Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 755-760

TUBULIN DOES NOT BIND GLYCEROL IRREVERSIBLY

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Received October 2, 1979

SUMMARY: Tubulin incorporates radioactivity when incubated with preparations of [^{14}C]-glycerol, but the material that binds to tubulin is a contaminant rather than glycerol itself. The apparent binding of glycerol by tubulin was not stoichiometric, nor was it significantly affected by dilution of the radioactive glycerol preparation with unlabelled glycerol. Radioactive glycerol purified by prior exposure to protein was an order of magnitude less effective in binding to tubulin than unpurified glycerol. Cytochrome-C and bovine plasma albumin, as well as tubulin, incorporated radioactivity from isotopically labelled glycerol. The amount of radioactivity incorporated into tubulin was unaffected by assembly-disassembly.

Glycerol is routinely employed in the purification of tubulin (1), to stabilize this protein and to enhance its *in vitro* assembly into microtubules. Indeed, for some biological sources (2) glycerol appears to be a requirement for tubulin isolation by the polymerization technique. Where procedures (3,4) have been developed for tubulin preparation in the absence of glycerol, the microtubular proteins obtained differ in some of their physico chemical properties and in their content of accessory proteins (5). The use of glycerol in purification and other experiments with tubulin and microtubules may therefore be viewed with some concern over the nature of its interaction with this protein (6,7).

Recently, Detrich *et al.* (8) have reported that the tubulin dimer binds 5 moles of glycerol; 2 irreversibly and 3 that are exchangeable upon polymerization/depolymerization. They also reported that when tubulin aggregated into rings of 26 dimers, each dimer bound 22 glycerol molecules. If irreversible binding actually occurs, glycerol might distort protein conformations and alter their interactions in such a way as to give misleading results. This would call into question previous conclusions drawn from studies of tubulin which had been exposed to glycerol. Since irreversible

0006-291X/79/230755-06$01.00/0

binding did not seem likely, we undertook experiments that provided a different explanation for the data that were previously interpreted as irreversible glycerol binding by tubulin.

MATERIALS AND METHODS

The tubulin used was isolated from bovine brains using DEAE cellulose (Whatman DE-52) chromatography according to Weisenberg et al. (9), and stored at -20° C in phosphate-magnesium buffer (10 mM phosphate, 5 mM $MgCl_2$, pH 7.0) containing 1 M sucrose. Prior to use, tubulin was freed from sucrose by dialysis overnight against a 400 fold excess of buffer. Protein samples were incubated with [^{14}C]-glycerol (Amersham, CFB 174 Batch 4) for 30 to 60 min at 4° C or for 30 min at 37° C. Unbound [^{14}C]-glycerol was then separated from the protein either by chromatography on Sephadex G-25 (1.0 x 27 cm; phosphate-magnesium buffer) or by extensive (72 hr) dialysis at 4° C against 3 changes of a 400 fold excess of phosphate-magnesium buffer. On some occasions dialysis was also performed against phosphate-magnesium buffer containing 3.4 M glycerol.

For assembly into microtubules, tubulin was dialysed overnight against assembly buffer (10 mM [2-N-morpholino) ethansulfonic acid], 1 mM EGTA, 5 mM $MgCl_2$, 3.4 M glycerol, pH 7.0) and then GTP (to 1 mM) and [^{14}C]-glycerol (10 μCi) were added. Polymerization was monitored by light scattering at 350 nm when the temperature was raised to 37° C. Control samples that avoided polymerization used identical conditions except for the omission of GTP.

Horse heart cytochrome C was obtained from Sigma and crystallized bovine plasma albumin was from Armour Pharmaceutical. Radioactivity bound to protein was measured in a Searle Mark II liquid scintillation counter and protein concentration was determined by the method of Lowry et al. (10).

RESULTS AND DISCUSSION

Table 1 gives the results of an experiment in which tubulin was incubated with [^{14}C]-glycerol and then dialysed to remove excess glycerol. Although a small amount of radioactivity indeed appeared to be bound irreversibly, if it was calculated as moles of glycerol, it was not stoichiometric. In repeating this experiment a number of times (using different batches and amounts of [^{14}C]-glycerol) the apparent binding of glycerol ranged from 0.2 to 0.9 moles per mole tubulin. Unless it was binding covalently, any radioactive glycerol bound to tubulin ought to be displaced by high concentrations of unlabelled glycerol according to the law of microreversibility, but the results in Table I show that such was not the case. These observations indicate that the radioactivity bound by tubulin was not glycerol.

Table 1

Apparent Stoichiometry of Glycerol Binding

Protein (2-4 mg/ml) was incubated with 90 mM glycerol (1.05×10^{-2} mM Ci/mmole and then dialysed against 3 changes of a 400 fold excess of buffer for 72 h at 4° C.

