Binding Sites for Calcium on Tubulin†

Frank Solomon

ABSTRACT: Calcium ions can inhibit the in vitro assembly of microtubules and, therefore, may play a role in the regulation of microtubule formation in vivo. In order to test the validity of this hypothesis, the interaction between calcium and purified brain microtubular protein has been investigated by standard binding assays. We have detected and characterized two classes of binding sites for calcium on tubulin, the major component of cytoplasmic microtubules. There is a single high-affinity site per tubulin molecule, characterized by a dissociation constant of 3.2×10^{-6} M. That site is inhibited by magnesium ($K_I = 5 \times 10^{-5}$ M) and potassium chloride. There are approximately 16 low-affinity sites which have a dissociation constant of 2.8×10^{-4} M, and which are also inhibited by potassium chloride. Binding at the low-affinity sites is slightly enhanced by low magnesium concentrations. Both classes of sites are distinguishable from the colchicine binding site, and are apparently also distinct from the vinblastine and guanine nucleotide sites. The characteristics of the calcium binding activity of tubulin are similar to those found for the calcium-binding proteins of sarcoplasmic reticulum. The results are consistent with a physiological role for calcium in the regulation of microtubule assembly.

The ability of cells to regulate the state of assembly of the microtubules has been suggested as an important part of the expression of various differentiated functions (Olmsted and Borisy, 1973). Cytoplasmic microtubules in cultured cells are able to assemble without the synthesis of new protein (Seeds et al., 1970; Hsie and Puck, 1971; Patterson and Waldren, 1973). They are composed primarily, if not exclusively, of tubulin, a 120 000-dalton protein containing two different polypeptide chains. The cell could regulate the assembly of a subunit pool either by covalent modification of the tubulin to a form with an altered ability to assemble, or by a noncovalent interaction with some regulatory species. The most frequently proposed mechanism of covalent modification is phosphorylation (Goodman et al., 1970). Phosphate has been found associated with purified tubulin (Eipper, 1972), although the nature of that association is in doubt (Shelanski, 1973; Solomon et al., 1976). From studies of the requirements for in vitro formation of microtubules from brain extracts, possibilities for noncovalent regulators have arisen. For example, macromolecules which promote in vitro assembly, either by interacting with tubulin itself or with species which inhibit assembly, have been detected in tissue extracts (Weingarten et al., 1975; Bryan et al., 1975; Murphy and Borisy, 1975; Sloboda et al., 1975). An absolute requirement for some of those factors has recently been questioned using highly purified tubulin (Lee and Timasheff, 1975).

Another possibility was suggested by Weisenberg, whose report of the first in vitro assembly of microtubules noted inhibition by calcium ions (Weisenberg, 1972). Kirschener has since expanded that model to include destabilization of intact microtubules by calcium (Kirschener et al., 1974; Kirschener and Williams, 1974). Subsequently, other groups have demonstrated inhibition by calcium ions, at widely different concentrations.

A necessary condition for calcium inhibition in vivo is that calcium interact with a component of the microtubules under conditions likely to be pertinent in vivo. We have found that microtubular protein preparations can bind calcium. The activity is primarily, if not exclusively, associated with tubulin, and is similar to the activity of other calcium-binding proteins. We have characterized the binding sites and tested the effect of other compounds which interact with microtubules on the calcium binding. The possible role of these sites in the control of assembly in vivo and in vitro is discussed. A preliminary report of this work has been made (Solomon, 1975).

Experimental Section

Preparation of Microtubular Protein. Mouse brain microtubular protein was prepared by three cycles of temperature- and glycerol-dependent assembly and disassembly (Sloboda et al., 1975). The final pellet was resuspended in either reassembly buffer (10^{-1} M Pipes[1] buffer, 2.0×10^{-3} M EGTA, 10^{-3} M $MgSO_4$, 10^{-3} M GTP, 4 M glycerol, pH 6.9) or 5×10^{-3} M Tris-HCl, 4 M glycerol, pH 7.5, and stored at 4 °C. The presence of glycerol prevents the decay of the colchicine binding activity (Frigon and Lee, 1972; Solomon, et al., 1973). To determine the purity of the protein preparations, sodium dodecyl sulfate-polyacrylamide slab gels were run according to the procedure of Laemmli (Laemmli, 1970; Maizel, 1971). The gel was stained with Coomassie blue in 50% methanol–7% acetic acid, destained in the same solvent without dye, and then stained and destained to detect minor bands.

