BINDING OF GLYCEROL BY MICROTUBULE PROTEIN

H. William Detrich, III, Steven A. Berkowitz, Helen Kim, and Robley C. Williams, Jr.

Department of Biology, Yale University, New Haven, CT 06520

Received December 10, 1975

SUMMARY: When microtubules are purified by polymerization and depolymerization in a buffer containing glycerol, some glycerol becomes bound to the microtubule protein and is not removable by gel filtration or by prolonged dialysis. Both 6s tubulin and larger aggregates containing tubulin and accessory proteins bind glycerol. The 6s fraction has associated with it about 5 moles of glycerol per mole of tubulin dimer; 3 moles are exchangeable upon polymerization-depolymerization and 2 moles are not. The aggregate fraction has associated with it about 22 moles of glycerol per mole of tubulin dimer; approximately 11 moles are exchangeable and 11 moles are not.

Both sucrose and glycerol have been found to stabilize microtubule protein (tubulin and associated proteins) from several sources (1-3) and to enhance yield in the assembly-disassembly process (3). The use of glycerol in the preparation of microtubules *in vitro* has become a common practice, and the possibility that its presence may introduce artifacts into the study of microtubule polymerization merits assessment. The sedimentation coefficient and structure of the "ring" aggregates of microtubule protein prepared in glycerol appear to differ from those of proteins prepared in its absence (4-8). Glycerol present during polymerization or added just prior to depolymerization causes the partial disappearance of a 30s ring structure and the appearance of new sedimenting boundaries at 8s to 18s or at 20s (9, 10). Glycerol at concentrations of 4 to 6 M has been reported to induce the formation of rings from 6s tubulin at 0° and to cause the assembly of microtubules from this otherwise inactive material at 35° (5). The observations suggest a strong and specific interaction between glycerol and microtubule protein. We report here preliminary data showing that glycerol, when it is used in the preparative procedure, binds to microtubule protein.

MATERIALS AND METHODS

Calf brains were obtained from freshly slaughtered animals, were kept on ice, and were used within one hour after slaughter. Glycerol [2-^{3}H(N)] , 200 mCi/mmole, glycerol [2-^{14}C] , 10.6 mCi/mmole, and Aquasol liquid scintillation

cocktail were obtained from New England Nuclear, Inc. GTP was Sigma grade II-S. Bio-Gel A15m was purchased from Bio-Rad Laboratories. The buffers employed were 100 mM PIPES, 2mM EGTA, 1 mM $MgSO_4$, 0.5 mM GTP, pH 6.9 (abbreviated PM), and the same buffer made 8 M in glycerol (abbreviated PMG and used as a diluent to prepare solutions at a final glycerol concentration of 4 M).

Microtubule protein was purified by a modification of the method of Shelanski, et al. (3), as summarized in detail in Figure 1. Preparation and subsequent chromatography were carried out continuously over a period of 42 hours. Brains were homogenized in a Sorvall Omni-Mixer for 50 sec at speed 3 and 10 sec at speed 9. The homogenate was then centrifuged twice, as shown. GTP was added to the supernatant of the second centrifugation (designated S-0) to a concentration of 0.5 mM, and the mixture was then diluted 1:1 with PMG buffer containing 0.02 mCi/ml of [^{3}H] glycerol and incubated at 30° to allow assembly of microtubules. The tubules were harvested by centrifugation, resuspended in PM buffer at 0° (1/5 the volume of the S-0), dispersed by gentle shearing in a Dounce homogenizer, and incubated at 0° to depolymerize the tubules. The solution was centrifuged to pellet undissociated tubules and the supernatant (de-

