Seo, H., Vassart, G., Brocas, H., & Refetoff, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2054–2058.
Shapiro, L. E., Samuels, H. H., & Yaffe, B. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 45–49.
Siddiqui, U. A., Goldflam, T., & Goodridge, A. G. (1981) *J. Biol. Chem. 256*, 4544–4550.
Simat, B. M., Towle, H. C., Schwartz, H. L., & Oppenheimer, J. H. (1980) *Endocrinology (Baltimore) 107*, 1338–1344.
Solomon, J., & Greep, R. O. (1959) *Endocrinology (Philadelphia) 65*, 158–164.
Sonenshein, G. E., & Brawerman, G. (1977) *Biochemistry 16*, 5445–5448.
Tata, J. R., & Widnell, C. C. (1966) *Biochem. J. 98*, 604–620.
Tepperman, H. M., & Tepperman, J. (1964) *Am. J. Physiol. 206*, 357–361.
Tomkins, G. M. (1975) *Science (Washington, D.C.) 189*, 760–763.
Towle, H. C., Dillmann, W. H., & Oppenheimer, J. H. (1979) *J. Biol. Chem. 254*, 2250–2257.
Towle, H. C., Mariash, C. N., & Oppenheimer, J. H. (1980) *Biochemistry 19*, 579–585.
Towle, H. C., Mariash, C. N., Schwartz, H. L., & Oppenheimer, J. H. (1981) *Biochemistry 20*, 3486–3492.
Tsai, J. S., & Samuels, H. H. (1974) *Biochem. Biophys. Res. Commun. 59*, 420–428.
Turyn, D., & Dellacha, J. M. (1978) *Endocrinology (Philadelphia) 103*, 1190–1195.
Wise, E. M., & Ball, E. G. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 1255–1263.

Tubulin–Zinc Interactions: Binding and Polymerization Studies†

Geoffrey R. Eagle, Randall R. Zombola, and Richard H. Himes*

ABSTRACT: The binding of Zn^{2+} to tubulin and the ability of this cation to promote the polymorphic assembly of the protein were examined. Equilibrium binding showed the existence of more than 60 potential Zn^{2+} binding sites on the dimer, including a number of high-affinity sites. The number of high-affinity sites, estimated by using a standard amount of phosphocellulose to remove more weakly bound Zn^{2+}, reached a maximum of 6–7.5 with increasing levels of Zn^{2+} in the incubation solution. The number also increased with time of incubation at a single Zn^{2+} concentration. It is suggested that tubulin is slowly denatured in the presence of Zn^{2+}, exposing more binding sites. Cu^{+} and Cd^{2+} were effective inhibitors of Zn^{2+} binding; Mg^{2+}, Mn^{2+}, and Co^{2+} were much less effective, and Ca^{2+} was without effect. Zn^{2+} does not replace the tightly bound Mg^{2+}. GTP reduces the amount of Zn^{2+} binding under equilibrium conditions and the amount bound to high-affinity sites. Zinc-induced protofilament sheets are produced at a Zn^{2+}/tubulin ratio of 5 in the presence of 0.5 mM GTP, conditions where about two to three Zn^{2+} ions would be bound to the dimer. At higher GTP concentrations, less assembly occurred, and the products were narrower sheets and microtubules. Zn^{2+}–tubulin, isolated from phosphocellulose, will not assemble unless Mg^{2+} and dimethyl sulfoxide (Me_2SO) or more Zn^{2+} is added. Broad protofilament sheets, formed from Zn^{2+}–tubulin in the presence of Mg^{2+} and Me_2SO, contain slightly more than one Zn^{2+} per dimer. It is concluded that Zn^{2+} stimulates tubulin assembly by binding directly to the protein, not via a ZnGTP complex.

Assembly of the tubulin dimer into protofilamentous structures is stimulated by a variety of agents, including microtubule-associated proteins, organic solvents, and divalent metal ions. Stimulation by divalent cations can lead to microtubules or a variety of polymorphic structures. Microtubules are usually produced in the presence of Mg^{2+} (Lee & Timasheff, 1975; Herzog & Weber, 1977) and Mn^{2+} (Buttlaire et al., 1980), although at high concentrations of Mg^{2+} double rings (Frigon & Timasheff, 1975), latticelike arrays of rings (Voter & Erickson, 1979; Zabrecky & Cole, 1980; Haskins, 1981), ribbons, and multiple tubule structures (Carlier & Pantaloni, 1978; Haskins, 1981) are also produced.

Co^{2+} and Zn^{2+} can also promote the assembly of microtubules (Haskins et al., 1980; Gaskin, 1981) but more generally lead to the formation of several types of polymorphic structures (Larsson et al., 1976; Wallin et al., 1977; Gaskin & Kress, 1977; Gaskin, 1981; Haskins et al., 1980). The major structural forms produced in the presence of these two cations are sheets or ribbons containing variable numbers of protofilaments. The structure of sheets produced by Zn^{2+} has been examined by image reconstruction, and the sheets apparently are constructed from protofilaments arranged in an alternating antiparallel fashion (Baker & Amos, 1978; Tamm et al., 1979). Structures morphologically similar to the Zn^{2+} sheets have been observed in both Zn^{2+}-treated cultures of dorsal root ganglion (Gaskin et al., 1978) and swollen dendrites, when zinc wires were implanted into the brains of rats (Kress et al., 1981).

At present, information pertaining to the number of Zn^{2+} binding sites on tubulin is not available. The purpose of this study was to examine the binding of Zn^{2+} to tubulin and relate this binding to the ability of Zn^{2+} to promote the polymorphic assembly of tubulin.

Experimental Procedures

Materials. GTP, EGTA, Pipes, and Mes were purchased from Sigma Chemical Co. In most experiments, buffer and GTP solutions were passed through phosphocellulose columns to remove divalent cations. $^{65}ZnCl_2$ was a product of New England Nuclear Corp.

† From the Department of Biochemistry, University of Kansas, Lawrence, Kansas 66045. *Received July 2, 1982.* This work was supported by National Institutes of Health Grant NS11360, by American Cancer Society Grant CH 98, and by a postdoctoral fellowship to R.R.Z. from the Kansas Cancer Society.

0006-2960/83/0422-0221$01.50/0

Preparation of Tubulin. Bovine brain tubulin was prepared by the polymerization–depolymerization procedure of Shelanski et al. (1973). The protein was stored at −70 °C in 1-mL portions at a concentration of 10–15 mg/mL in buffer containing 100 mM 2-(*N*-norpholino)ethanesulfonic acid (Mes),[1] 0.5 mM $MgCl_2$, 1 mM EGTA, and 2 M glycerol, pH 6.5. On the day of the experiment, further purification was done to obtain the tubulin dimer. The protein solution was thawed and an equal volume of 0.8 M Pipes–20% Me_2SO, pH 6.9, added. The protein was then polymerized in the presence of 0.5 mM GTP. This procedure results in tubulin largely depleted of associated proteins (Himes et al., 1977). The remaining associated proteins and lower molecular weight buffer components were removed by passing the polymerized protein through a 5 × 1.2 cm column of phosphocellulose (Whatman P11) that was placed on top of a Sephadex G-75 column (25 × 1.2 cm). The column had been previously washed with 20 mM Pipes, pH 6.9, and the same buffer was used to elute the protein.