Assays of Calcium and Colchicine Binding Activity. Assays of the calcium-binding activity were performed on aliquots of the stored tubulin after column chromatography or dialysis against four changes of 100 volumes of assay buffer (5×10^{-3} M Tris-HCl, pH 7.5, with or without 10^{-1} M KCl). All solutions were made with double-distilled water, which had been previously deionized (Hydroponics, Inc.). The buffers used have been analyzed by atomic absorption spectroscopy. The standard buffer, 5×10^{-3} M Tris-HCl, contains less than 1×10^{-6} M calcium, although the level is too low to be measured precisely. Because of that uncertainty and because the con-

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[1] Abbreviations used are: Pipes, 1,4-piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Ellman's reagent, 5,5′-dithiobis-2-nitrobenzoic acid.

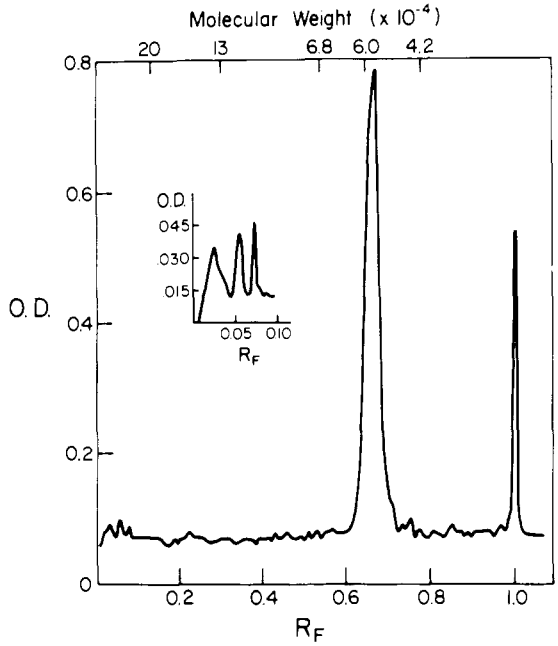

FIGURE 1: Scan of Coomassie blue stained sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of mouse brain microtubular protein. The gel technique used is described under Experimental Section. The standards used to determine the molecular weight were run in adjacent lanes: myosin (mol wt 200 000); β-galactosidase (mol wt 130 000), bovine serum albumin (mol wt 68 000), catalase (mol wt 60 000), and actin (mol wt 42 000). The peak at R_f = 1.0 represents the dye front. Inset: scan of high-molecular-weight region of the gel at threefold scale expansion.

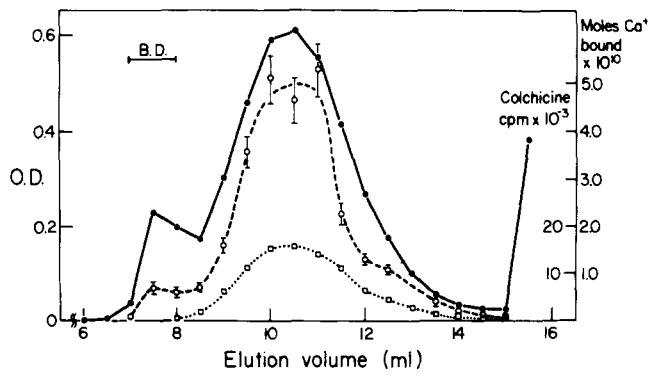

FIGURE 2: Chromatography of microtubular protein, preincubated with tritiated colchicine (2×10^{-5} M, 60 min, 37 °C) on Bio-Gel agarose A 0.5 M, 0.9 × 28 cm, flow rate 8 ml/h, equilibrated with 5×10^{-3} M Tris-HCl, 10^{-1} M KCl, pH 7.5, at 25 °C. Fractions of 0.5 ml were collected and assayed for optical density at 280 nm (closed circles), calcium-binding activity (open circles), and counted for tritium (squares). The calcium-binding data are averages ± standard deviation of triplicate determinations at 10^{-4} M Ca^{2+}. The OD 280 absorbing material at the inclusion volume is GTP from the reassembly buffer. "B.D." represents fractions containing Blue Dextran 2000 in a separate run.

