24 min after reaching full speed.

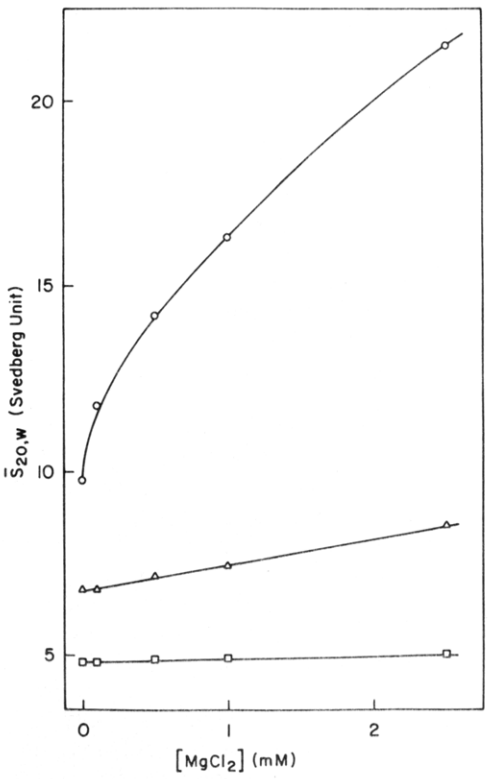

FIGURE 3: Dependence of the weight-average sedimentation coefficient of tubulin on magnesium ion concentration. The weight-average sedimentation coefficients were calculated by numerical integration of the schlieren boundaries. The vinblastine concentrations were 5×10^{-5} M (○), 1×10^{-5} M (△), and zero (□).

became increasingly concave. Inset a of Figure 1 shows the root mean squares of deviations between the ordinate of the experimental data and the best fit theoretical binding isotherm as a function of the product K_1K_2. The minimum of this curve indicates that the binding data can be described best by a ligand binding isotherm with $K_1 = 3.8 \times 10^5\ M^{-1}$ and $K_2 = 5.2 \times 10^5\ M^{-1}$, depicted by the solid curve of Figure 1. However, it is evident that these intrinsic association constants have very large ranges of error as demonstrated by the relatively small differences between the three theoretical binding isotherms shown in Figure 1 calculated with quite different values of K_1 and K_2. One result is evident, however: that the addition of magnesium ions increases the value of the product K_1K_2. Thus, the binding of vinblastine to tubulin and/or the vinblastine-induced tubulin self-association must be linked thermodynamically to the interaction of the divalent cation with the tubulin–vinblastine system.

Effects of Magnesium Ions on Vinblastine-Induced Self-Association of Tubulin. To characterize the effect of magnesium ions on the vinblastine-induced tubulin self-association, sedimentation velocity experiments were performed with calf brain tubulin equilibrated by gel filtrations with PG buffer containing 1×10^{-5} and 5×10^{-5} M vinblastine. Prior to centrifugation, $MgCl_2$ was added to the protein solution to give various concentrations of the divalent cation salt. As shown by pattern F of Figure 2, in 5×10^{-5} M vinblastine–PG buffer, tubulin sedimented as a single forward-skewed peak characteristic of an isodesmic indefinite self-associating system (Na & Timasheff, 1980a). With the introduction of increasing concentrations of magnesium ions, the boundary became increasingly skewed and sharpened, as reflected by patterns B–E of Figure 2. Such a change in the shape of the sedimentation boundary induced by the magnesium ions is similar to that induced by increasing the protein concentration in the absence of magnesium ions (Na & Timasheff, 1980a). The weight-average sedimentation coefficients $\bar{s}_{20,w}$ of the boundaries were calculated and are shown in Figure 3. At 5×10^{-5} M vinblastine, $\bar{s}_{20,w}$ increased from 10S in the absence of magnesium ions to 21S in the presence of 2.5 mM $MgCl_2$. A further increase of the magnesium ion concentration to 5 mM caused the formation of very large tubulin aggregates which sedimented completely to the bottom of the cell during rotor acceleration as indicated by the flat schlieren profile of pattern A in Figure 2.