Self-Assembly Reaction. Assembly reactions were carried out in 0.1 M Pipes, pH 6.9 at 37 °C. Other components such as $MgCl_2$, $ZnCl_2$, Me_2SO, and GTP were added at concentrations given in the text. The reaction was followed by measuring the apparent absorbance at 350 nm as a function of time.

Zn^{2+} Binding. Equilibrium binding studies were performed by using $^{65}ZnCl_2$ with the gel filtration technique of Hummel & Dreyer (1962). Columns of Sephadex G-75 (28 × 0.7 cm) were equilibrated at room temperature with various concentrations of $^{65}ZnCl_2$ in buffers described in the text. The protein solution (2–3 mg/mL) was adjusted to the same $^{65}ZnCl_2$ concentration as in the equilibrium buffer, and, after 30 min at room temperature, 0.5 mL of this solution was applied to the column. Elution was with the equilibrating buffer, and 0.5-mL fractions were collected. Samples were taken for protein determination and counted directly in a Packard Model 500C γ counter. K_d values and the number of binding sites were estimated from a Scatchard plot by using the graphical method of Rosenthal (1967) and assuming two classes of independent sites.

Zn^{2+} binding was also measured by passing a preincubated solution containing $^{65}ZnCl_2$ and tubulin through a column of phosphocellulose. Usually 1 mL of a solution containing 1.5–3.0 mg of protein was added to phosphocellulose previously stored in 20 mM Pipes, pH 6.9, and packed by centrifugation using a clinical centrifuge. The amount of phosphocellulose normally used was sufficient to give a 2.5-mL gravity-packed column in a Pasteur pipet. The phosphocellulose–protein slurry was then placed in a Pasteur pipet and the protein eluted with 20 mM Pipes, pH 6.9. Control studies showed that the ion exchanger retained all of the $^{65}Zn^{2+}$ from a solution lacking protein, in the presence and absence of GTP. When EDTA was present, gel filtration was used because phosphocellulose does not remove Zn^{2+} from the ZnEDTA complex.

In some experiments, Zn^{2+} binding to polymerized tubulin was determined. A portion of the reaction mixture was layered on top of 6 mL of 50% sucrose in reaction buffer at 37 °C. After being centrifuged at 140000*g* for 90 min and at 37 °C, the supernatants were carefully removed with a Pasteur pipet. The pellets were rinsed with warm (37 °C) buffer and then suspended in cold buffer. The suspended pellets were centrifuged at 48000*g* for 10 min at 5 °C. The Zn^{2+} and protein contents of the supernates were then determined.

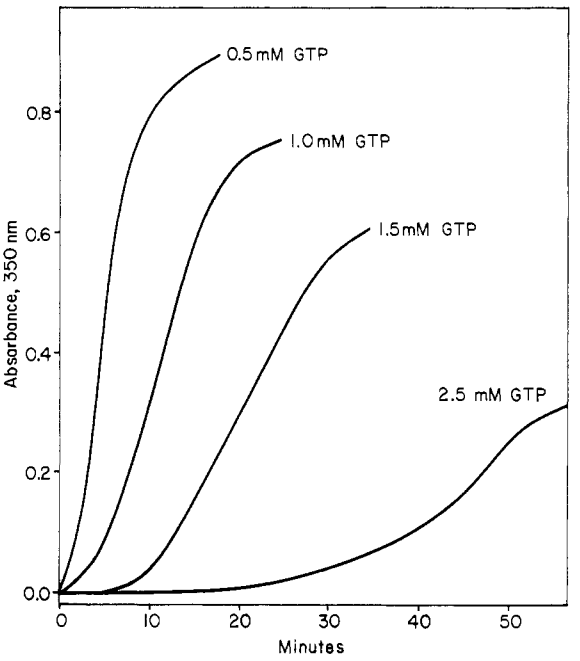

FIGURE 1: Effect of GTP concentration on the assembly of tubulin induced by Zn^{2+}. Tubulin (18 μM) was assembled in 0.1 M Pipes, pH 6.9, 75 μM $ZnCl_2$, and the GTP concentrations shown. Assembly was initiated by the addition of GTP.

Protein, Magnesium, Nucleotide, and Free Sulfhydryl Determinations. Protein concentrations were determined by the method of Bradford (1976). Magnesium was analyzed by atomic absorption spectroscopy using an Instrumentation Laboratory Model 151 spectrometer. Bound guanine nucleotide was determined by precipitating the protein with 5% perchloric acid and measuring the absorbance at 252 nm of the supernate after neutralization with KOH (Weisenberg et al., 1968). A molar extinction coefficient of 13 700 M^{-1} cm^{-1} was used. The free sulfhydryl group content of tubulin was estimated by using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

Electron Microscopy. Samples were negatively stained on carbon-coated grids with 2% uranyl acetate and viewed in a Philips 300 electron microscope. Some samples were fixed with 2.5% glutaraldehyde and stained with tannic acid, after which they were fixed with osmium tetraoxide, embedded in Araldite, sectioned, and stained again with methanolic uranyl acetate and lead citrate, as described previously (Burton & Himes, 1978).

Analytical Ultracentrifugation. Analytical ultracentrifugation analysis of tubulin treated with $ZnCl_2$ was performed on a Beckman Model E analytical ultracentrifuge by using schlieren optics.

Results

Effect of GTP and EDTA on Zn^{2+}-Induced Assembly. Much of the work on the promotion of polymorphic assembly products by Zn^{2+} has been done with microtubule protein preparations, i.e., tubulin preparations which contain associated proteins. However, it has been shown that Zn^{2+}, at low concentrations, induces the assembly of the pure tubulin dimer in the absence of associated proteins or organic solvents (Haskins et al., 1980). This implies that direct binding of Zn^{2+} to the protein is responsible for the stimulation of assembly. It is of interest, therefore, to determine the effects of Zn^{2+} chelators on the assembly reaction, especially GTP, since it

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid.