tribution of such a contamination to the value of the binding constant found is small, it has been ignored in the calculations. The addition of 10^{-1} M potassium ions or of 10^{-4} M magnesium ions to the Tris buffer results in calcium concentrations of 3×10^{-6} M and less than 2.2×10^{-6} M, respectively, too low to account for the effects of those ions on calcium binding. For low concentrations of calcium—below 5×10^{-5} M—binding data were obtained by equilibrium dialysis of 0.5 ml of protein solution (0.20–1.0 mg) against 200 volumes of assay buffer containing ^{45}Ca. The dialysis reaches equilibrium in 8 h. After 16 h, the solutions inside and outside the dialysis tubing were counted to determine the distribution of calcium, and the inside solution was assayed for protein using bovine serum albumin as standard (Lowry et al., 1951). Above 10^{-5} M, calcium binding activity was determined by a rapid ultrafiltration procedure using PM-10 Amicon disks and an apparatus manufactured by Metallo-Glass, Inc., Boston, Mass. (Paulus, 1969). Assays by both techniques were performed in triplicate, at 22 °C, and the binding was expressed as the average value ± standard error of the moles of calcium bound per mole of tubulin. Binding values given by the two techniques at the same calcium concentration agree within experimental error. The data were fitted to the Scatchard equation by successive trials.

Colchicine binding activity was measured using a DEAE-cellulose Whatman filter disk assay (Borisy, 1972).

Materials. Radioactive calcium was purchased from New England Nuclear as the chloride salt. Tritiated colchicine (2 Ci/mmol, The Radiochemical Centre), colchicine (Fisher Scientific Co.), vinblastine sulfate (Sigma), and Trizma base (Sigma) were all used without further purification.

Results

Calcium-Binding Activity Associated with Tubulin. Gel electrophoresis of the microtubular protein, prepared as described above, shows that about 90% of the material stained by Coomassie blue migrates with tubulin (Figure 1). The major contaminants are the high-molecular-weight microtubule-associated proteins, which are present to varying extents in all such preparations (Dentler et al., 1974; Gaskin et al., 1974; Erickson, 1974). The purity of the preparation was also confirmed by the specific activity of colchicine binding; freshly purified material bound 0.90 ± 0.02 mol of colchicine/120 000 g of protein. This figure is a minimum estimate of the tubulin concentration in the preparation, since some of the colchicine sites may have been inactivated during purification.

To determine whether these protein preparations were competent to bind calcium, and which of the components was active, gel filtration was used to separate the tubulin and the high-molecular-weight proteins. Microtubular protein was incubated with tritiated colchicine and the reaction mixture was then centrifuged at 100 000*g* at 4 °C to remove aggregates formed during the warm incubation. The supernatant was applied to a column of Bio-Gel A-0.5 m, equilibrated with 5×10^{-3} M Tris-HCl, 10^{-1} M KCl, pH 7.5. The eluant fractions were assayed for protein, for associated colchicine, and for calcium binding activity at 10^{-4} M calcium. Two peaks of protein were eluted from the column (Figure 2). The minor peak, at the void volume of the column, has calcium binding activity but contains no associated colchicine. Gel electrophoresis of this material shows that it is primarily composed of the tubulin polypeptides, as well as the high-molecular-weight components. The second peak is eluted from the column at a position consistent with a molecular weight of 120 000. Throughout that peak, the relationships between protein concentration, colchicine, and calcium-binding activity are constant. The greater part of the calcium-binding activity of our preparations is, therefore, associated with tubulin. The first excluded peak probably consists primarily of tubulin aggregates not removed by centrifugation and which are not competent to bind colchicine, although they can bind calcium under the conditions of the assay. We can not exclude the possibility that the high-molecular-weight proteins contribute to the total calcium-binding activity to some extent.