In 1×10^{-5} M vinblastine–PG buffer, the protein exhibited a bimodal sedimentation boundary characteristic of a Cann–Goad type strong ligand-induced self-association (Cann & Goad, 1970a,b, 1972; Cann & Kegeles, 1974). The sedimentation boundary emerged first as a single peak. It then gradually resolved first into a broad shoulder and then a bimodal reaction boundary as the centrifugation proceeded (Na & Timasheff, 1980a). As shown in patterns G–K of Figure 2, at 1×10^{-5} M vinblastine, the effect of magnesium ions is manifested in the enhanced resolution of the bimodal boundary. In the absence of magnesium ions, only a shoulder appeared in the sedimentation boundary after 24 min of centrifugation at 60 000 rpm. In the presence of 2.5 mM $MgCl_2$, the bimodal nature of the sedimentation boundary was already clearly evident after the same extent of centrifugation. The weight-average sedimentation coefficients of the bimodal sedimentation boundaries were calculated and are presented in Figure 3. At 1×10^{-5} M vinblastine, magnesium ions induced a much smaller increase in the sedimentation coef-

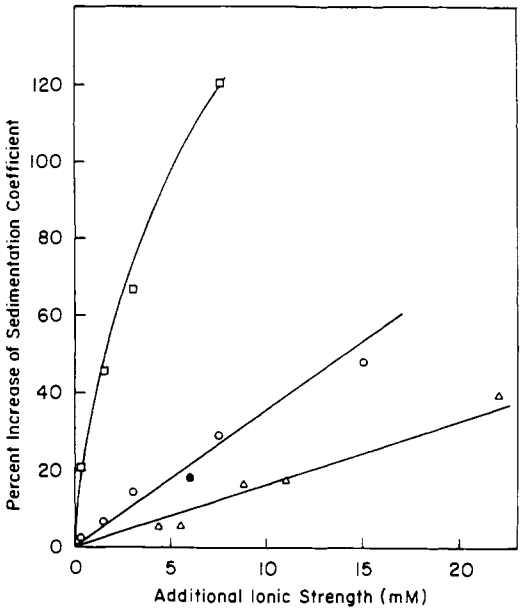

FIGURE 4: Effects of salts on the sedimentation velocity of tubulin in PG–5 × 10^{-5} M vinblastine buffer. Weight-average sedimentation coefficients were used for $MgCl_2$ (□), while sedimentation coefficients of the apex of the boundary were used for NaCl (○), NaP_i (△), and GTP (●). The results are expressed as a function of the ionic strength contributed by the extra salt to the PG buffer.

ficient of the protein than at 5 × 10^{-5} M vinblastine. The results of the control experiments, also shown in Figure 3, indicate that, within the concentration range of 0–2.5 mM, magnesium ions per se had no observable effects on either the shape or the sedimentation coefficient of the boundary.

Effect of Other Salts on Vinblastine-Induced Tubulin Self-Association. Two other salts, NaCl and NaP_i, were examined for their effects on the vinblastine-induced tubulin self-association to determine whether the enhancing effect of magnesium chloride was due simply to the increase of the solution ionic strength. Figure 4 presents the effects of NaCl, NaP_i, and $MgCl_2$ on the sedimentation coefficient of tubulin in PG–5 × 10^{-5} M vinblastine buffer. Although all three salts examined did increase the sedimentation coefficient of the protein, the effect of $MgCl_2$ was by far the strongest, NaCl was next, and NaP_i was the least effective. GTP was also examined at a single concentration, and its effect was similar to that of NaCl.

