INTERACTION OF MICROTUBULE PROTEINS WITH PHOSPHOLIPID VESICLES

JOAN M. CARON and RICHARD D. BERLIN. From the Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06032

ABSTRACT

We have examined the interaction of unilamellar dimyristoyl phosphatidylcholine liposomes with the high-speed supernate of brain homogenate and with tubulin purified through one or two cycles of microtubule assembly-disassembly. Tubulin and certain high molecular weight proteins are selectively adsorbed from these mixtures onto liposomes. The composition of adsorbed proteins is similar to that obtained during corresponding cycles of microtubule assembly, suggesting the equivalency of these processes. Adsorption induces stacking and/or fusion of liposomes into multilamellar structures indicating strong protein-lipid interaction. In addition, liposome-adsorbed tubulin forms extensive intermolecular disulfide bridges that are inert to reducing agents in the aqueous medium. The observations form a basis for further study of the distribution, function, and properties of membrane-bound tubulin.

KEY WORDS tubulin · membrane · disulfides · phospholipid · microtubules · liposomes

Pharmacological evidence has implicated microtubules in the control of a wide range of surface phenomena. For example, microtubule disassembly by colchicine enables the movement of surface-bound concanavalin A into caps in leukocytes (1, 35) and prevents the segregation of membrane proteins (26, 32) and probably lipids (4) that normally accompanies phagocytosis in neutrophils. In addition, studies of neutrophils from mice with the Chedak-Higashi syndrome show that failure of microtubule assembly causes colchicine-like effects but in the absence of drugs (25). These results indicate that microtubule assembly is required to control and direct the distribution of membrane components.

We have suggested that microtubule regulation of surface events may occur indirectly through control of the microfilament-contractile system (5). On the other hand, there is increasing evidence for a direct association of tubulin (the major subunit component of microtubules) with membranes. For example, there is specific colchicine binding to isolated membrane fractions from brain (6, 10); and tubulin has been identified in synaptic vesicles by polyacrylamide gel electrophoresis (7, 11, 19), by reaction with specific antibodies (33), and recently by analysis on two-dimensional electropheretograms and by tryptic peptide mapping (17). Stephens' (30) analyses of membranes of the scallop have also supported the existence of membrane-bound tubulin: it was clearly shown that tubulin is associated with ciliary but not flagellar membranes. Finally, previous in vitro studies in our laboratory demonstrated fluorescence resonance energy transfer between fluorescein-labeled neutrophil membranes and rhodamine-labeled brain tubulin (2). At 37°C, a high degree of transfer indicated close approximation of the membrane and tubulin. At 0°C, transfer did not occur.

While these studies indicate the presence of tubulin in isolated membrane fractions, they indicate neither the molecular nor functional basis of the tubulin-membrane interaction. In particular, a major difficulty in establishing the molecular

basis of interaction is the complexity of biological membranes, and the presence of associated structures, such as cilia and soluble phases that contain abundant tubulin including, perhaps, the lumen of synaptic vesicles.

Many known membrane proteins can be functionally reconstituted in simple artificial membranes (9, 13, 28). We have investigated the possibility that tubulin shares with such integral membrane proteins the ability to interact with artificial phospholipid vesicles. We show first that phosphatidylcholine unilamellar liposomes selectively adsorb tubulin and associated high molecular weight proteins from either crude soluble supernates of brain, or tubulin purified through one or two cycles of microtubule assembly and disassembly. Adsorption may be governed by forces similar to those involved in microtubule assembly since the composition of adsorbed proteins is essentially equivalent to that achieved in parallel cycles of assembly and disassembly. Adsorption leads to highly ordered aggregation or fusion of liposomes into stacks or multilamellar structures. In addition, we show that adsorption is associated with the formation of intermolecular disulfide bonds. These bonds of the liposome-protein structure are inert to reducing agents in the external aqueous milieu but are readily reduced after detergent extraction. The evidence is consistent with strong and specific tubulin interactions with simple bilayer membrane structures.