Stoichiometry and Affinity of Calcium Binding. Calcium-binding proteins from several sources have been assayed in 5×10^{-3} M Tris-HCl, pH 7.5, with and without 0.1 M KCl

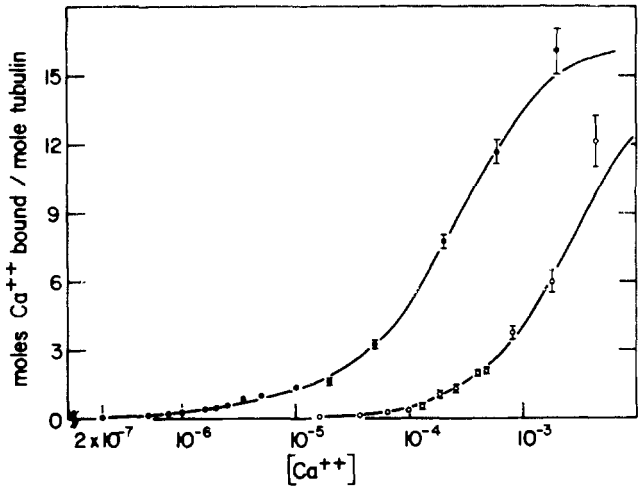

FIGURE 3: Calcium binding of tubulin as a function of calcium concentration and presence of salt. Closed circles: assays in 5×10^{-3} M Tris-HCl, pH 7.50. Open circles: assays performed in 5×10^{-3} M Tris-HCl, 10^{-1} M KCl, pH 7.50. The curves are the theoretical lines generated by the parameters: no salt, $K_1 = 3.57 \times 10^{-6}$ M, $n_1 = 1$; $K_2 = 2.83 \times 10^{-4}$ M, $n_2 = 16$; with salt, $K = 2.5 \times 10^{-3}$ M, $n = 16$.

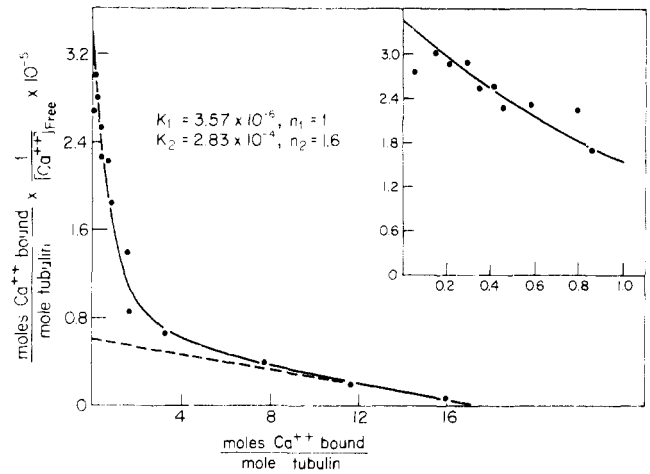

FIGURE 4: Scatchard analysis of binding in the absence of KCl. Average values from Figure 3 have been replotted according to the Scatchard equation. The curve is the theoretical line for the parameters in Figure 3. Inset: Binding at low concentrations replotted on an expanded scale.

(MacLennan, 1974). A typical assay of the tubulin-binding activity under those conditions is shown in Figure 3. In the absence of KCl, there is detectable binding at very low calcium concentrations. Analysis using the Scatchard equation shows that the binding data is consistent with two classes of binding sites (Figure 4). There is a single high-affinity site per mole of tubulin, with a dissociation constant, K_d, averaged from five separate determinations, of 3.2×10^{-6} M (Table I). If the contaminating calcium concentration noted under the Experimental Section is at its maximal value—about 1×10^{-6} M—then the true constant is somewhat lower, about 2×10^{-6} M. There are also approximately 16 low-affinity sites, with $K_d = 2.5 \times 10^{-4}$ M. In the presence of KCl, there is no detectable binding at low calcium concentrations. The affinity of the low-affinity sites is an order of magnitude weaker than in the absence of salt, although their stoichiometry is apparently the same. Therefore, in the presence of high salt, the high-affinity site is either totally inhibited, or its dissociation constant is shifted such that the site can no longer be separated from the low-affinity binding. The stoichiometry data are not sufficiently precise to distinguish between those possibilities. Both the high- and low-affinity sites are associated with the included tubulin peak in Figure 2.