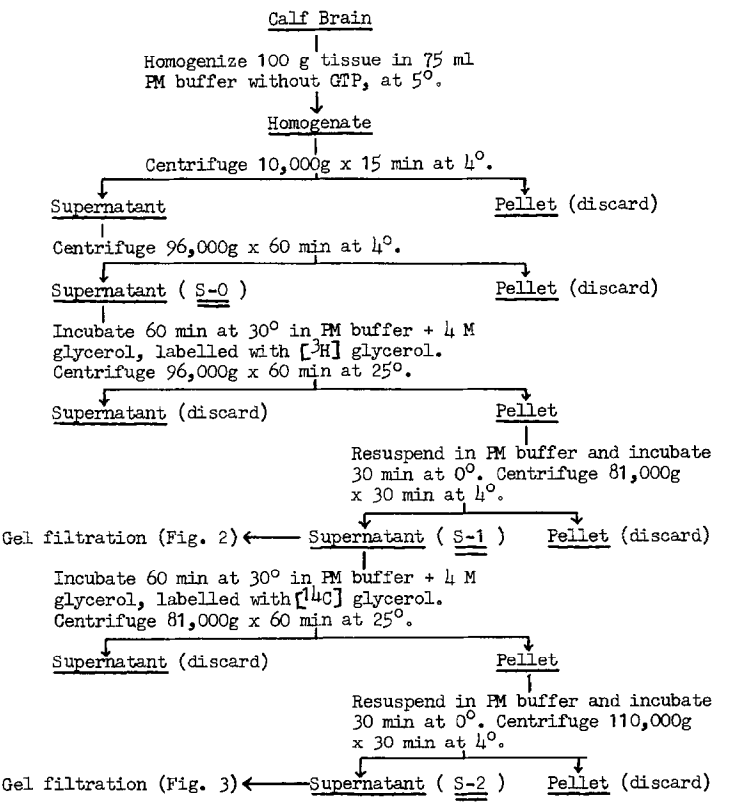

Figure 1. Detailed summary of experimental protocol for preparation of microtubule protein in labelled glycerol.

Abbreviations: EGTA, ethylene glycol bis-(β-aminoethyl ether) N,N'-tetraacetic acid; PIPES, piperazine-N,N'-bis-(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate.

signated S-1) was retained. Three ml of the S-1 were reserved for gel filtration and assay, and the remaining material was diluted 1:1 with PMG buffer containing 0.02 mCi/ml of [^{14}C] glycerol. Twice polymerized microtubules were prepared from this material by a further cycle of polymerization-depolymerization, as shown. The final supernatant (designated S-2) contained microtubule protein that had been exposed to two glycerol labels.

A "ring" fraction of relatively high molecular weight can be separated from 6s tubulin in preparations of depolymerized microtubules by the use of columns of Bio-Gel A-15m (4, 11). In order to assess separately the binding of glycerol to each of these two components, layered columns, the bottom 1/3 consisting of Sephadex G-25 and the top 2/3 of Bio-Gel A-15m, were prepared. A test separation of a mixture of bromophenol blue, human hemoglobin A, and blue dextran showed the composite column to have a separation range large enough to resolve the three components. Presence of ^{14}C and ^{3}H in the column eluate was assayed by liquid scintillation counting in a Nuclear-Chicago Mark I counter. Protein solution (3.5 ml) was counted in Aquasol (11.5 ml). SDS-polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (13). The gels were stained with Coomassie Brilliant Blue R.

RESULTS

When [^{3}H] glycerol is added at the first or second polymerization step in preparation protocols otherwise identical to that shown in Figure 1, and the S-2 supernatant is subjected, at 4°, to gel filtration on a 1.5 x 30 cm column of Sephadex G-75, an unexpectedly large fraction of the label appears within the protein peak (data not shown). The results of such simple experiments led to the more complex experiments described below.

The results of gel filtration of the S-1 supernatant on a 1.6 x 90 cm

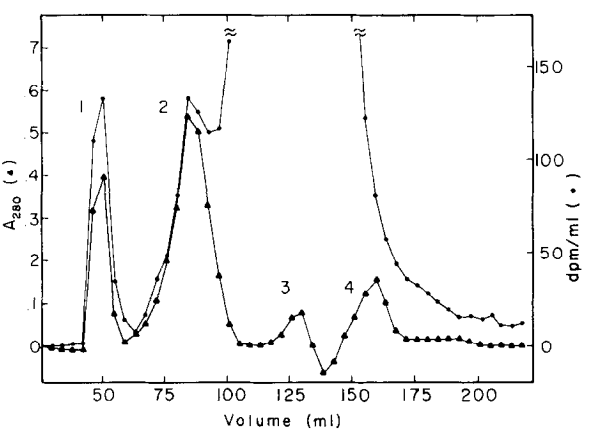

Figure 2. Elution pattern of the S-1 supernatant from a 1.6 x 90 cm layered column (see text) equilibrated and eluted with PM buffer. A_{280} (▲); dpm of ^{3}H (●). The sample contained 17.4 mg of protein in a volume of 2.0 ml, and the recovery was 97%. Flow rate: 10.1 ml/hr. Fraction volume: 4.2 ml. Temperature: 4°.