Vinblastine–Tubulin Aggregation in the Presence of High Concentrations of Magnesium Ions. The formation of large tubulin aggregates induced by vinblastine in PG buffer containing more than 2.5 mM $MgCl_2$ were further investigated by turbidimetry.[3] Aggregates formed immediately on introduction of the drug into the solution. These aggregates were amorphous when examined in the optical microscope. Figure 5 shows turbidimetric titrations of a 1.1 × 10^{-5} M tubulin solution in the presence of different concentrations of magnesium ions. In PG buffer, the protein solution did not display any detectable turbidity up to 2 × 10^{-4} M vinblastine. In the presence of 5 mM $MgCl_2$, however, the solution turbidity

[3] It has been demonstrated that when the associated species assume the shape of long thin rods, such as is true in microtubule and actin filament assemblies, the solution turbidity measured at 350 nm is proportional to the mass concentration of the aggregates (Gaskin et al., 1974; Berne, 1974; Timasheff, 1981). Such a relationship has not been established in the vinblastine–induced tubulin aggregation. Nevertheless, the solution turbidity can be used as a valid semiquantitative measurement of the growth of the aggregates (Ventilla et al., 1975; Andreu & Timasheff, 1982b).

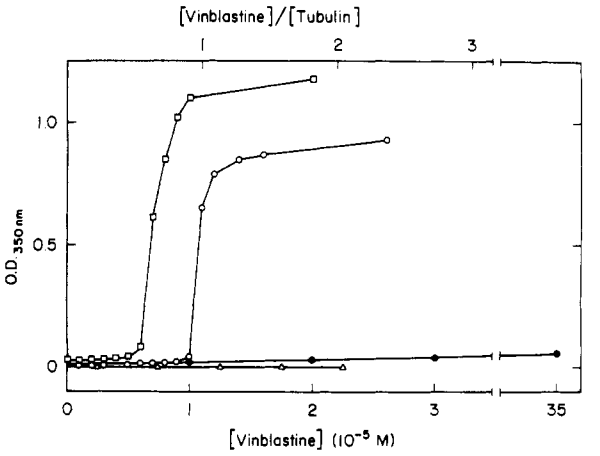

FIGURE 5: Turbidimetric titration of tubulin with vinblastine at different magnesium ion concentrations. Tubulin was equilibrated in PG buffer containing 1 × 10^{-2} M (□), 5 × 10^{-3} M (○), and no (△) $MgCl_2$. The protein concentration was adjusted to 1.1 × 10^{-5} M and then titrated by adding small aliquots of 10^{-2} M vinblastine. The solution turbidities at 350 nm were recorded 5 min after each addition of the drug. A similar titration performed in the presence of 5 mM $MgCl_2$ and 0.1 M NaCl is depicited by the closed circles (●).

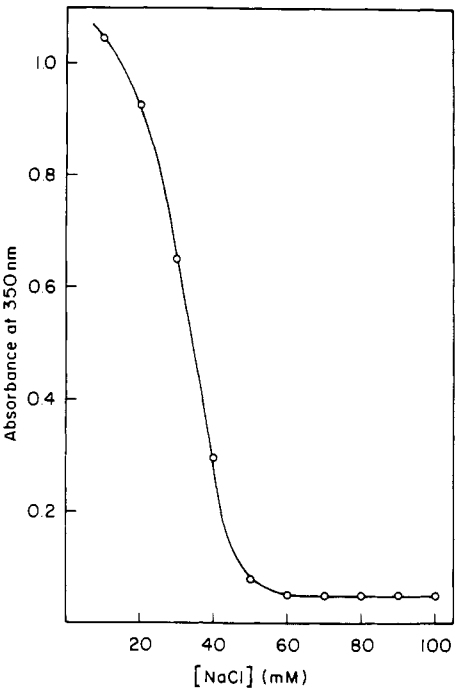

FIGURE 6: Effect of NaCl on the vinblastine-induced tubulin aggregation. Tubulin equilibrated in PG buffer by column gel filtration was adjusted to a concentration of 1.3 × 10^{-5} M. NaCl was then added to the specified concentration followed by vinblastine to a final concentration of 2 × 10^{-5} M. The mixtures were allowed to stand at room temperature for 20 min before their turbidity was measured.

increased sharply at ca. 1 × 10^{-5} M total vinblastine and then plateaued around 1.5 × 10^{-5} M vinblastine. At 10 mM $MgCl_2$, a similar sharp sigmoidal growth of the solution turbidity took place around 7 × 10^{-6} M vinblastine. It plateaued around 1 × 10^{-5} M vinblastine and at a higher final turbidity than that at 5 mM $MgCl_2$.