TABLE I: Values for Dissociation Constant (K_d) and Number of Sites (n) under Different Conditions.[a]

Additions to Assay Buffer	n_1	K_{d_1} (M)	n_2	K_{d_2} (M)
None[b]	1	3.2×10^{-6}	16	2.5×10^{-4}
10^{-1} M KCl[c]			16	2.5×10^{-4}
10^{-6} M $MgSO_4$[c]	1	3.6×10^{-6}	16	1.4×10^{-4}
6×10^{-5} M $MgSO_4$[c]	1	9.4×10^{-6}	16	2.5×10^{-4}

[a] The values for n and K_d were determined by fitting theoretical curves to data in Scatchard plots. [b] Values average of five determinations. [c] Values average of three determinations.

TABLE II: Calcium Binding in the Presence of Colchicine.

Calcium Concn (M)	Colchicine in Preincubation[a]	Mol of Calcium/ Mol of Tubulin[b]	Mol of Colchicine/ Mol of Tubulin[b]
2.0×10^{-7}	+	0.039 ± 0.003	0.82 ± 0.12
2.0×10^{-7}	−	0.036 ± 0.004	
1.6×10^{-6}	+	0.440 ± 0.020	0.82 ± 0.13
1.6×10^{-6}	−	0.410 ± 0.002	

[a] Microtubular protein which had been stored in 4 M glycerol was diluted into 5×10^{-3} M Tris-HCl, pH 7.5, and incubated for 90 min at 37 °C with and without 2×10^{-4} M tritiated colchicine. [b] The binding data are reported as moles bound per 120 000 g of protein, corrected to 90% purity.

Relationship to Nucleotide and Alkaloid Binding Sites. Specific binding sites for colchicine, guanine nucleotides, and vinblastine have been detected on tubulin (Bryan, 1972; Wilson et al., 1975). Since colchicine is an inhibitor of in vitro assembly and binds with the same stoichiometry as the high-affinity calcium binding, we tested the possibility that there was a common site for colchicine and calcium. We have previously reported that the colchicine and calcium sites are at least partially separable, since the calcium-binding activity is not affected by treatments which destroy the colchicine-binding activity (Solomon, 1975), such as blocking free sulfhydryl groups with Ellman's reagent or prolonged incubation without GTP or glycerol (Solomon et al., 1973). However, a more rigorous test of whether the sites share a common region on the protein is to ask whether prior binding of colchicine inhibits the extent of calcium binding at equilibrium. Tubulin was preincubated with tritiated colchicine and aliquots were then assayed for calcium-binding activity by equilibrium dialysis at two concentrations of calcium (Table II). Under these conditions, the colchicine concentration during the dialysis assay is sufficient to maintain the colchicine binding. After equilibrium dialysis, the preferential binding of both colchicine and calcium to the protein was determined. As shown in Table II, colchicine does not interfere with the binding of calcium at either 2×10^{-7} M, where the high-affinity site is measured, or at 1.6×10^{-6} M, where the contribution of the low-affinity sites becomes considerable.

The possibility that calcium binds at the nucleotide or vinca alkaloid site has also been tested. Assays at 5×10^{-7} M calcium in the presence of 10^{-4} M GTP or after preincubation

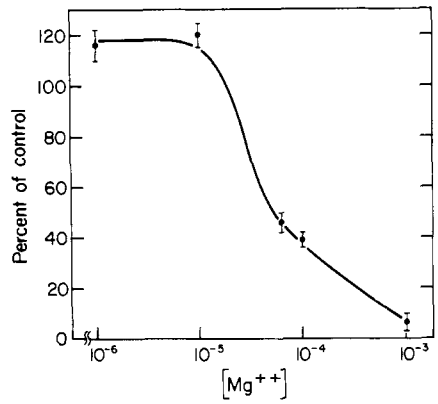

FIGURE 5: Calcium binding of tubulin at 5×10^{-7} M calcium as a function of magnesium concentration.