column are shown in Figure 2. Electrophoresis of the two major peaks showed that peak 2 contained predominantly tubulins 1 and 2, while peak 1 contained the high molecular weight accessory proteins MAP-1 and MAP-2 (14), five bands with mobilities intermediate between tubulin and the MAPs, traces of two proteins of lower molecular weight, and tubulins 1 and 2. The MAPs were enriched in peak 1 compared to their fractional amount in the material loaded onto the column. Based on this evidence, peak 1 was judged to correspond to the "ring" fraction (4, 5, 11, 14, 15), while peak 2 corresponds to tubulin dimer. Peaks 3 and 4 were found by electrophoresis to contain no detectable protein. Figure 3 shows the elution profile resulting from gel filtration of the S-2 supernatant on a 2 x 100 cm layered column under conditions identical to those employed with the S-1 supernatant. Electrophoresis showed that peak 2 contained tubulins 1 and 2 with minor impurities visible only when the gel was greatly overloaded. Peak 1 contained tubulins 1 and 2, MAPs 1 and 2, and traces of the same high and low molecular weight proteins observed in peak 1 of Figure 2. It is qualitatively clear both from Figure 2 and from Figure 3 that not all of the labelled glycerol introduced at the first and second polymerization steps is removed from the protein by gel filtration.

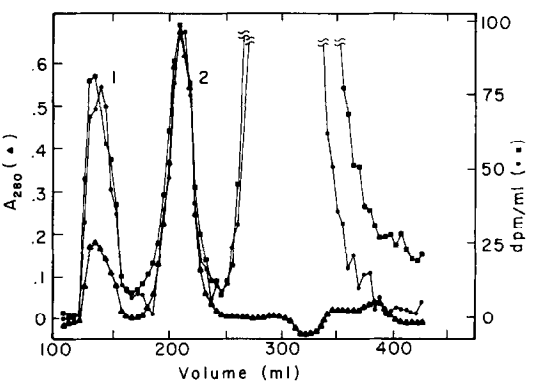

Figure 3. Elution pattern of the S-2 supernatant from a 2.0 x 100 cm layered column (see text). A_{280} (▲); dpm of ^{3}H (●); dpm of ^{14}C (■). The sample contained 26.2 mg of protein in a volume of 2.0 ml, and the recovery was 112%, The high value probably results from overestimation of the protein in peak 1 due to light scattering by the aggregated microtubule protein. Flow rate: 11.4 ml/hr. Fraction volume: 4.75 ml. Temperature: 4°.

It can be seen from Figure 3 that some of the [^{3}H] glycerol bound at the first polymerization step is displaced by the [^{14}C] glycerol introduced at the second step. The simplest interpretation of this result is that there exist a number of ("strong") glycerol-binding sites which are slowly enchangeable or which bind glycerol with a large association constant, or both, and that there are also ("weak") rapidly exchangeable or low affinity sites. Quantitative estimates, contingent upon this assumption, of the number of strong and weak sites are shown in Table I. Since peak 2 of the S-2 eluate was essentially 100% tubulin, the 2.2 moles of glycerol per mole of 110,000 dalton material that are carried into peak 2 from the first polymerization step represent a maximum estimate of the number of strong sites per tubulin dimer. The 2.6 moles of glycerol per mole of 110,000 daltons carried into this peak from the second step represent a minimum estimate of the number of weak sites per tubulin dimer. There thus appear to be about 5 sites per tubulin dimer that are capable of binding glycerol with a high enough association constant or a slow enough dissociation rate to avoid removal by gel filtration. There is no detectable dissociation of glycerol from tubulin under the conditions of the gel

TABLE I

Quantitative Results of Gel Filtration Experiments

Supernatant	Peak	Fraction of Total Protein[a]	Bound [^{3}H] glycerol[a] (dpm)	Bound [^{14}C] glycerol[a] (dpm)	Total Protein[a] (mg)	Apparent Molar Ratio[b] (glycerol/dimer) Traced by ^{3}H	Apparent Molar Ratio[b] (glycerol/dimer) Traced by ^{14}C
S-1	1	23%	1201	--	4.0	8.5	--
S-1	2	77%	2130	--	12.9	4.0	--
S-2	1	18%	2012	2182	5.3	10.8 ±3	11.7 ±3
S-2	2	82%	2729	3160	24.2	2.2 ±0.2	2.6 ±0.3

[a]Protein concentrations in the eluants were measured by determination of A_{280}. The extinction coefficients (0.84 ml/mg for the leading peak and 0.72 ml/mg for the trailing peak) reported by Kirschner, et al. (4) were employed to convert absorbance to concentration. Protein concentrations initially applied to the columns were measured by the method of Lowry, et al. (12). Observed counts/min were reduced to dpm by means of barium-133 external standardization quench correction. The amounts of radioactive label and protein corresponding to each peak were determined by trapezoidal integration.