MATERIALS AND METHODS

Dimyristoyl phosphatidylcholine (DMPC) was purchased from Sigma Chemical Co., St. Louis, Mo. The purity of the DMPC was checked by thin-layer chromatography. Lipids from sonicated liposomes alone and sonicated liposomes incubated with brain homogenate for 15 min at 30°C were extracted with chloroform: methanol (2:1) according to the method of Folch et al. (12) and run on silica-coated glass plates (EM Laboratories) in chloroform:methanol:H_2O (68:25:1). A single spot was identified by iodine vapor under all conditions, indicating the absence of phospholipid degradation. Similarly, no fatty acid degradation products were shown by gas-liquid chromatography. Unilamellar phospholipid vesicles were prepared under nitrogen according to the method of Rogers and Strittmatter (28).

Crude brain supernate was prepared by homogenizing fresh bovine brain in the cold in 0.1 M Na glutamate, 0.02 M phosphate, pH 6.75 (23). The homogenate was centrifuged at 40,000 *g* for 45 min at 4°C. Tubulin was prepared by incubating the bovine brain supernate with 2.5 mm GTP, 1 mm EGTA, 0.5 mm $MgCl_2$ for 20 min at 30°C, and centrifuging at 40,000 *g* for 30 min at 30°C. The pellet was resuspended in one-tenth volume of the phosphate-glutamate buffer, gently homogenized, incubated on ice for 30 min, and recentrifuged at 40,000 *g* for 30 min at 4°C. The cold-soluble supernate is designated "once cycled." Twice-cycled tubulin was prepared by repeating the assembly-disassembly procedure.

All incubations and centrifugations with liposomes were carried out at 30°C, i.e., above the transition temperature of the DMPC (24°C). The various protein samples were incubated with the liposomes in 0.1 M NaCl, 0.02 M phosphate, pH 6.75, 2 mM EDTA. In some experiments, liposomes were prepared in buffered 1.0 M NaCl, and protein and liposomes were incubated in the same high salt solution. It was found that the composition of adsorbed proteins was unchanged from 15 min to 6 h of incubation. Therefore, the incubation was routinely terminated at 15 min by addition of an equal volume of Ficoll of density 1.10. This was layered within a discontinuous Ficoll gradient of the following densities: 1.08, ~1.05 (sample), 1.04, 1.03, 1.007. The liposomes were separated from the nonadsorbed proteins and washed by centrifuging upward through this gradient at 100,000 *g* for 2 h. The liposome band at the 1.03–1.007 interface was removed with a pasteur pipette, diluted in the salt buffer, and centrifuged at 100,000 *g* for 1 h. This represented all of the added liposomes, which thus had been floated and washed through layers of 1.04 and 1.03 specific gravity. The supernate was aspirated, and the pellet was resuspended in buffer.

SDS polyacrylamide gel electrophoresis using gradient slab gels (13 × 0.2 × 0.90 cm) of 5–15% acrylamide with a 3% stacking gel was performed according to the method of Laemmli (21). All samples were boiled for 2 min in SDS immediately before application. After electrophoresis, the gels were fixed and stained in 0.025% Coomassie blue, 10% glacial acetic acid, 25% isopropanol, and destained in 10% methanol, 10% glacial acetic acid. Protein concentrations were determined by the method of Lowry et al. (22).

For negative staining, a drop of the liposome incubation mixture was placed on a carbon-coated formvar grid for 30 s, drained, stained with 1% phosphotungstic acid, pH 6.5, and examined in a Philips 300 electron microscope.