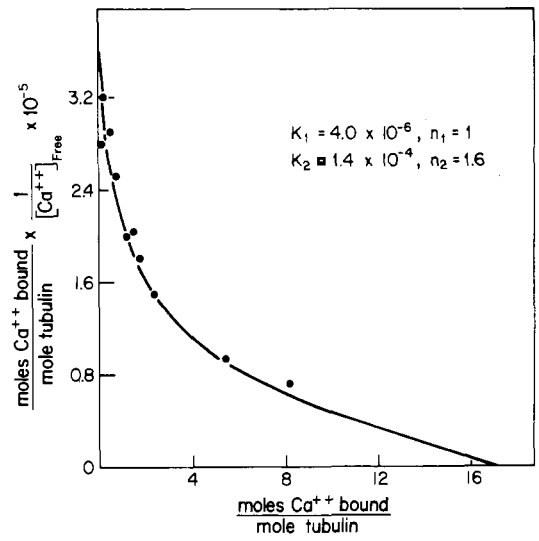

FIGURE 6: Scatchard analysis of calcium binding in the presence of 10^{-6} M magnesium. The curve is the theoretical line for $K_1 = 4.0 \times 10^{-6}$ M, $n_1 = 1$, $K_2 = 1.4 \times 10^{-4}$ M, $n_2 = 16$.

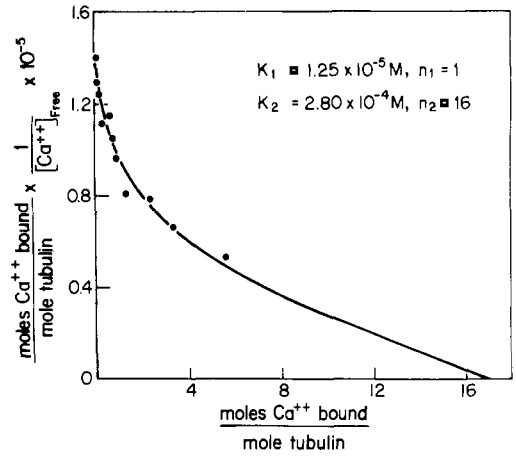

FIGURE 7: Scatchard analysis of calcium binding data in the presence of 6×10^{-5} M magnesium. The curve is the theoretical line for $K_1 = 1.25 \times 10^{-5}$ M, $n_1 = 1$, $K_2 = 2.8 \times 10^{-4}$ M, $n_2 = 16$.

with 10^{-5} M vinblastine show no effect of these compounds. The possibility of overlap of binding sites can not be rigorously ruled out in these cases, since the precise extent of nucleotide and vinblastine binding was not directly measured.

The Effect of Magnesium on Calcium Binding. We have previously reported that calcium-binding activity can be either enhanced or inhibited by low or high concentrations of magnesium, respectively (Solomon, 1975). At 10^{-3} M magnesium, <10% of the calcium binding at 5×10^{-7} M calcium remains. At that calcium concentration, 10^{-7} to 10^{-6} M magnesium increases calcium binding by about 25% (Figure 5). These effects could be the result of a change in the affinity constants of either site, or in the number of sites available. To distinguish between these possibilities, Scatchard analysis of calcium binding at 10^{-6} and 6×10^{-5} M magnesium was performed in the absence of added KCl (Figures 6 and 7); the results are summarized in Table I. At the low concentration of magnesium, the increases in binding observed can all be accounted for by an effect on the K_d of the low-affinity sites, and not the number of such sites. The inhibition of binding by higher magnesium concentrations can be explained by an effect on the apparent K_d at the high-affinity binding site (Table I). This behavior is consistent with competition of magnesium for the high-affinity site. Half-maximal calcium binding at that site occurs at about 5×10^{-5} M magnesium.

Discussion

Results of the in vitro assembly experiments suggest that calcium might interact with a component of microtubules, or with the intact microtubule itself, to shift the equilibrium of assembly toward the dissociated state. The results reported here demonstrate that calcium can bind to tubulin with high affinity. Previous reports of interactions between calcium and tubulin have been reported. Relatively high concentrations of calcium can induce tubulin aggregates (Weisenberg and Timasheff, 1970) and precipitate tubulin along with other proteins (Wilson et al., 1970).

The studies of calcium-binding activity have provided evidence for two distinct interactions between magnesium and tubulin. Magnesium inhibits the high-affinity site reaction by affecting the binding constant rather than the stoichiometry. Apparently, then, magnesium may bind to the same site as calcium, although with an order of magnitude lower affinity. The enhancement of calcium binding detected in the presence of very low concentrations of magnesium, however, suggests the presence of sites that do not bind calcium, since there is no evidence for a cooperative effect of calcium concentration on observed binding. It was not possible to demonstrate that magnesium is an absolute requirement for the calcium-binding reaction at either site, since extensive dialysis of protein against EDTA did not lower the binding activity. This result might be explained by the presence of trace amounts of magnesium in the buffers (see Experimental Section), which are tightly bound by the protein when the chelater is removed. Based on the estimates of the in vivo magnesium concentration discussed below, it is unlikely that the enhancement represents a physiologically significant event. The possibility that both magnesium and calcium can bind to the same site, as in the inhibition reaction, suggests a model for regulation of microtubule assembly, which depends upon which species occupies that site.