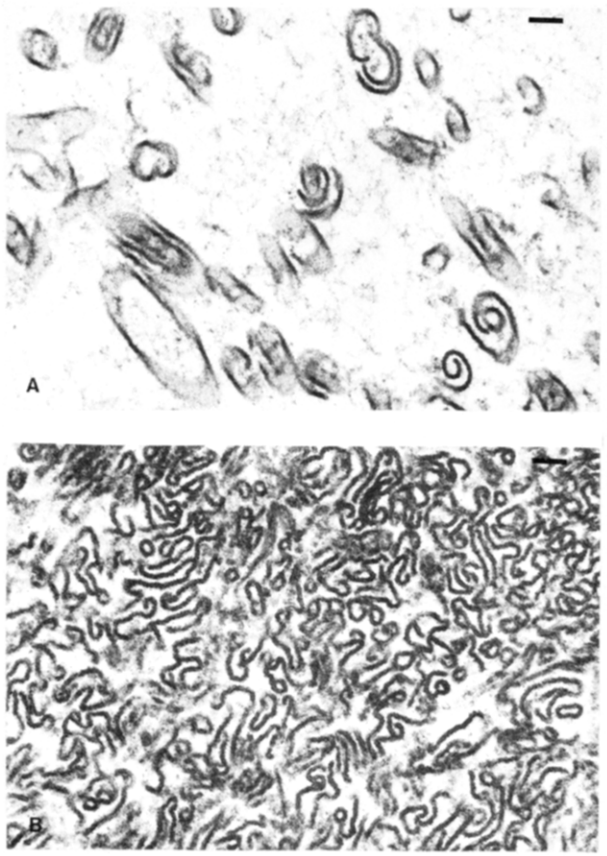

FIGURE 2: Effect of GTP concentrations on tubulin assembly induced by Zn^{2+}. Upon completion of the reactions shown in Figure 1, samples were fixed for thin sectioning as described under Experimental Procedures. (A) In the presence of 0.5 mM GTP; bar line = 91 nm. (B) In the presence of 2.5 mM GTP; bar line = 57 nm.

is a component of the reaction solution.

The presence of 1 mM EDTA completely abolished any assembly. GTP also inhibited assembly, but to a lesser extent (Figure 1). That the decrease in apparent absorbance was indeed due to a decreased amount of polymer formed was demonstrated by centrifuging samples and determining the amount of cold-soluble pelleted material. In the experiment shown in Figure 1, the amount of cold-soluble polymer formed in the presence of 0.5, 1, 1.5, and 2.5 mM GTP was 1.58, 1.49, 1.20, and 0.82 mg/mL, respectively. Of further interest was the effect of increasing GTP concentration on the morphology of the polymers produced. Negatively stained samples showed that in 0.5 mM GTP only wide sheets of protofilaments were formed, whereas in 2.5 mM GTP, less complex sheet structures and some microtubules were observed. Sectioned material demonstrated the differences in sheet structure more clearly (Figure 2).

The addition of 1 mM EDTA to preformed polymer caused a rapid and total disassembly (Figure 3A). Recently Banerjee et al. (1982) also reported disassembly of Zn^{2+}-induced polymers by EDTA. GTP was less effective in producing depolymerization, consistent with the fact that the equilibrium constant for the formation of ZnEDTA is 10^4 times higher than that for ZnGTP (Sigel, 1977; Sillen & Martell, 1971).

Binding of Zn^{2+} to Tubulin under Equilibrium Conditions. The actual amount of Zn^{2+} binding was measured in order to obtain a clear view of Zn^{2+}-induced tubulin assembly and the effects of GTP. Quantitation of Zn^{2+} binding to tubulin was performed by gel filtration under different buffer conditions, using various concentrations of $^{65}ZnCl_2$. Curve 1 of Figure 4 shows the Scatchard plot of the binding data obtained

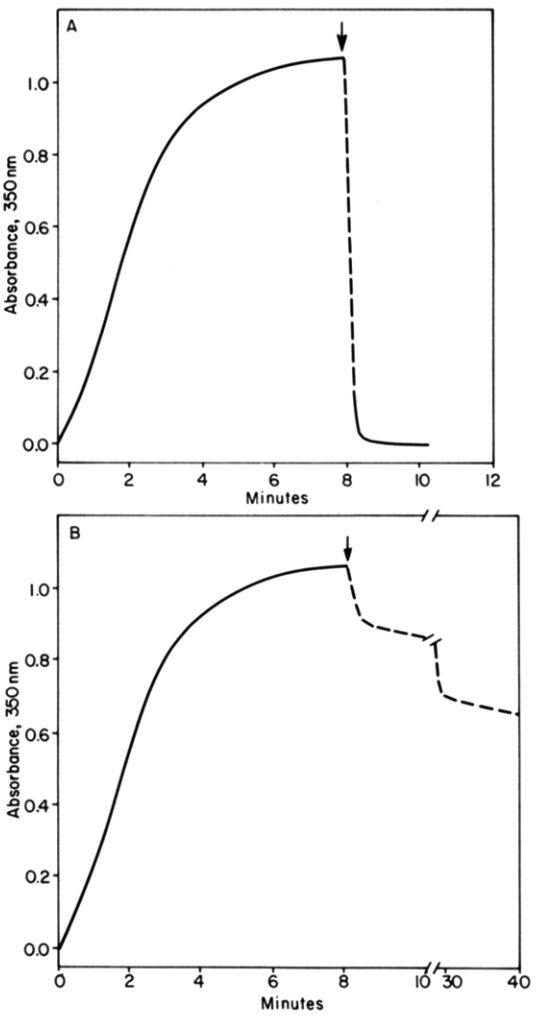

FIGURE 3: Depolymerization of Zn^{2+}-induced assembly by EDTA and GTP. Tubulin (20 μM) was polymerized in 0.1 M Pipes, pH 6.9, 100 μM $ZnCl_2$, and 0.5 mM GTP. At the arrow, the solution was made either 1 mM in EDTA (panel A) or 5 mM in GTP (panel B).

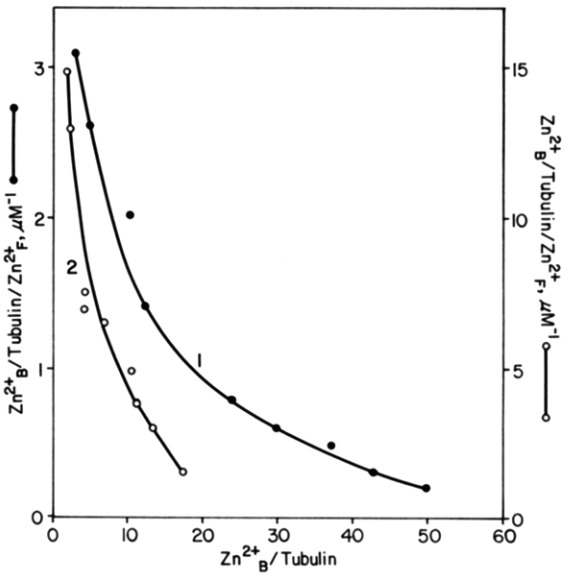

FIGURE 4: Scatchard plot of Zn^{2+} binding. The binding experiments were performed as described under Experimental Procedures. (Curve 1) In 20 mM Pipes, pH 6.9; (curve 2) in 0.1 M Pipes, pH 6.9, containing 0.5 mM GTP. Zn^{2+}_B is bound Zn^{2+} and Zn^{2+}_F is free Zn^{2+}. So that data could be obtained for curve 2, the free Zn^{2+} concentration was calculated by using a formation constant for ZnGTP of 91.2 mM^{-1} (Sigel, 1977).