RESULTS

A comparison of whole brain supernate (Fig. 1 *a*) with liposome-adsorbed proteins (Fig. 1 *b*) shows selective uptake into liposomes of proteins from the 100,000 *g* supernates. The selective enrichment in synthetic DMPC liposomes of tubulin (separated as the 52–55,000 dalton α and β monomers) from the crude brain supernate is clearly demonstrated. Nearly all of the α- and β-monomers and high molecular weight components normally associated with tubulin assembly are taken up into the liposomes while several other components are

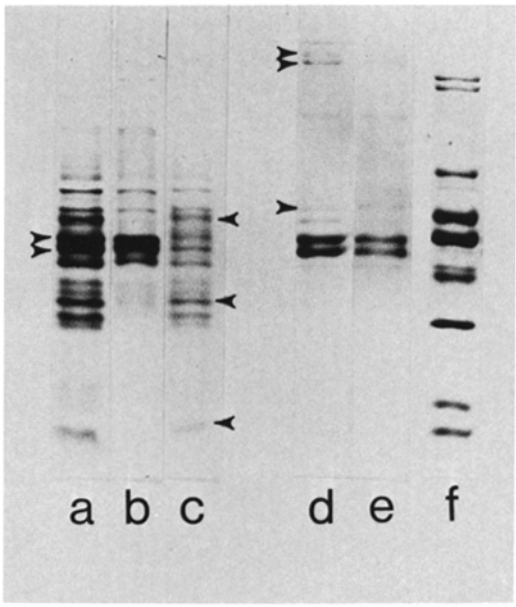

FIGURE 1 Selective adsorption to liposomes of proteins from brain homogenate and once cycled tubulin. SDS polyacrylamide gel electrophoresis (see Materials and Methods) of whole brain homogenate (*a*), liposome-adsorbed proteins (*b*), and proteins not adsorbed to the liposomes (*c*). 1 mg of the whole brain homogenate was incubated with 10 mg DMPC vesicles in 0.6 ml salt buffer. The arrows (*a*) indicate the α- and β-monomers of tubulin. Selective adsorption of tubulin and associated HMW proteins to liposomes is clearly demonstrated in (*b*). The arrows in (*c*) indicate several of many proteins which do not associate with the liposomes, including hemoglobin (bottom arrow). The electrophoretic pattern of once-cycled tubulin and the corresponding liposome-adsorbed proteins are shown in (*d*) and (*e*), respectively. 0.5 mg once-cycled tubulin was incubated with 10 mg of DMPC vesicles in 0.6 ml of salt buffer. Again, the selective uptake of the α- and β-monomers of tubulin and the associated HMW proteins is demonstrated. The arrows (*d*) indicate microtubule-associated proteins, Map's 1 and 2 (29) and intermediate proteins, probably tau (34). Standards (*f*) of increasing molecular weight are lysozyme (14.4 K) soybean trypsin inhibitor (21), carboanhydrase (31), peroxidase, (40), pyruvate kinase (57), bovine serum albumin (68), phosphorylase *b* (92) and spectrin (230,265). Samples for electrophoresis contained 1% SDS, 1% β-mercaptoethanol and 20 μg of protein (except [*c*] where the protein concentration was not determined).

entirely excluded. This is emphasized by the complementary gel pattern (Fig. 1 *c*) of supernatant proteins remaining after the incubation with liposomes. Virtually all tubulin and high molecular weight components have been removed. However, many other bands are present that do not appear on the liposome pattern. A few proteins of relatively poor affinity for the lipid are present in patterns of both liposome and residual supernatant proteins.

When the homogenate is purified through one cycle of microtubule assembly-disassembly and then incubated with liposomes, selective uptake is again seen with complete adsorption of tubulin and high molecular weight components. Thus, Fig. 1 *d* shows the tubulin-enriched, but still heterogeneous, electrophoresis pattern of once cycled tubulin, while Fig. 1 *e*, the pattern for the liposome-adsorbed fraction of once cycled tubulin, is clearly dominated by tubulin and its associated, high molecular weight components. Twice-cycled tubulin showed a similar extreme affinity for liposomes (Fig. 4 *a*, and *c*).

Essentially all of the tubulin from fresh homogenates or after one or two cycles of assembly of brain supernate could be adsorbed onto liposomes. This excludes the possibility that only denatured tubulin associates with the lipid bilayer.