The relationship between calcium binding and an altered ability to assemble, if it exists at all, is apparently not directly related to the effects of other compounds which can bind to tubulin and affect assembly. We have been unable to demonstrate any relationship between the calcium-binding sites and those for colchicine and vinblastine, which inhibit assembly, or GTP, which is necessary for assembly (Weisenberg, 1972). Wilson et al., using radioactive vinblastine, have reported that

alkaloid binding is not affected by 10^{-3} M calcium under conditions where only binding to the low-affinity sites would have been observed (Wilson et al., 1975). Also, the local anesthetic Procaine, at concentrations up to 10^{-3} M, does not inhibit calcium binding (F. Solomon, unpublished observations). This finding argues against the suggestion that the effects of this compound on cell shape are mediated by its effect on the tubulin-calcium interaction (Nicolson et al., 1976).

The major issue is whether the binding reaction is significant for events in the intact cell. The sites for calcium on tubulin are similar to sites on the sarcoplasmic reticulum proteins. In particular, the 55 000-dalton "high-affinity calcium-binding protein" has a high-affinity site with a comparable $K_d = 3 \times 10^{-6}$ M (Ostwald and MacLennan, 1974). Like tubulin, this protein also contains several sites of lower affinity—about 25/mol—with a $K_d = 1.2 \times 10^{-4}$ M. In general, all the sarcoplasmic reticulum proteins (Ostwald and MacLennan, 1974), both forms of calsequestrin (MacLennan, 1974), and calcium-binding proteins from rat liver (Towry, 1973) all have a lower affinity for calcium in the presence of 10^{-1} M KCl, as does tubulin. Myosin also has two classes of calcium-binding sites, with stoichiometries and affinities similar to those of tubulin; those sites are also inhibited by KCl and magnesium (Beinfeld et al., 1975). This comparison implies that tubulin could be a member of a class of proteins which presumably function in vivo to bind calcium. The tubulin binding is, however, three orders of magnitude weaker than the tight binding sites on troponin (Potter and Gergely, 1975). Magnesium binding to tubulin has been previously reported (Frigon and Timasheff, 1975; Olmsted and Borisy, 1975). The characteristics of magnesium enhancement suggest that it may occur at the same site as the extremely tight binding, at 1 mol/mol, of magnesium detected by Olmsted and Borisy.

Comparison of the binding reaction with specific conditions likely to pertain inside the cell is complicated by the fact that measurements in vivo of both overall and local ion and protein concentrations are, at best, problematical. Figures reported for the relevant ion concentrations vary considerably. The free calcium concentration in red blood cells is estimated to be lower than 4×10^{-5} M (Schatzman and Vincenzi, 1969), although somewhat higher values have been reported for tissue culture cells (Borle, 1968). The concentration of free magnesium has been estimated at $0.6\text{-}1.0 \times 10^{-3}$ M (Veloso et al., 1973) and intracellular potassium in muscle has been measured as 1.6×10^{-1} M (Waddel and Bates, 1969). At that concentration of potassium ions, 1 mol of calcium would not be bound per mole of tubulin until a calcium concentration of greater than 10^{-4} M, or somewhat higher than the values reported. The figures do argue against a physiological role for the high-affinity site per se. However, there may be other factors which are present in the cell and which, like magnesium, enhance the calcium binding. There is also considerable doubt as to the meaning of the nominal intracellular ion concentrations. To what extent those concentrations are averages of widely different compartments inside the cells, and to what extent the ions are loosely complexed as counterions to polyionic macromolecules, simply is not known.