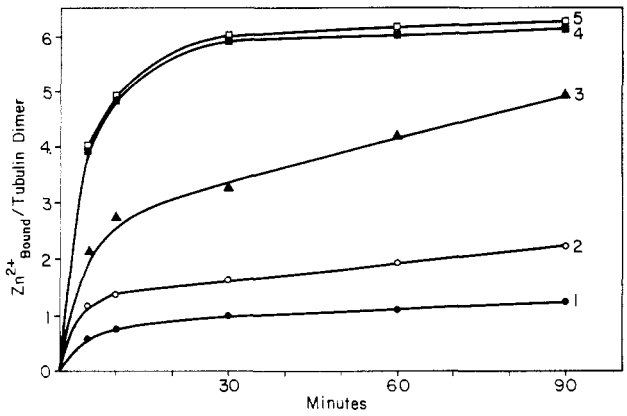

FIGURE 5: High-affinity binding of Zn^{2+} to tubulin. $^{65}ZnCl_2$ was incubated with 18 μM tubulin at different molar ratios for varying periods of time. The solutions were then added to phosphocellulose. The phosphocellulose–protein slurry was then placed in a Pasteur pipet column (2.5-mL packed volume) and the protein eluted with 20 mM Pipes, pH 6.9. The eluates were examined for $^{65}Zn^{2+}$ and protein contents. The initial molar ratios of Zn^{2+}/tubulin were (1) 2.5, (2) 5, (3) 10, (4) 15, and (5) 20.

in 20 mM Pipes, pH 6.9. Treating the data as if there are two classes of independent binding sites, we estimate that there are about 10 sites having an apparent K_d of 2.6 μM and 55 sites with a K_d of 85 μM. To examine for competition by other metals, we determined the binding of 0.1 mM Zn^{2+} in the presence of 1 mM Mg^{2+}, 1 mM Mn^{2+}, and 100 μM Co^{2+}. At these concentrations of divalent cations, no reduction in bound Zn^{2+} occurred.

We also examined the binding under conditions that approximate those in assembly reactions. Curve 2 of Figure 4 shows that in the presence of 0.1 M Pipes, pH 6.9, and 0.5 mM GTP there is a substantial reduction in the amount of Zn^{2+} binding. Under these conditions, there are 3 sites with an apparent K_d of 0.9 μM and 17 sites with an apparent K_d of 16 μM. A separate experiment demonstrated that increasing the Pipes concentration from 20 mM to 0.1 M decreased the binding only by 38%. Therefore, the presence of GTP is the main contributing factor to the decrease in Zn^{2+} binding. Increasing the GTP concentration higher than 0.5 mM further decreased the amount of bound Zn^{2+}. For example, when 100 μM $ZnCl_2$ was used, the amounts bound at 0, 0.5, 1.0, 2.5, and 5.0 mM GTP were 37.1, 10.5, 3.1, 2.2, and 2.0, respectively.

High-Affinity Binding of Zn^{2+} to Tubulin. In studying Co^{2+} binding to tubulin, Himes et al. (1982) found that after passage of a mixture of $CoCl_2$ and tubulin through ion exchangers, one Co^{2+} remained bound to the protein. Incubation periods of 0.5–90 min and wide ranges of Co^{2+}/tubulin ratios had little effect on the amount of tightly bound cobalt. We have done similar studies with Zn^{2+} and found a different picture. Incubation of tubulin with increasing molar excesses of Zn^{2+}, followed by treatment with phosphocellulose under a defined set of conditions, led to an increase in the amount of bound Zn^{2+}, up to a intial molar ratio of 15. Above this ratio, no further increase in binding was observed (Figure 5). In addition, increasing lengths of time led to more binding (Figure 5). The largest amount of Zn^{2+} binding found under these conditions was 6 mol/mol of tubulin dimer. In other experiments, values as high as 7.5 mol/mol of tubulin dimer were obtained. The Zn^{2+} bound to these sites must dissociate slowly since it remained bound after passage through a cation exchanger under *nonequilibrium conditions*. Considering the initial concentrations of Zn^{2+} and protein used, and the amount of Zn^{2+} bound, it is apparent that the Zn^{2+} must be bound

Table I: Dependence of High-Affinity Zn^{2+} Binding on the Amount of Phosphocellulose[a]

phosphocellulose (mL)	Zn^{2+} bound/tubulin (a)	(b)	(c)
2.5	2.0	3.4	7.5
5.0	2.0	3.0	6.7
7.5	1.7	2.9	6.0
12.5	1.3	2.2	4.5
17.5	0.9	1.4	3.8

[a] Tubulin solutions (18 μM) in 20 mM Pipes, pH 6.9, were incubated for 30 min at room temperature with (a) 90 μM $^{65}ZnCl_2$, (b) 180 μM $^{65}ZnCl_2$, and (c) 270 μM $^{65}ZnCl_2$. The solutions were then added to different amounts of packed phosphocellulose. The protein–phosphocellulose slurries were then placed in a Pasteur pipet (milliliters of packed phosphocellulose given in the table), and the protein was eluted with 20 mM Pipes, pH 6.9. The eluates were examined for $^{65}Zn^{2+}$ and protein contents.

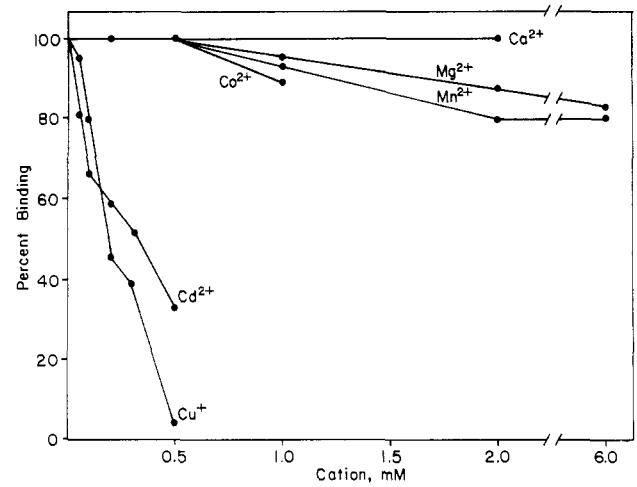

FIGURE 6: Competition by cations for high-affinity Zn^{2+} binding. $^{65}ZnCl_2$ (180 μM) was incubated with 18 μM tubulin in 20 mM Pipes, pH 6.9, and the chloride salts of the cations were shown, in a 0.5-mL volume. The $^{65}ZnCl_2$ was added last. After 30 min at room temperature, the solutions were added to phosphocellulose. The protein–phosphocellulose solutions were placed in Pasteur pipets (2.5-mL packed volume), and the protein was eluted with 20 mM Pipes, pH 6.9. The elutates were examined for $^{65}Zn^{2+}$ and protein contents. The curves have been normalized by setting the value of the control in the absence of cation at 100%. Concentrations of Co^{2+} exceeding 1 mM led to precipitation.

to fairly high affinity sites. The increase in binding with time suggests that the structure of the protein may have been changing, exposing more sites. The actual number of sites occupied by Zn^{2+} could be manipulated by increasing the amount of cation exchanger used in the binding experiment. As the results in Table I show, increasing the phosphocellulose concentration 7-fold caused a decrease in the amount of Zn^{2+} retained by the protein. This simply reflects the competition of the protein and phosphocellulose for Zn^{2+} and the slow rate of dissociation of the cation from the protein. Although the amount of ion exchanger used affects the final value, by using a defined set of conditions, it was possible to examine the effects of other components on the number of Zn^{2+} cations bound.