We particularly emphasize from the data in Fig. 1 the strikingly similar purification of brain proteins resulting from liposome adsorption and the conventional cycles of microtubule assembly and disassembly. Thus, proteins from the crude supernate adsorbed on liposomes (Fig. 1 *b*) are highly similar to the once-cycled tubulin (Fig. 1 *d*), and tubulin adsorbed from the latter (Fig. 1 *e*) is virtually identical to twice-cycled tubulin (Fig. 4 *a*). We emphasize, however, that adsorption to liposomes occurs under conditions that are not compatible with tubulin polymerization: GTP is not present and EDTA is added to remove any residual divalent cations. Moreover, microtubules are not seen by electron microscopy of negatively stained preparations.

Despite the absence of microtubule assembly, morphological examination by negative staining suggests a specific interaction of tubulin with the liposomes. The small insert, Fig. 2 *a*, shows control liposomes (no added protein). Single lipid vesicles are of a uniform diameter of ~290 Å. Fig. 2 *b* shows liposomes incubated with twice-cycled tubulin for 15 min at 30°C and then diluted 1:10 with the salt buffer before staining. Fig. 2 *c* shows the same liposome-tubulin incubation mixture without dilution before negative staining. In the presence of tubulin the liposomes stack into regularly ordered arrays or columns (Fig. 2 *b*). This stacking pattern resembles that obtained from nas-