Effects of Solution Ionic Strength on Vinblastine-Induced Tubulin Aggregation. The solution ionic strength affected both the rate and the morphology of the vinblastine-induced tubulin aggregation. In PG buffer containing 5–10 mM $MgCl_2$, the aggregates formed immediately after introduction of vinblastine into the solution. The rate of aggregation decreased

gradually with an increase in the solution ionic strength. As shown in Figure 5, in PG buffer containing 5 mM $MgCl_2$ and 0.1 M NaCl, there was no solution turbidity observed up to 3.5×10^{-4} M vinblastine if the mixture was incubated for only 5 min after each addition of the drug. Figure 6 depicts the ionic strength dependence of the solution turbidity observed 20 min after the introduction of 2×10^{-5} M vinblastine to a tubulin solution in PG–5 mM $MgCl_2$ buffer. It is evident that the amount of aggregates formed within this time period decreased with an increase in the solution ionic strength. Above 0.05 M NaCl, the protein solution became turbid only after ca. 30 min of incubation, and optical microscopic examination of the turbid solution revealed that the aggregates formed were paracrystalline, rather than amorphous (Na & Timasheff, 1982).

Discussion

The binding of vinblastine to tubulin and the drug-induced self-association of the protein have been investigated by several laboratories, and a consensus had been reached that each tubulin α–β dimer contains two strong vinblastine binding sites. The values of the drug binding constants reported from the different laboratories, however, differed greatly from one another (Lee et al., 1975; Wilson et al., 1975; Bhattacharyya & Wolff, 1976), and there was a disagreement on the question of which of the two binding sites is linked to the self-association of the protein. Bhattacharyya and Wolff (1976) claimed that the second i.e., weaker, specific binding site was responsible for the tubulin self-association induced by vinblastine; we concluded (Na & Timasheff, 1980b) that the self-association was linked to the first site. Furthermore, in the present studies we have demonstrated that tubulin contains only one strong vinblastine binding site, additional binding being to a number of weak nonspecific loci (see preceding paper in this issue).

What is the cause of these disagreements? During an analysis of the equilibrium linkage of the vinblastine-induced tubulin self-association, we noticed that the media used in the binding studies performed in the different laboratories contained different concentrations of magnesium ions. We reasoned in accordance with the Wyman (1964) theory of linkages that, if magnesium ions can enhance the vinblastine-induced tubulin self-association, they should also enhance the binding of vinblastine to the association-linked binding site (Na & Timasheff, 1980b). The results shown in Figure 1 clearly demonstrate that 1 mM $MgCl_2$ can cause a striking strengthening of the binding of vinblastine to the first binding site on the protein. In fact, the binding isotherm obtained in PG–1 mM $MgCl_2$ buffer is quite similar to the one obtained by Bhattacharyya and Wolff (1976) in a similar phosphate–GTP buffer containing 10 mM magnesium ions. It is evident, then, that the difference in the vinblastine binding isotherms reported from the various laboratories can be attributed primarily to the differences in the magnesium ion concentrations used in the buffers.