The relationship between the in vitro assembly experiments and the calcium-binding sites is more easily examined. It is generally concluded that assembly absolutely requires the presence of low concentrations of calcium or magnesium, and that high concentrations of calcium can destabilize the microtubules once they are formed (Haga et al., 1974; Olmsted and Borisy, 1975; Rosenfeld and Weisenberg, 1974). The inhibition of assembly, however, apparently depends upon the precise conditions of the reaction. Values for the concentration of calcium required for inhibition vary from about 10^{-5} M or essentially equimolar with respect to tubulin (Haga et al., 1974; Lee and Timasheff, 1975), to about 10^{-3} M (Rosenfeld and Weisenberg, 1974; Olmsted and Borisy, 1975). In fact, this discrepancy can be explained by our findings on the effect of salt concentration on the calcium-binding activity, since the higher values were obtained from reassembly experiments at much higher ionic strength. This conclusion is supported by the fact that increasing potassium chloride decreases the ability of calcium to inhibit (Haga et al., 1974). Finally, relatively high magnesium concentrations—between 5×10^{-3} M (Rosenfeld and Weisenberg, 1974) and 2×10^{-2} M (Olmsted and Borisy, 1975)—enhance the inhibitory effect of calcium. This cooperativity may be a reflection of the magnesium stimulation on calcium binding, which is illustrated in Figure 5, although under the conditions of the binding reaction magnesium inhibits binding at those high concentrations.

These considerations taken together are consistent with, but do not prove, a physiological role for calcium in the regulation of microtubule assembly. Such a model predicts that factors which affect the intracellular ion concentrations will also affect the state of assembly of microtubules. We are presently testing those predictions in appropriate tissue culture systems.

Acknowledgments

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Metal Site Conformational States of Vanadyl(IV) Human Serotransferrin Complexes†

N. Dennis Chasteen,* Lawrence K. White, and Robert F. Campbell‡

ABSTRACT: This study was undertaken to investigate the conformational states of the two metal sites in the human serum transferrin molecule. The 9.2 GHz electron paramagnetic resonance spectra of frozen solutions of divanadyl(IV) transferrin consist of a superposition of two sets of resonances, A and B, due to the magnetically nonequivalent binding environments of the VO^{2+} ion. Examination of the intensities of the A and B resonances as a function of pH from 6.0 to 10.7 reveals that they arise from two conformational states of the metal sites in which the geometrical arrangement and/or identity of one or more ligands in the first coordination sphere are different. From pH 7.5 to 9.0, the metal sites exist in A and B conformations but above pH 9.0 the A conformation undergoes a transition to the B conformation. This transformation is coupled to the ionization of an apparently noncoordinating protein functional group with a $pK = 10.0 \pm 0.1$. Below pH 7.0, binding in the B conformation is rapidly lost, driven in part by the protonation of a functional group, possibly the anion, with a $pK = 6.6 \pm 0.1$. In 90% D_2O, this pK is elevated to 7.8 ± 0.1. At pH 6.0 in H_2O, essentially one VO^{2+} ion remains bound to the protein with the metal site in the A conformation. Experiments with mixed VO^{2+}–Fe^{3+} transferrin complexes indicate that the same may be true of Fe^{3+}. At pH 10.7, a new set of VO^{2+} resonances, labeled C, are observed; they possibly arise from a third conformation of the metal site. One bicarbonate or carbonate is required per VO^{2+} ion bound to the protein. 2.7 H^+ are released per VO^{2+} bound in either the A or B conformations. The above results are discussed in terms of the "equivalence" and "nonequivalence" of the metal sites.

The iron transport protein transferrin has two Fe(III) binding sites which exhibit similar spectroscopic and thermodynamic properties (Gafni and Steinberg, 1974; Luk, 1971; Binford and Foster, 1974; Aasa and Aisen, 1968; Aisen et al., 1969; Aasa, 1972; Price and Gibson, 1972a). Transferrin has a molecular weight of around 80 000 and consists of a single polypeptide chain (Mann et al., 1970) with two identical carbohydrate side chains (Spik et al., 1975). Human serotransferrin has been recently reviewed (Chasteen, 1977).

A suitable anion must be present in a 1:1 stoichiometric ratio with the iron in order for the metal to bind (Aisen et al., 1967;

† From the Department of Chemistry, University of New Hampshire, Durham, New Hampshire 03824. *Received June 17, 1976.* This work was supported by the National Institute of General Medical Sciences, Grant GM-20194-04.

‡ Present address: Department of Chemistry, Cornell University, Ithaca, N.Y.