Incubation Temp.	Dialysis Buffer	moles glycerol / moles dimer
4°	Phosphate-magnesium	0.7
4°	Phosphate-magnesium Glycerol[a]	0.7
37°[b]	Phosphate-magnesium	1.8

(a) When glycerol was used in dialysis the concentration was 3.4 M.
(b) Incubation at 37° was under conditions that avoided polymerization.

A further indication that the radioactivity bound to tubulin was not glycerol is the fact that the amount bound was approximately proportional to the amount of radioactive glycerol in which the protein was incubated rather than the amount of total glycerol. The results in Table 1 for incubation at 37° were obtained by use of 1.5 μCi [^{14}C]-glycerol plus 10.5 μl unlabelled glycerol. When this experiment was repeated with the omission of the unlabelled glycerol, this had the effect of increasing the specific activity of the glycerol about 10^4 fold but the radioactivity bound increased only by a factor of 2.5.

The suppliers (Amersham) list the radiochemical purity of their [^{14}C]-glycerol as 98%, and since the amount of radioactivity incorporated into protein represented less than 0.1% of the total radioactivity added, contaminants in the glycerol could easily have accounted for all the incorporation. If radiochemical decomposition products accounted for the incorporation of radioactivity into tubulin, other proteins ought to be similarly labelled. This proved to be the case. When 1.0 μCi [^{14}C]-glycerol was added to 0.5 ml of 1% cytochrome C and incubated for 30 min in the cold or at 37°, the results shown in Table II were obtained. Sephadex G-25 chromatography showed radioactivity associated with the protein peak. As in the case of tubulin,

Table II

Retention of Radioactivity by Protein

Protein was incubated with [^{14}C]-glycerol preparations as described in text, and separated from excess glycerol by dialysis or chromatography on Sephadex G-25 (at 4° when samples had been incubated at that temperature, otherwise at room temperature). Protein concentrations and radioactivity were determined for fractions across chromatographic peaks and averaged to give the value presented in CPM/mmol of 110,000 dalton dimer.

	Incubation Temp.	Separation Method	Specific Act. (mCi/mmol)	CPM/mmole (x 10^{-7})
Tubulin	4°	Dialysis	$1.05.10^{-2}$	1.4
	37°	Dialysis	$1.05.10^{-2}$	3.6
	37°	Chromatography	175	8.9
Tubulin[a]	37°	Chromatography	175	0.4
Cytochrome C	4°	Chromatography	175	0.2
	37°	Chromatography	175	1.5

(a) [^{14}C]-glycerol used in this experiment was first incubated with bovine plasma albumin 18 hr at 4° and separated on Sephadex G-25 (see text).

the level of incorporation was greater at the higher temperature, and it was again, less than stoichiometric when calculated as glycerol.

The binding contaminant in preparations of [^{14}C]-glycerol could be removed by prior exposure of the preparations to protein. Incubation of 2 μCi [^{14}C]-glycerol with 0.5 ml of 10% bovine plasma albumin for 18 h in the cold was followed by Sephadex chromatography to separate the protein from free glycerol. As expected, radioactivity was found in the unretained, protein peak. The glycerol from the retained fraction, was incubated with tubulin for 30 min at 37°. The tubulin subsequently isolated by Sephadex chromatography had incorporated less radioactivity, by nearly an order of magnitude, than when glycerol was used that had not been exposed previously to protein (Table II).

The process of assembly and disassembly of microtubules from tubulin did not affect the incorporation of radioactivity from [^{14}C]-glycerol. Tubulin was put through a cycle of polymerization/depolymerization in the

Table III

Binding During Microtubule Assembly

Tubulin (ca. 4 mg/ml) was incubated about 10 min in 10 mM [2-(N-morpholino) ethanesulfonic acid], 3.4 M glycerol, with and without 1 mM GTP. The final specific activity of [^{14}C]-glycerol was $2.9 \cdot 10^{-3}$ μCi/mmol. The incubation at 37° C was sufficient for light scattering (350 nm) to reach a plateau. The mixtures were then dialyzed against 3 changes of a 400 fold excess of phosphate-magnesium buffer for at least 72 hrs.

	CPM/mmol Tubulin
Assembly (+GTP)	1.4×10^8
No assembly (-GTP)	1.5×10^8

presence of [^{14}C]-glycerol and compared to a control sample for which polymerization was prevented by the omission of GTP from the assembly buffer.

In conclusion, we find that it is an impurity in preparations of [^{14}C]-glycerol that binds irreversibly to tubulin (and other proteins) rather than glycerol itself. Of course our experiments do not bear on the notion that glycerol interacts reversibly with tubulin to affect its thermodynamic behavior as discussed, for example, by Lee and Timasheff (11). Our findings show that the useful effects of glycerol on stabilization and self assembly of tubulin may be exploited without the worry of permanent modification of the protein.

ACKNOWLEDGEMENTS: This work was supported by National Institutes of Health Grants GMS 20338, EHS P30 ES01896, GM) 7232. The costs of the publication of this article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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