The observation that the binding of only the first vinblastine molecule is enhanced by magnesium ions supports our previous conclusion that it is this binding site that is linked to the self-association of the protein (Na & Timasheff, 1980b, 1986). In fact, the vinblastine–tubulin binding data, shown in Figure 1, can be fitted quite well by a theoretical binding isotherm calculated according to the one-ligand-mediated plus -facilitated isodesmic indefinite self-association mechanism described in the preceding paper (Na & Timasheff, 1986). The only difference is found in the higher values of the intrinsic association constants in the presence of magnesium ions. This probably reflects the fact that, in the presence of magnesium ions, both the drug binding and the protein self-association are strengthened, reflecting linkages of the magnesium ion interaction to both processes. In the presence study, the fitting of the data did not permit the determination of the intrinsic association constants with good accuracy. A more exact determination would require measurements of the dependence of ligand binding on protein concentration and of the dependence of the self-association on ligand concentration, both in the presence of magnesium ions. Nevertheless, the present data demonstrate further the linkage between the binding of the first vinblastine molecule to tubulin and the protein self-association and the strong effect of magnesium ions on that linkage.

The results of the sedimentation velocity studies, shown in Figures 2 and 3, indicate that magnesium ions can strongly enhance the vinblastine-induced tubulin self-association. At 5×10^{-5} M vinblastine, the effect of the magnesium ions on the shape of the sedimentation boundary of tubulin is quite similar to that caused by increasing the protein concentration in the absence of magnesium ions. This suggests that magnesium ions do not change the tubulin association stoichiometry, i.e., the self-association in the presence of the divalent cations still proceeded through the same isodesmic indefinite mechanism (Na & Timasheff, 1980a). The sedimentation coefficient of tubulin in the presence of 5×10^{-5} M vinblastine and 2.5 mM $MgCl_2$ is higher than that obtained at a saturating vinblastine concentration in the absence of magnesium ions (Na & Timasheff, 1980a). Since the sedimentation coefficients measured at saturating ligand concentrations reflect the intrinsic self-association constant K_2 (Na & Timasheff, 1980a), these results indicate that the value of K_2 must be increased in the presence of magnesium ions.

At 1×10^{-5} M vinblastine, the same concentration of magnesium ions caused a much smaller increase of the sedimentation coefficient of tubulin. This reflects the method used for sample preparation. In these experiments, the protein solution was first equilibrated in PG–vinblastine buffer by gel filtration. Then, the appropriate amount of $MgCl_2$ was added. Thus, the total amount of vinblastine in the solution was limited. At 5×10^{-5} M vinblastine, which is the stoichiometric amount relative to the concentration of tubulin in the solution, the introduction of magnesium ions could cause saturation of the self-association-linked site on the protein and result in the strong enhancement of the self-association of the protein. On the other hand, at 1×10^{-5} M vinblastine, which is substoichiometric to the protein concentration, even the introduction of magnesium ions could not cause the saturation of the association-linked binding site, and therefore, the sedimentation coefficient was increased only slightly. This whole phenomenon is, therefore, a clear manifestation of the linkage relationship and a further indication that the self-association requires that a stoichiometric amount of vinblastine be bound to the protein.

In PG buffer containing more than 2.5 mM magnesium ion, vinblastine induces tubulin to self-associate into large amorphous aggregates at a drug stoichiometry of 0.7–1.0 mol of vinblastine per mole of tubulin. These results indicate that this aggregation is induced by the binding of the first vinblastine molecule to each tubulin α–β dimer just as is the isodesmic indefinite self-association of tubulin in the absence of magnesium ions. This conclusion is different from that of Bhattacharyya and Wolff (1976), who, on observation of tubulin aggregation at a vinblastine concentration between 10^{-5} and 10^{-3} M, concluded that the aggregation is caused by the binding of vinblastine to the weaker, or second, binding site

Table I: Vinblastine-Induced Self-Associations of Tubulin in PG Buffer under Different Conditions

other components	temp (°C)	vinblastine concn (M)	nature of association	site	ref
0–2.5 mM $MgCl_2$	10–30	10^{-6} to 2×10^{-4}	isodesmic, indefinite	first	*a, c*
>2.5 mM $MgCl_2$	10–30	10^{-6}–10^{-5}	large amorphous aggregates,	first	*c*
>2.5 mL $MgCl_2$, 0.05–0.1 M NaCl	4–30	4×10^{-5} to 10^{-3}	paracrystals	first	*b*
5×10^{-4} M $MgCl_2$, 3 M glycerol	30–37	10^{-6}	inhibition of microtubule assembly	unknown	*d*