The presence of GTP in the incubation mixture greatly diminished the number of Zn^{2+} ions remaining bound after phosphocellulose treatment. In an experiment done in 20 mM Pipes, pH 6.9, and with a Zn^{2+}/tubulin molar ratio of 5.5, 3.1 Zn^{2+} ions were bound in the absence of GTP. This was reduced to 1.1 and 0.9 in the presence of 0.5 and 2.5 mM GTP, respectively. When 1 mM EDTA was included in the incu-

bation mixture, 0.1 Zn^{2+} remained bound after gel filtration.

The chloride salts of a number of cations were used to determine if they could compete for the Zn^{2+} binding sites. The results presented in Figure 6 show that Cu^{+} and Cd^{2+} are effective competitors. However, Mg^{2+}, Mn^{2+}, and Co^{2+} compete poorly. Ca^{2+} was unable to prevent Zn^{2+} binding at the concentrations used.

^{65}Zn–tubulin (20 μM) isolated from phosphocellulose was incubated with other divalent cations or GTP and rechromatographed on phosphocellulose to determine whether the firmly bound Zn^{2+} could be removed by other agents. Mg^{2+}, Mn^{2+}, and Co^{2+}, at 0.5 mM, removed between 0 and 25% of the bound Zn^{2+}. Even 0.5 mM Zn^{2+} removed only 57% of the bound Zn^{2+}. GTP (0.5 mM) removed all but one firmly bound Zn^{2+}, and 1 mM EDTA reduced the Zn^{2+} content to 0.1. Dialysis against 20 mM Pipes for 16 h at room temperature had no effect on the Zn^{2+} content. Inclusion of 10 mM 2-mercaptoethanol resulted in a 50% decrease. In the presence of 8 M urea and 2-mercaptoethanol, the content of all samples was reduced to one Zn^{2+} per dimer. With 1 mM EDTA in the buffer, all of the bound Zn^{2+} was removed.

Effect of Zn^{2+} on the Properties of Tubulin. It was noted in the binding studies that Zn^{2+}/tubulin molar ratios in excess of 15 caused aggregation and precipitation of tubulin. Therefore, analytical ultracentrifugation was used to show that in the presence of Zn^{2+} several tubulin oligomers appear. The formation of oligomers produced by incubation of Zn^{2+} with tubulin containing associated proteins has also been reported (de la Torre et al., 1981). Upon incubation of tubulin in 20 mM Pipes, pH 6.9, with a 15-fold molar excess of Zn^{2+} at 20 °C, 70% of the protein sedimented with a coefficient of 18 S. Reducing the Zn^{2+}/tubulin ratio to 5 decreased the aggregated form to 30%. In the absence of added Zn^{2+}, only the 6S dimer was observed. Observation of schlieren patterns before the centrifuge reached the final speed showed no rapidly sedimenting components. The addition of 1 mM EDTA to the incubation mixture before centrifugation failed to reduce the amount of aggregated protein. When the sedimentation experiments were done in the presence of 0.5 mM GTP and 0.1 M Pipes, somewhat different results were obtained. At a Zn^{2+}/tubulin ratio of 5 (180 μM $ZnCl_2$ and 36 μM tubulin), no aggregates were observed at 5 °C. At 20 °C, large components were formed, which sedimented as the centrifuge reached speed. Under these conditions, assembly was probably taking place. It would appear that the reduction in Zn^{2+} binding caused by the presence of GTP prevents the formation of the aggregates.

For examination of the possible interaction of Zn^{2+} with sulfhydryl groups, the free sulfhydryl group content of tubulin was determined in the presence and absence of Zn^{2+}. In the presence of 20 mM Pipes, pH 6.9, 16 ± 0.2 sulfhydryl residues/tubulin dimer were obtained in control preparations. This value was unaltered in preparations containing up to a 15-fold molar excess of Zn^{2+} over tubulin. Similar results were obtained in the presence of 0.1 M Pipes, pH 6.9, containing 0.5 mM GTP.

The tubulin dimer contains one tightly bound Mg^{2+} (Olmsted & Borisy, 1975; Himes et al., 1977). The incorporation of Zn^{2+} into tubulin was sometimes accompanied by a slight decrease in Mg^{2+} content, with values ranging from 0.7 to 1.0 atom per dimer. It is possible that the firmly bound Mg^{2+} is complexed by the nonexchangeable nucleotide. Therefore, we determined whether the decrease in Mg^{2+} content was also accompanied by a decrease in guanine nucleotide content. Routinely, two guanine nucleotides per tubulin dimer are found in our preparations, as determined from the absorption spectrum of supernates after precipitation with perchloric acid. We found that a lower Mg^{2+} content was associated with a decreased GTP content. For example, in one experiment, the dimer contained 2.0 mol of guanine nucleotide and 0.95 mol of Mg^{2+} per dimer. After treatment with a 15-fold molar excess of Zn^{2+} for 30 min and isolation from phosphocellulose, the dimer contained 1.5 mol of nucleotide, 0.71 mol of Mg^{2+}, and 6.0 mol of Zn^{2+}. In those cases where the incorporation of Zn^{2+} resulted in only a small decrease in Mg^{2+} content, little decrease in nucleotide content was observed.

Table II: Zn^{2+} Binding under Assembly Conditions[a]

addition after polymerization	Zn^{2+}/tubulin ratio: gel filtration	Zn^{2+}/tubulin ratio: phosphocellulose
none	2.5	1.6
GTP, 5 mM	1.5	1.6
EDTA, 1 mM	0.2	ND

[a] Tubulin (20 μM) was assembled in the presence of 0.1 mM $^{65}Zn^{2+}$, 0.5 mM GTP, and 0.1 M Pipes, pH 6.9, and divided into three portions. One was placed on ice. A second was made 5 mM in GTP and after 40 min was placed on ice. The third was made 1 mM in EDTA and after completion of disassembly was placed on ice. Samples of the three were chromatographed on Bio-Gel P10. The control and GTP-treated preparations were also chromatographed on 2.5 mL of packed phosphocellulose.

Relationship between the Amount of Zn^{2+} Bound and Assembly. Although the data presented in Figure 4 showed the existence of multiple binding of Zn^{2+}, under the conditions used for assembly in most of the experiments in this work, a maximum of five Zn^{2+} ions could be bound to the dimer, since 100 μM Zn^{2+} and 20 μM tubulin were usually used. In an attempt to estimate the amount of bound Zn^{2+} needed to stimulate assembly and produce sheet structures, we performed two types of experiments.

The results of one experiment are presented in Table II. After polymerization of tubulin with a 5-fold excess of Zn^{2+} and in the presence of 0.5 mM GTP, followed by cold depolymerization, 2.5 Zn^{2+} ions were bound as determined by gel filtration. Ion-exchange chromatography reduced this to 1.6 ions. The addition of 5 mM GTP caused slight disassembly, as described in Figure 3, and reduced the Zn^{2+} content after gel filtration to about 1.5. EDTA, which causes rapid disassembly, removed essentially all of the bound Zn^{2+}. This experiment provided information on the Zn^{2+} content of total tubulin under assembly conditions. A sample of the assembled protein was also centrifuged through warm 50% sucrose and was found to contain one Zn^{2+} per dimer.