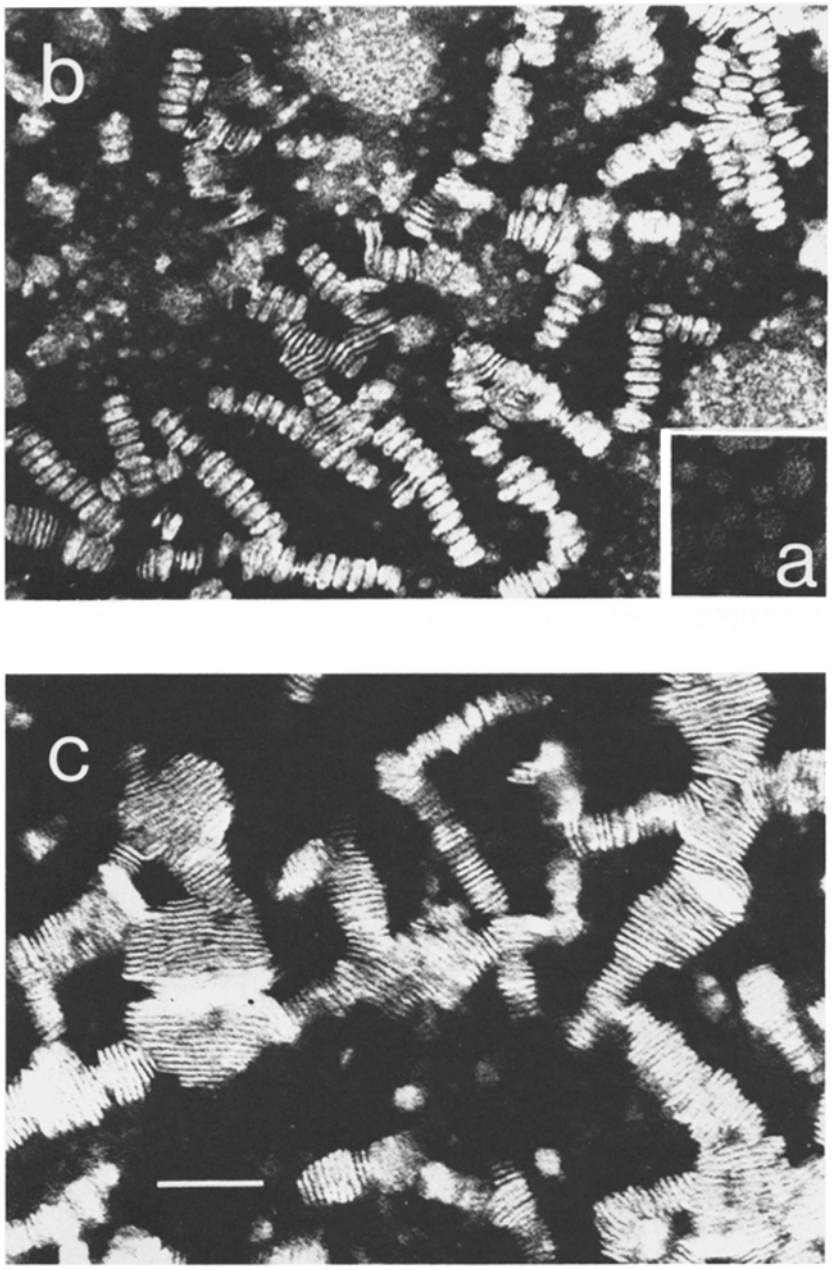

FIGURE 2 Electron micrographs of negatively stained liposomes. The control liposomes (*a*—insert) were formed from 10 mg of DMPC in 0.6 ml of salt buffer and diluted 1:10 before staining. Uniform diameters of 290 Å are shown. No stacking or aggregation occurs. In (*b*) and (*c*), 10 mg of DMPC vesicles were incubated with 0.5 mg of twice-cycled tubulin in 0.6 ml of salt buffer. Immediately after 15 min at 30° the sample was either diluted 1:10 with the salt buffer, placed on a carbon-coated formvar grid and stained (*b*) or placed directly onto the grid without dilution and stained (*c*). In *b* stacks of liposomes have diameters of 340 Å and a longitudinal periodicity of ~120Å. The multilamellar sheets (*c*) have a periodicity of ~70 Å. Bar (*c*), 1,000 Å. × 139,000.

cent high density lipoproteins (HDL) (15). At higher concentrations the vesicles appear to fuse into multilamellar sheets (Fig. 2*c*). The highly ordered tubulin-induced aggregates of liposomes are consistent with the development of strong protein-lipid interactions. Such interaction might involve conformational changes or altered reactivities of functional groups as the protein is taken from a polar, aqueous environment to a nonpolar lipid environment.

Electrophoretic profiles of soluble and liposome-adsorbed tubulin run in the absence of reducing agents revealed the first major difference between these proteins. Unlike soluble tubulin, adsorbed tubulin (and certain other proteins) rapidly formed intermolecular disulfide bonds. These disulfides could form in the presence of water-soluble reducing agents. They were readily reduced in the same reagents after detergent-solubilization of the liposome-adsorbed protein. Fig. 3 shows the electrophoretic pattern of whole brain homogenate run on a gel either with (Fig. 3*a*) or without (Fig. 3*b*) β-mercaptoethanol added immediately before electrophoresis. The incubation of homogenate with an equal volume of Ficoll at density 1.10 without liposomes results in the same gel pattern whether or not β-mercaptoethanol is present. These identical gel patterns suggest that intermolecular disulfides are not present to a significant degree in the soluble proteins. The same observations can be made using twice-cycled tubulin: Fig. 4*a* shows twice-cycled tubulin with β-mercaptoethanol added to the electrophoretic sample, and Fig. 4*b* shows the identical pattern obtained from tubulin with no reducing agent present.

In contrast, the electrophoretic pattern of detergent-solubilized liposome protein is markedly dependent on the presence of reducing agents. Fig. 3*c* and *d* shows liposome proteins from brain homogenate. In Fig. 3*c* the liposomes were extracted with SDS and β-mercaptoethanol, and in Fig. 3*d* extraction was with SDS only. Fig. 4*c* and *d* show liposome-proteins from twice-cycled tubulin extracted under the same conditions. In both cases, gel samples without β-mercaptoethanol show very little protein entering the gel. This indicates disulfide bond formation between lipid-adsorbed proteins. Most of the protein under non-reducing conditions failed to enter the 3% stacking gel, indicating the presence of very large (>500,000 dalton) cross-linked complexes.