[a] Na and Timasheff (1980a,b). [b] Na and Timasheff (1982). [c] This paper. [d] G. C. Na and S. N. Timasheff (unpublished results).

on the protein described in their study, characterized by a drug binding constant of 8×10^4 M^{-1}. Closer examination of their experimental conditions revealed, however, that these authors had used buffers of different compositions in their drug binding and turbidity titration experiments: The formation of aggregates was followed in a buffer of much higher ionic strength, but containing only $^1/_{20}$th the concentration of $MgCl_2$ than the buffer used in their drug binding experiments. Our results indicate clearly that both the strength of the binding of the first vinblastine molecule to each tubulin dimer and that of the ensuing self-association are strongly dependent on the solution ionic strength and magnesium ion concentration. Therefore, the conclusions of Bhattacharyya and Wolff (1976), based on the comparison of results of two different experiments carried out at drastically different buffer conditions, must be regarded as being invalid.

At an ionic strength >0.05, the vinblastine-induced tubulin self-association proceeded at a slower rate and only at vinblastine concentrations from 10^{-5} to 10^{-3} M. Optical microscopic examinations of the slowly formed aggregates at higher ionic strengths have shown these to be large paracrystals rather than amorphous structures. Since these paracrystals contain only one vinblastine molecule per tubulin α–β dimer (Na & Timasheff, 1982), the self-association of the protein in high ionic strength buffers is still linked to the binding of the first vinblastine molecule to the protein. The high drug concentrations needed for the formation of paracrystals may be the consequence of the weakening of the drug affinity for tubulin in a buffer of high ionic strength. Such a reduction in drug affinity could be caused by several factors. First, vinblastine exists in solution as a cation, and ionic interactions are possibly involved in its binding to the protein. Then an increase in solution ionic strength could weaken the binding by simple Debye–Hückel screening. Second, since the very strong binding of vinblastine to the protein is caused by the presence of magnesium ions, a reduction of the apparent drug binding constant could result from a weaker binding of magnesium ions to the protein at high ionic strength. Such an ionic strength effect can explain the lower vinblastine binding constant of $(3–5) \times 10^5$ M^{-1} reported by Wilson et al. (1975) in a buffer containing 5 mM $MgCl_2$ and 0.15 M NaCl.

The molecular mechanism through which magnesium ions bring about the enhancement of the vinblastine-induced tubulin self-association is not clear. As shown in Figure 4, both NaCl and NaP_i can enhance the vinblastine-induced tubulin self-association. In fact, in the absence of vinblastine, 0.1 M NaCl can induce a weak self-associtin in tubulin (Frigon & Timasheff, 1975a). It is likely that these effects are caused by nonspecific protein–ion interactions. NaCl and NaP_i increase the sedimentation coefficient of tubulin in PG–5×10^{-5} M vinblastine buffer much less than do magnesium ions. Thus, their effect is most likely due to nonspecific interactions. Furthermore, magnesium ion, by itself, can induce in tubulin a specific self-association into a double ring structure containing 26 ± 2 tubulin dimers (Frigon & Timasheff, 1975a,b). In the presence of GTP, this self-association of tubulin becomes manifest in sedimentation velocity at 5–16 mM $MgCl_2$. It should be quite weak in the range of 0.1–2.5 mM $MgCl_2$ employed in the present study. If in the presence of the two ligands the two different types of ligand-induced self-associations can take place simultaneously with the formation of a single associated product, there should be a synergistic effect. This linkage should result in a final free energy change that is not just the sum of the free energy changes of the two separate self-associations, but there should be an additional negative free energy contribution due to the fact that the individual macromolecules need to be brought together only once (Andreu & Timasheff, 1982a). The possibility of such a linked two-ligand-induced self-assocation still needs to be explored.