[b]The apparent molar ratio of glycerol to tubulin dimer was calculated by assuming that 70% of the material in peak 1, and 100% of the material in peak 2, was tubulin. The number of moles of glycerol present in each peak was obtained by dividing the integrated number of dpm by the specific activity of the buffer (5.56×10^9 dpm/mole glycerol).

filtration experiment, since such a process would give rise to a trailing shoulder of radioactivity on peak 2. No such shoulder is seen, so the exchange of [^{14}C] glycerol for [^{3}H] glycerol at the weak sites must either occur primarily during the depolymerization-polymerization cycle, or the exchange may somehow be stopped by the conditions employed in gel filtration. Table I and Figure 3 also show that a higher ratio of bound glycerol to protein is observed for peak 1 than for peak 2. Other investigators (5, 15) have shown that high molecular weight components comprise 25% to 30% of the peak 1 fraction. These values are consistent with observation of our own nonquantitatively stained gels. Using 70% as the proportion by weight of tubulin in peak 1 of the S-2 fraction, one finds approximately 11 moles of glycerol bound strongly and another 12 moles bound weakly per mole of tubulin dimer. The possibility that the 30% of non-tubulin protein in peak 1 contributes heavily to the observed binding cannot, of course, be ruled out.

Preliminary dialysis experiments in which microtubule protein, polymerized once in the presence of [^{3}H] glycerol in PM buffer + 4 M glycerol, indicated that 19±6 moles of glycerol per mole of 110,000 dalton protein remain bound after dialysis for 96 hours at 4° against 4 changes of a 100-fold excess of PM buffer. These data are in qualitative agreement with the gel filtration data.

DISCUSSION

The results show clearly that about 5 moles of glycerol bind to one mole of tubulin dimer in such a way that they are not removed by gel filtration. Of these 5 moles, 2 to 3 appear to exchange with glycerol in the buffer during the depolymerization-polymerization cycle, while about 2 moles remain tightly bound. The results also suggest, but do not prove, that tubulin in the "ring"-aggregated state may bind about 22 moles of glycerol per mole of dimer, approximately half of it exchangeably. Therefore, tubulin prepared with the aid of glycerol has glycerol bound to it, even after passage through a gel filtration column, and differs in that way at least from tubulin as it exists *in vivo*. This glycerol-tubulin may differ in its polymerization properties from tubulin

prepared without the aid of glycerol. It is possible that glycerol-tubulin may form the double-ring structure (4, 6), while glycerol-free tubulin forms the disc (single-ring) structure (8). Indeed, R. B. Scheele and G. G. Borisy (personal communication) have found that when depolymerized microtubules prepared in the presence of glycerol by the method of Weingarten, et al. (11) are examined by sedimentation velocity, boundaries of $6s^{\circ}_{20,w}$ and $36s^{\circ}_{20,w}$ are observed, while under identical conditions material prepared without glycerol by the method of Borisy, et al. (8) yields boundaries of $6s^{\circ}_{20,w}$ and $31s^{\circ}_{20,w}$.

The details of the relationship of the observed binding to the stabilizing and polymerization-enhancing effects of glycerol are not obvious. It has been proposed that glycerol exerts its effects through alteration of the structure of the water in the solvent (3). Nothing in the present data can render this explanation incorrect. However, the observation of glycerol binding renders plausible mechanisms in which stabilization and enhancement of polymerization are the result of small conformational changes (cf. 16, 17) induced by the binding.

ACKNOWLEDGEMENTS: We thank Dr. Joel L. Rosenbaum for advice and encouragement. This study was supported by Grant HL12901 of the National Institutes of Health, and by USPHS Training Grants GM02299 (H.W.D.) and HD00032 (S.A.B.)

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