Addition of β-mercaptoethanol to SDS extracts

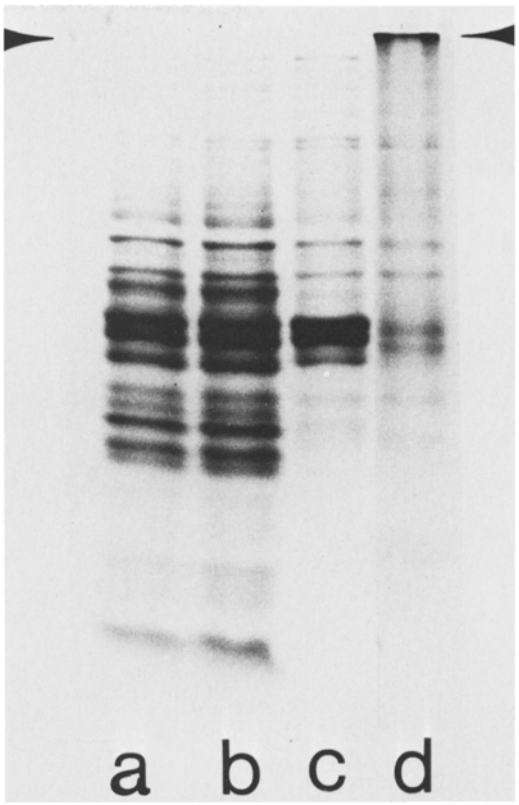

FIGURE 3 Effect of β-mercaptoethanol on SDS electrophoresis of brain homogenate versus liposome-adsorbed proteins. Whole brain supernate was electrophoresed in the presence (*a*) or absence (*b*) of 1% β-mercaptoethanol. The patterns are essentially identical. 1 mg of this whole brain homogenate was incubated with 10 mg of DMPC vesicles for 15 min. The liposome-adsorbed proteins were extracted with 1% SDS, boiled, and electrophoresed either with (*c*) or without (*d*) β-mercaptoethanol. In all cases, 20 μg of protein in 1% SDS was applied. Most of the protein in (*d*) failed to enter the 3.0% stacking gel (not shown).

of liposomes allowed free penetration of proteins into the gel, and a pattern identical to that obtained when the reducing agent was present during extraction. However, the presence of β-mercaptoethanol during liposome adsorption in concentrations as high as 0.14 M (1%) did not prevent formation of intermolecular disulfide bonds between liposome-adsorbed proteins. Furthermore, inclusion of 0.14 M β-mercaptoethanol or 20 mM N-ethyl maleimide in the Ficoll wash-gradient also did not prevent intermolecular disulfide formation. Since tubulin was the predominant adsorbed protein, it is likely that tubulin was also the prin-