The results of in vitro studies of vinblastine-induced tubulin self-associations under different conditions and their linkages to vinblastine binding are summarized in Table I. In PG buffer with <2.5 mM $MgCl_2$, vinblastine induces an isodesmic indefinite self-association of tubulin. Above 2.5 mM $MgCl_2$, the self-association of the protein leads to the formation of large amorphous aggregates at low ionic strength and of paracrystalline aggregates at high ionic strength. It is evident that the different vinblastine-induced tubulin self-associations can all be attributed to the binding of the first vinblastine molecule to the protein. It is most likely that this is the same binding site of the protein. In fact, it seems most probable that the three types of vinblastine-induced tubulin self-associations, namely, the isodesmic, indefinite association in the absence of magnesium ions, aggregation into large amorphous structures in the presence of magnesium ions, and the formation of paracrystals in the presence of magnesium ion and high ionic strength, are all derived from the same tubulin self-association reaction. We propose that addition of a high concentration of magnesium ions strengthens the isodesmic indefinite self-association of tubulin induced by vinblastine, leading to the formation of large aggregates. Reduction of the rate of aggregation at high ionic strength permits perfect alignment of the linear aggregates and the formation of ordered paracrystalline structures. This notion is supported by the observation that the tubulin–vinblastine paracrystals are made of intertwined linear polymers of tubulin (Fujiwara & Tilney, 1976; Amos et al., 1984).

The identity of the drug binding site linked to the vinblastine inhibition of microtubule assembly remains unclear. This is an important question in the unraveling of the mechanism of the antimitotic activity of the drug. It is known that substoichiometric amounts of vinblastine, at a concentration of 10^{-6} M or less, are sufficient to inhibit the in vitro microtubule assembly reaction. The system is complicated by the simultaneous occurrence of two different types of self-associations of tubulin, the vinblastine-induced tubulin self-association and the self-assembly of tubulin into microtubules. It is quite possible that the vinblastine inhibition of the in vitro microtubule assembly and the drug-induced tubulin self-association are not independent of each other. One attractive mechanism relating these two events is through the so-called "poisoning effect" in which the vinblastine-induced tubulin self-association proceeds from microtubule ends, thus blocking the normal

growth of microtubules (Wilson et al., 1976, 1982; Na & Timasheff, 1982a). If this is correct, the vinblastine inhibition of microtubule assembly must be linked to the same vinblastine binding site on the protein as the self-association reaction. This mechanism is rendered even more plausible now in view of the strong enhancement by magnesium ions of the vinblastine-induced tubulin self-association reported here.

Registry No. Mg, 7439-95-4; $MgCl_2$, 7786-30-3; vinblastine, 865-21-4.

References

Amos, L. A., Jubb, J. S., Henderson, R., & Vigers, G. (1984) *J. Mol. Biol. 178*, 711-729.

Andreu, J. M., & Timasheff, S. N. (1982a) *Biochemistry 21*, 534-543.

Andreu, J. M., & Timasheff, S. N. (1982b) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6753-6756.

Berne, B. J. (1974) *J. Mol. Biol. 89*, 755-758.

Bhattacharyya, B. & Wolff, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2375-2378.

Cann, J. R., & Goad, W. B. (1970a) in *Interacting Macromolecules*, Academic Press, New York and London.

Cann, J. R., & Goad, W. B. (1970b) *Science (Washington, D.C.) 170*, 441-445.

Cann, J. R., & Goad, W. B. (1972) *Arch. Biochem. Biophys. 153*, 603-609.

Cann, J. R., & Kegeles, G. (1974) *Biochemistry 13*, 1868-1874.

Frigon, R. P., & Timasheff, S. N. (1975a) *Biochemistry 14*, 4559-4566.