Another type of experiment was designed to answer the question whether tubulin containing only tightly bound Zn^{2+} could assemble and, if so, what products would be produced. We found that tubulin containing up to six Zn^{2+} ions per dimer, isolated from phosphocellulose, would not assemble (at concentrations of 3 mg/mL) in the presence of 100 mM Pipes, pH 6.9, and 0.5 mM GTP and in the absence of additional Zn^{2+}. Of course, under these conditions, the bound Zn^{2+} content would be reduced by the GTP. Tubulin containing tightly bound Zn^{2+} could assemble, however, in the presence of Mg^{2+} and Me_2SO. Tubulin, containing various amounts of bound Zn^{2+}, was isolated from phosphocellulose and assembled in the presence of 0.5 mM $MgCl_2$, 0.5 mM GTP, and 10% Me_2SO. The results presented in Figure 7 demonstrate that assembly decreased with increasing Zn^{2+} content of the dimer. The reduced polymerization, which was confirmed by

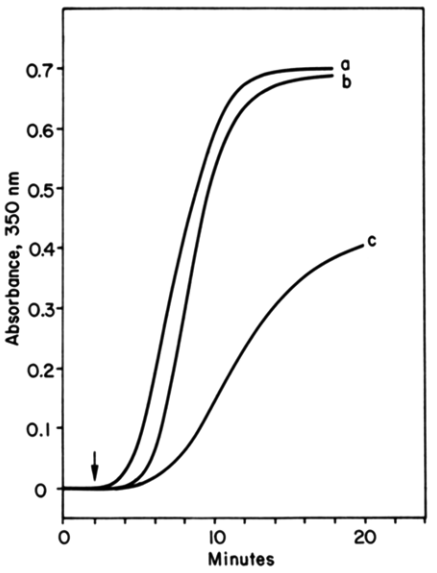

FIGURE 7: Assembly of tubulin containing tightly bound Zn^{2+}. Tubulin solutions (32 μM) in 0.1 M Pipes, pH 6.9, were incubated on ice (a) without Zn^{2+}, (b) with 160 μM $^{65}ZnCl_2$ for 30 min, and (c) with 320 μM $^{65}ZnCl_2$ for 60 min. The solutions were then added to phosphocellulose. The protein–phosphocellulose slurries were placed in Pasteur pipets (2.5-mL packed volume), and the protein was eluted with 0.1 M Pipes, pH 6.9. The eluted protein contained (a) 0 Zn^{2+}/dimer, (b) 2.1 Zn^{2+}/dimer, and (c) 4.4 Zn^{2+}/dimer. The Zn^{2+}–tubulin solutions, at a concentration of 18 μM, were incubated in 0.1 M Pipes, pH 6.9, 0.5 mM $MgCl_2$, and 10% Me_2SO in a 2.0-mL volume. At the arrow, 50 μL of a 20 mM GTP solution was added. Upon completion of the reaction, samples were fixed for thin sectioning. The remaining solution was centrifuged through 50% sucrose to determine the Zn^{2+} content of the polymerized material.

Table III: Assembly of Zn^{2+}–Tubulin in Me_2SO[a]

Zn^{2+}/tubulin ratio			
initial	after PC[b]	after assembly	assembly products
0	0	0	microtubules, some sheets
5	2.1	0.54	narrow sheets, some microtubules
10	4.4	1.30	wide sheets

[a] The experimental details are given in Figure 7. [b] PC = phosphocellulose.

measuring the amount of polymer formed after centrifugation, is probably due to some denaturation of the protein as suggested by the results in Figure 5. The polymer was also isolated by centrifugation through sucrose and the Zn^{2+} content determined. The results in Table III demonstrate that the Zn^{2+} content of polymerized tubulin was decreased and that sheet structures are observed with little Zn^{2+} binding; in fact, the broad sheets contain slightly more than one Zn^{2+} ion per dimer. Electron micrographs of sectioned material from the experiment in Figure 7 and Table III, presented in Figure 8, show the formation of wider sheets at the higher Zn^{2+} content. These wide sheets tend to wrap around themselves.

Inactivation of Tubulin by Zn^{2+}. The binding results shown in Figure 5 and the results presented in Figure 7 indicated that Zn^{2+} has a denaturing or inactivating effect on tubulin. This was shown more directly by an experiment in which tubulin was preincubated with 0.1 mM $ZnCl_2$ for varying periods of time before assembly was initiated (Figure 9). With increasing preincubation periods, a decisive increase in the lag period preceding assembly was seen. Moreover, the final absorbance value decreased. The amount of cold-soluble pelletable material assembled after the 90-min preincubation

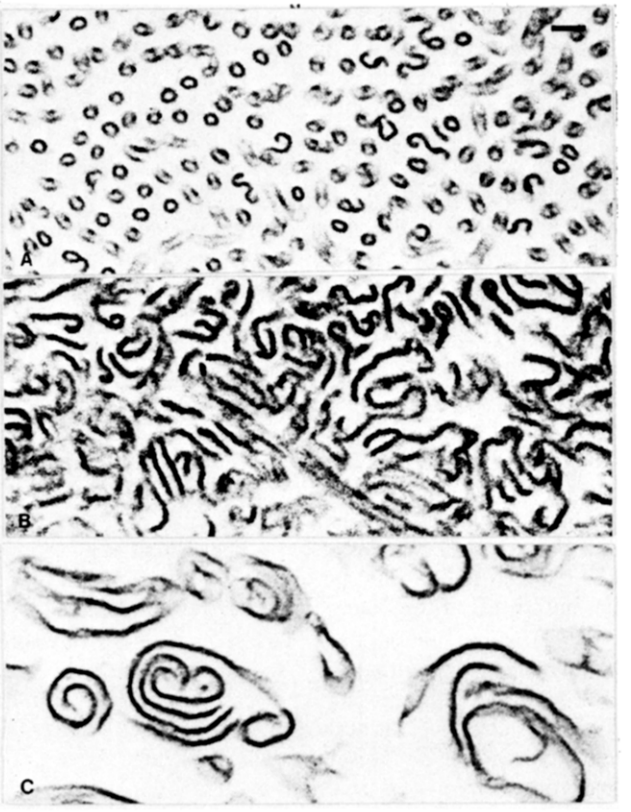

FIGURE 8: Tannic acid stained sections of Zn^{2+}–tubulin polymerized in Me_2SO. Upon completion of the reactions, 0.5-mL samples from the experiment described in Figure 7 were fixed, stained, and sectioned as described under Experimental Procedures. Initial Zn^{2+} content of tubulin: (A) 0; (B) 2.1; (C) 4.4. Bar line = 60 nm for all micrographs.