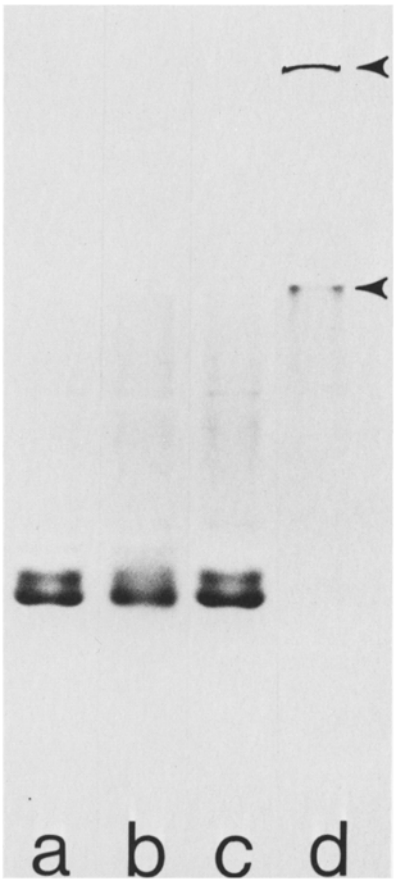

FIGURE 4 Effect of β-mercaptoethanol on SDS electrophoresis of twice-cycled tubulin versus liposome-adsorbed proteins. Twice-cycled tubulin was electrophoresed in the presence (*a*) or absence (*b*) of 1% β-mercaptoethanol. The patterns are essentially identical. 0.5 mg of this twice-cycled tubulin was incubated with 10 mg of DMPC vesicles for 15 min. After washing and pelletting the liposomes, the lipid was extracted and the proteins precipitated by addition of ice-cold acetone. Acetone did not affect the protein content or distribution by electrophoresis but improved the resolution of liposome-adsorbed proteins run without reducing agent. Proteins were taken up in 1% SDS, boiled, and electrophoresed either with (*c*) or without (*d*) β-mercaptoethanol. In all cases, 20 μg of protein in 1% SDS was applied. Most of the protein in (*d*) failed to enter the 3.0% stacking gel. The arrows (*d*) indicate the top of the 3.0% stacking gel and the interface between the stacking gel and the 5–15% gradient gel.

cipal protein involved in the formation of intermolecular disulfides.

DISCUSSION

We have demonstrated a direct and specific interaction of tubulin and associated high molecular weight components with synthetic phospholipid bilayers. The electrophoretic pattern of crude brain homogenate adsorbed to liposomes clearly demonstrates a selective uptake of proteins of molecular weight 52–55,000, the same as the α- and β-monomers of tubulin. Liposome-adsorbed proteins from brain homogenate carried through one or two cycles of microtubule assembly-disassembly confirm that tubulin and associated high molecular weight proteins are selectively taken up by the lipid vesicles. Experiments with freshly twice-cycled tubulin where virtually all of the tubulin adsorbs to the lipid bilayer eliminates the possibility that a subpopulation, for example, denatured or incompetent protein, interacts with the liposomes.

The resemblance between the liposome-adsorbed proteins from crude brain homogenate and once-cycled tubulin and those obtained from one and two cycles of microtubule assembly-disassembly, respectively, suggests that the interaction of tubulin with the lipid bilayer may be governed by forces similar to those determining assembly of tubulin into microtubules. However, lipid association can occur in the absence of nucleotides or divalent cations and so appears to be energetically more favorable than assembly.

The possibility that DMPC was contaminated with charged lipids or was cleaved to lysolecithin by enzymes in the supernates was ruled out by thin-layer chromatography. Therefore, adsorption is to neutral lipids. Adsorption occurs from buffers with a nominal ionic strength of ~0.12. In addition, virtually identical adsorption of proteins from twice-cycled tubulin or whole brain homogenate to liposomes occurs in the presence of 1.0 M NaCl. This indicates that ionic (salt) bonds are probably not of primary importance in adsorption of tubulin to liposomes, in contrast to the binding of the highly basic protein cytochrome *c* (8, 14, 18) or albumin at pH's below its isoelectric point (31) to negatively charged phospholipids.

An alternative explanation for the adsorption is hydrophobic bonding between a portion of the protein and the lipid bilayer. The apparent paradox of a soluble protein interacting with the lipid bilayer can be explained by supposing a cooperative interaction of a small hydrophobic region of the molecule with the interior of the bilayer. Such an interaction might be expected to lead to significant conformational changes in the adsorbed protein (27).

The formation of intermolecular disulfide bonds

possibly within hydrophobic regions of the lipid-protein complex is consistent with the anticipated conformational change. Our preliminary data on the sulfhydryl content of liposome-adsorbed tubulin indicates that only a small fraction of the total reduced sulfhydryl groups is involved in disulfide bonding. Kelly and Cotman (16) found that the proteins of isolated rat brain synaptic densities which include tubulin are also extensively cross-linked by disulfide bonds.

Our results indicate that tubulin binds strongly to simple phospholipid bilayers and so is very likely capable of association with all cellular membranes. However, if intermolecular disulfide bonds are an obligatory consequence of this association, important constraints are likely placed on its functional capacity. In particular, sulfhydryl reagents markedly affect the assembly of microtubules from tubulin even without the hindrance of intermolecular bonding (20, 24). Further study of the liposome-tubulin system will, hopefully, allow precise description of the structure and functional competence of "membrane tubulin" and the development of new experimental approaches to tubulin in biological membranes.

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