Frigon, R. P., & Timasheff, S.N. (1975b) *Biochemistry 14*, 4567-4573.

Fujiwara, K., & Tilney, L. G. (1976) *Ann. N.Y. Acad. Sci. 253*, 27-50.

Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737-758.

Lee, J. C., Harrison, D., & Timasheff, S. N. (1975) *J. Biol. Chem. 250*, 9276-9282.

Na, G. C., & Timasheff, S. N. (1980a) *Biochemistry 19*, 1347-1354.

Na, G. C., & Timasheff, S. N. (1980b) *Biochemistry 19*, 1355-1365.

Na, G. C., & Timasheff, S. N. (1980c) *American Chemical Society Meeting*, Las Vegas, NV, Abstract Biol. 170.

Na, G. C., & Timasheff, S. N. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1548.

Na, G. C., & Timasheff, S. N. (1982) *J. Biol. Chem. 257*, 10387-10391.

Na, G. C., & Timasheff, S. N. (1986) *Biochemistry* (preceding paper in this issue).

Timasheff, S. N. (1981) in *Protein-Protein Interactions* (Frieden, C., & Nichol, L. W., Eds.) pp 315-336, Wiley, New York.

Ventilla, M., Cantor, C. R., & Shelanski, M. L. (1975) *Arch. Biochem. Biophys. 171*, 154-162.

Weisenberg, R. C., & Timasheff, S. N. (1970) *Biochemistry 9*, 4110-4116.

Wilson, L., Creswell, K. M., & Chin, D. (1975) *Biochemistry 14*, 5586-5592.

Wilson, L., Anderson, K., & Chin, D. (1976) *Cold Spring Harbor Conf. Cell Proliferation 3*, 1051-1064.

Wilson, L., Jordan, M. A., Morse, A., & Margolis, R. L. (1982) *J. Mol. Biol. 159*, 125-149.

Wyman, J. (1964) *Adv. Protein Chem. 19*, 224-285.

Nucleotide Sequence and Nuclease Hypersensitivity of the Chinese Hamster Dihydrofolate Reductase Gene Promoter Region†

Jane Clifford Azizkhan,‡,§,‖ James P. Vaughn,‡ Robert J. Christy,§ and Joyce L. Hamlin*,‡

Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908, and Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Received March 31, 1986; Revised Manuscript Received June 9, 1986

ABSTRACT: We have sequenced the 1240 base pairs (bp) upstream from the translation start site of the hamster dihydrofolate reductase (DHFR) gene. The DNA in the 5′ flanking region contains several elements that are homologous in both sequence and relative location to corresponding elements in the human and murine DHFR genes: an 11-bp element adjacent to the ATG codon, a 19-bp element that coincides with the major transcription start site, and two 29-bp upstream elements that are represented 4 times in the murine DHFR gene but only once in the human gene. Two clusters of short, G/C-rich elements conforming to the consensus binding sequence for the transcription factor Sp1 are located in the upstream region in all three genes. The symmetrical placement of the G/C boxes coincides with a symmetrical DNase I hypersensitive pattern in the chromatin, suggesting that the Sp1 protein may be involved in maintaining chromatin structure in this region.

Dihydrofolate reductase (DHFR)[1] catalyzes the reduction of folate to dihydrofolate and, subsequently, to tetrahydrofolate. Reduced folates are required in the biosynthesis of purine nucleotides, thymidine, and glycine. The gene encoding DHFR is expressed in all tissues, and its product has therefore been termed a "housekeeping" enzyme.

Several lines of evidence suggest that the transcription of

† This study was supported by a grant from the NIH and an American Cancer Society Faculty Research Award to J.L.H.

* Address correspondence to this author.

‡ University of Virginia School of Medicine.

§ The Johns Hopkins University.

‖ Present address: Department of Pediatrics, Lineberger Cancer Research Center, University of North Carolina, Chapel Hill, NC 27514.

[1] Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-6228$01.50/0