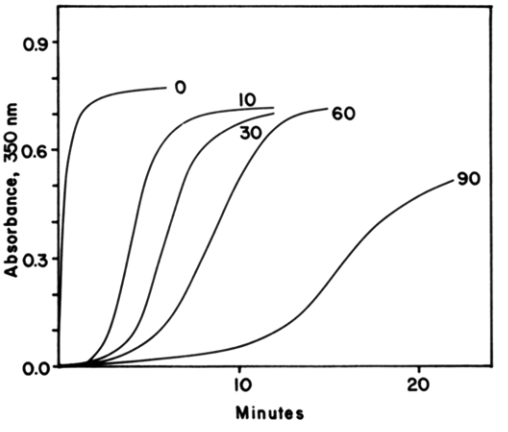

FIGURE 9: Inactivation of tubulin in the presence of Zn^{2+}. Tubulin (3.0 mg/mL) was incubated in the presence of 0.1 M Pipes, pH 6.9, and 0.5 mM GTP, with or without 0.1 mM $ZnCl_2$ at 0 °C. At the times shown, 0.5-mL samples were taken and warmed to 37 °C in a Gilford spectrophotometer. The reaction was followed by measuring the apparent absorbance at 350 nm as a function of time. In the absence of Zn^{2+}, no difference was observed between the 0- and 90-min samples when Zn^{2+} was added to initiate assembly.

was also decreased by 50%. This preincubation was done at 0 °C to prevent assembly, at a low Zn^{2+}/tubulin ratio, and in the presence of GTP which would decrease the amount of Zn^{2+} bound. Even under these conditions, significant inactivation of the dimer occurred.

High-Affinity Zn^{2+} Binding to Preformed Microtubules. Tubulin was polymerized in the presence of 10% Me_2SO and 50 μM $MgCl_2$. After completion of the reaction, a 5- or 10-fold molar excess of $^{65}Zn^{2+}$ over total tubulin was added. The reaction mixtures were centrifuged after 30 min to sed-

iment the polymerized material. The supernatant fluid, containing unpolymerized tubulin, was drawn off, and the sediment was redissolved in cold buffer. The resuspended pellet, the unpolymerized supernatant fraction, and tubulin from an unpolymerized control similarly treated with Zn^{2+} were added to phosphocellulose. Aliquots of the eluants were measured for Zn^{2+} and protein content. The tubulin in the supernates contained 1.6 and 2.7 Zn^{2+} per dimer in the two experiments compared to 2.6 and 3.8 in the unpolymerized tubulin. The polymerized tubulin contained 0.4 and 0.6 Zn^{2+} per dimer, indicating that some of the binding sites are buried in the microtubular structure. The small amount of bound Zn^{2+} could actually be due to some sheet formation upon the addition of the cation (Gaskin, 1981).

Discussion

In this study, we examined the binding of Zn^{2+} to tubulin and the ability of this cation to induce assembly of the protein. All of the studies were done with tubulin lacking microtubule-associated proteins. Although greater than 60 potential binding sites exist on the dimer, under conditions where zinc sheet formation occurs, much less is bound. For example, in the presence of 0.1 mM Zn^{2+}, 20 μM tubulin, and 0.5 mM GTP, conditions used to produce sheet structures, a maximum of five Zn^{2+} ions can be bound per dimer. In fact, the presence of GTP causes this number to be reduced. The results presented in Table II suggest that under these conditions about 2.5 Zn^{2+} ions are bound, one to a site which is not considered high affinity by the definitions used in this work. At the same time, tubulin containing Zn^{2+} bound only to high-affinity sites does not assemble unless either more Zn^{2+} or Mg^{2+} plus Me_2SO is added. The additional cation could be required to form a metal–GTP complex. Using tubulin containing Zn^{2+} bound to high-affinity sites and assembling in Me_2SO and Mg^{2+}, we showed that a Zn^{2+} content of about one per dimer is sufficient to cause sheet formation (Figures 7 and 8, Table III). A Zn^{2+} content of more than one results in the formation of wide sheets. We conclude that stimulation of assembly by Zn^{2+} is promoted by the binding of just a few cations and that sheet formation can result from tubulin containing only one Zn^{2+} per dimer.

Inhibition of Zn^{2+}-induced assembly by excess GTP suggests that the action of Zn^{2+} is due to direct binding of the cation to the protein and not via a ZnGTP complex, although this complex may also be required. A similar conclusion was reached by Gaskin (1981) using a different experimental approach. The change in morphology of the assembled product with increasing GTP concentrations (Figure 2) suggests that occupation of sites by the cation changes lateral interactions between tubulin dimers in protofilamentous structures; the more sites occupied, the greater is the possibility for sheet formation.

Our results show that Zn^{2+} does not replace the tightly bound Mg^{2+}. After incubations with up to a 20-fold molar excess of Zn^{2+}, only slight decreases in Mg^{2+} content were observed. These decreases were accompanied by similar decreases in guanine nucleotide content and were probably due to denaturation of a small amount of tubulin. Denaturation by Zn^{2+} was indicated by a loss in assembly activity with extended incubation periods with Zn^{2+} (Figures 7 and 9) and an increase in high-affinity binding sites with time (Figure 5).

The addition of Zn^{2+} to microtubules in vitro results in the loss of microtubules and the formation of wide sheets (Gaskin, 1981). This apparently is not a direct conversion of microtubules to sheets but occurs from the polymerization of tubulin present into sheets; as a consequence of the depletion of tubulin, microtubules depolymerize (Gaskin, 1981). This is consistent with our finding that the number of high-affinity sites for Zn^{2+} is greatly reduced, if not completely absent, in microtubules.

The interaction of tubulin with Zn^{2+} is similar to its interaction with Co^{2+} (Himes et al., 1982). Both cations reduce the critical protein concentration necessary for assembly and induce assembly at low protein and cation concentrations. With each cation, microtubules are observed at low cation/tubulin ratios but not at high ratios. Sheets of protofilaments are obtained at all ratios but become wider as the ratio is increased. A large number of Zn^{2+} or Co^{2+} ions can bind to tubulin, but under assembly conditions, this number is greatly reduced; in fact, assembly occurs when only a few are bound. Zn^{2+} does appear to bind to more sites with high-affinity than does Co^{2+}. In the presence of GTP, Co^{2+} binds to one or two sites with a K_d of about 1 μM (Himes et al., 1982) whereas Zn^{2+} binds to three such sites. One Co^{2+} is bound to tubulin after incubation with the cation in the absence of GTP followed by treatment with phosphocellulose; as many as seven Zn^{2+} ions are bound after a similar treatment. The larger amount of high-affinity binding by Zn^{2+} probably explains why the formation of microtubules can be observed only with a narrow range of Zn^{2+} concentrations but over a wider concentration range of Co^{2+} (Haskins et al., 1980).

Registry No. Zn, 7440-66-6.

References

Baker, T. S., & Amos, L. A. (1978) *J. Mol. Biol. 123*, 89–106.

Banerjee, A., Roychowdhury, S., & Bhattacharyya, B. (1982) *Biochem. Biophys. Res. Commun. 105*, 1503–1510.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Burton, P. R., & Himes, R. H. (1978) *J. Cell Biol. 77*, 120–133.

Buttlaire, D. H., Czuba, B. A., Stevens, T. H., Lee, Y. C., & Himes, R. H. (1980) *J. Biol. Chem. 255*, 2164–2168.

Carlier, M. F., & Pantaloni, D. (1978) *Biochemistry 17*, 1908–1915.

de la Torre, J., Villasante, A., Corral, J., & Avila, J. (1981) *J. Supramol. Struct. Cell. Biochem. 17*, 183–196.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

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

Gaskin, F. (1981) *Biochemistry 20*, 1318–1322.

Gaskin, F., & Kress, Y. (1977) *J. Biol. Chem. 252*, 6918–6924.

Gaskin, F., Kress, Y., Brosnan, C., & Bornstein, M. (1978) *Neuroscience 3*, 1117–1128.

Haskins, K. M. (1981) Ph.D. Dissertation, University of Kansas, Lawrence, KS.

Haskins, K. M., Zombola, R. R., Boling, J. M., Lee, Y. C., & Himes, R. H. (1980) *Biochem. Biophys. Res. Commun. 95*, 1703–1709.

Herzog, W., & Weber, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1860–1864.

Himes, R. H., Burton, P. R., & Gaito, J. M. (1977) *J. Biol. Chem. 252*, 6222–6228.

Himes, R. H., Lee, Y. C., Eagle, G. R., Haskins, K. M., Babler, S. D., & Ellermeier, J. (1982) *J. Biol. Chem. 257*, 5839–5845.

Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.

Kress, Y., Gaskin, F., Brosnan, C. F., & Levine, S. (1981) *Brain Res. 220*, 139–149.

Larsson, H., Wallin, M., & Edstrom, A. (1976) *Exp. Cell Res. 100*, 104–110.

Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14*, 5183-5187.
Olmsted, J. B., & Borisy, G. G. (1975) *Biochemistry 14*, 2296-3005.
Rosenthal, H. (1967) *Anal. Biochem. 20*, 525-532.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765-768.
Sigel, H. (1977) *J. Inorg. Nucl. Chem. 39*, 1903-1911.
Sillen, L. G., & Martell, A. E. (1971) in *Stability Constants*, Suppl. 1, The Chemical Society, London.
Tamm, L. K., Creapeau, R. H., & Edelstein, S. J. (1979) *J. Mol. Biol. 130*, 473-492.
Voter, W. A., & Erickson, H. P. (1979) *J. Supramol. Struct. 10*, 419-431.
Wallin, M., Larsson, H., & Edstrom, A. (1977) *Exp. Cell Res. 107*, 219-225.
Weisenberg, R. C., Borisy, G. G., & Taylor, E. W. (1968) *Biochemistry 7*, 4466-4479.
Zabrecky, J. R., & Cole, R. D. (1980) *J. Biol. Chem. 255*, 11981-11985.

lac Repressor Headpiece Binds Specifically to Half of the *lac* Operator: A Proton Nuclear Magnetic Resonance Study†

R. M. Scheek, E. R. P. Zuiderweg, K. J. M. Klappe, J. H. van Boom,‡ R. Kaptein,* H. Rüterjans,§ and K. Beyreuther⊥

ABSTRACT: The complex formation of the N-terminal domain (headpiece) of the *Escherichia coli lac* repressor and a synthetic 14-base-pair *lac* operator fragment has been investigated by ^{1}H NMR. Titration shifts in the imino-proton region of the DNA spectrum and in the aromatic region of the headpiece spectrum are examined in detail and interpreted where possible. The assignment of the resonances in the complex follows in part from the titration data and is completed by nuclear Overhauser measurements. The shift of the His-29 C-2 resonance has been used to assess the binding strength of the complex. Evidence is presented for the presence of a high-affinity site on the *lac* operator fragment ($K_D \leq 2 \times 10^{-5}$ M), which shows features in common with one of the specific binding sites on the complete *lac* operator, and for the presence of a second, nonspecific binding site with lower affinity. The influence of this second site on the interpretation of the binding data is discussed.

Since the development of the classical models for gene regulation by Jacob & Monod (1961), the *lac* operon of *Escherichia coli* has been the archetypal example of a negatively controlled operon in prokaryotic organisms. The regulation of the expression of the *lac* genes involves interaction of the *lac* repressor with *lac* operator DNA, which has been the subject of intensive research over the past two decades [for reviews, see Bourgeois & Pfahl (1976), Miller & Reznikoff (1978), and Caruthers (1980)]. Our interest in this system stems from the fact that it may serve as a model for specific DNA recognition by proteins, a process that is still ill understood at the molecular level.

The *lac* operator consists of 21-25 base pairs (see Figure 1). Many of the functional groups that are recognized by the *lac* repressor were identified by measuring changes in the stability of the specific complex upon modification of the operator sequence by a variety of chemical and genetic methods (Gilbert et al., 1975; Ogata & Gilbert, 1977; Goeddel et al., 1978; Caruthers, 1980). Additional information came from the methylation experiments of Ogata & Gilbert (1979). Binding of the *lac* repressor modifies the pattern of purine methylation in a highly characteristic manner. A very similar methylation pattern is obtained when the repressor is replaced by its N-terminal domain (residues 1-59 or 1-51, long and short headpiece, respectively). This observation implies that most of the contact sites on the protein are concentrated in its N-terminal domains, in full agreement with conclusions from studies of mutated *lac I* gene products (Adler et al., 1972; Miller, 1979).

The relatively small size of the *lac* repressor headpiece and its ready availability allow the study of its structure and interactions with operator DNA by using NMR techniques. High-resolution ^{1}H NMR studies of the headpiece (Wade-Jardetzky et al., 1979; Buck et al., 1978; Ribeiro et al., 1981a-c; Wemmer et al., 1981a,b; Arndt et al., 1981; Nick et al., 1982) and of nonspecific complex formation with poly[d(AT)] (Buck et al., 1980; Hogan et al., 1981) have already proven the great potential of NMR techniques in this area of molecular biology. We have chosen to study a chemically synthesized 14-bp[1] DNA fragment comprising half the *lac* operator (base pairs -2 to 12; see Figure 1) because there are strong reasons to believe that half of the *lac* operator is the smallest functional unit able to form a specific complex with headpiece. Several lines of evidence support the model that two subunits of the tetrameric *lac* repressor recognize the

† From the Department of Physical Chemistry, University of Groningen, Groningen, The Netherlands. *Received June 23, 1982.* This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

‡ Present address: Department of Organic Chemistry, University of Leiden, Leiden, The Netherlands.
§ Present address: Institute of Biophysical Chemistry, University of Frankfurt am Main, Frankfurt am Main, FRG.
⊥ Present address: Institute of Genetics, University of Cologne, Cologne, FRG.

[1] Abbreviations: bp, base pair; NOE, nuclear Overhauser effect; CIDNP, chemically induced dynamic nuclear polarization; DTE, dithioerythritol; PMSF, phenylmethanesulfonyl fluoride; TCMP, trichloromethylpropane; DSS, 2,2-dimethyl-2-silapentane-1-sulfonate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-0228$01.50/0