THE MECHANISM OF BREAKDOWN OF TUBULIN IN VITRO*

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1. Introduction

It is well established that in vitro tubulin is unstable in that it gradually loses its ability to bind colchicine [1,2]. The half time of this change has been estimated to be 11 h at 0°C [3]. During the course of experiments designed to study the assembly of microtubules, it was noted that incubation with dithiothreitol (DTT) decreased the extent of conversion of tubulin to the form that does not bind colchicine. This chance observation led to a systematic study of the effects of both reducing and oxidizing agents on the loss of colchicine binding activity by purified tubulin preparations. This loss of colchicine binding activity will be termed breakdown, although it apparently is unclear whether or not it is actually a result of the dissociation of the tubulin dimer into its two constituent monomer proteins.

In contrast to the inhibitory effect of DTT, reduced glutathione (GSH) modestly increased the breakdown of tubulin occurring in 90 min. Oxidized glutathione (GSSG) had no apparent effect alone, while combinations of GSH and GSSG increased the extent of this breakdown more than did GSH alone. These results suggest (1) that the mechanism of breakdown of tubulin in vitro involves both the cleavage and the formation of disulfide bonds, and (2) that the tubulin dimer can exist in vitro in several different biochemical forms.

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2. Methods

2.1. *Purification of tubulin*

Rats were lightly anesthetized with ether, decapitated and their brains homogenized in 100 mM piperazine-*N*-*N*^{1}-bis(2-ethane sulfonic acid) (PIPES), pH 6.4, containing 0.5 mM $MgCl_2$. After centrifugation at 4°C at 35 000 *g* for 20 min, the supernatant fraction was incubated for 15 min at 37°C with 1 mM GTP, 5 mM phosphoenolpyruvate and 8–10% dimethylsulfoxide (DMSO) at a final dilution of 1 gram brain/2.5 ml. The assembled microtubules were pelleted by centrifugation at 35 000 *g* for 10 min at 30°C and were immediately resuspended in a small vol of 100 mM PIPES, pH 6.4, containing 0.5 mM $MgCl_2$, 1 mM GTP, and 5% DMSO. After a second centrifugation, this last step was repeated with the DMSO either absent or present at a concentration of 2%. After a third centrifugation, the microtubule pellet was homogenized in 100 mM PIPES, pH 6.9, containing 0.5 mM $MgCl_2$, and microtubules were disassembled either by leaving them at 0°C for 45 min or by gentle sonication (Branson Sonifier Model W185, special microtip, 45 W for four 30-second periods). The tubulin preparation was again centrifuged at 35 000 *g* for 10 min at 4°C and the precipitate discarded.

The novel features of this purification procedure are the use of phosphoenolpyruvate and DMSO to increase initial assembly and the use of sonication for disassembly. Phosphoenolpyruvate was preferred to EGTA because in crude preparations it consistently leads to a much larger increase in assembly than does EGTA. DMSO also increases the yield, probably acting

like glycerol to non-specifically stabilize assembled microtubules. Its advantage over glycerol is that an ultracentrifuge is not needed to pellet the assembled microtubules. Disassembly on ice is slow and frequently incomplete while sonication has neither of these advantages.

2.2. *Measurement of colchicine binding activity and tubulin breakdown*

The extent of breakdown of tubulin was determined by comparing the amount of [^{3}H]colchicine bound by fresh tubulin in 15 min with that bound by tubulin first incubated for 75 min with or without test compounds. The colchicine concentration was 2.5×10^{-6} M, the incubation temperature 37°C throughout, and the medium 100 mM PIPES, pH 6.9, containing 0.5 mM magnesium and 3% bovine serum albumin. The albumin was added because it ensures complete recovery of low concentrations of purified tubulin from Sephadex columns. The small amount of [^{3}H]-colchicine that binds to albumin (< 1%) was measured and subtracted before the calculation of breakdown rates. Test compounds were always added during the first incubation of freshly prepared tubulin with colchicine. In no case did these compounds interfere with the binding of colchicine to tubulin. Following the various incubations with colchicine, drug bound to tubulin was separated from free drug by passage over 1 × 15 cm Sephadex G-50 columns as previously described [1,4].

3. Results and discussion

The effects of dithiothreitol alone and reduced and oxidized glutathione, alone and together, on the breakdown of tubulin in 90 min are shown in table 1. GSH caused a small and variable increase in breakdown while DTT consistently inhibited the process. Oxidized glutathione had no apparent effect alone yet the combination of GSSG and GSH markedly increased the extent of breakdown. In separate experiments (data not shown) various concentrations of reduced and oxidized glutathione (0.5 1 and 2 mM) were studied alone and in all possible combinations. All combinations of different concentrations of the two compounds led to greater breakdown (61 to 75%) than did any concentration of either compound alone (51–57%).

Table 1
Effects of reduced and oxidized glutathione and dithiothreitol on tubulin breakdown

Addition (1 mM)	% Breakdown in 90 min[a] (Average ± S.D.)			
Exp.	1	2	3	4
None	49	42	30 ± 3	39 ± 6
GSH	57	50	51 ± 3	48 ± 5
GSSG	50	45	30 ± 4	37 ± 6
GSH + GSSG	75	80	63 ± 3	62 ± 2
DTT	41	35	23 ± 3	18 ± 2

[a]Experiments were carried out in duplicate or quadruplicate. The difference between the effects of GSH alone and GSH combined with GSSG in experiments 3 and 4 is significant with a "p" value in both cases of < 0.001 (Student's t test).

The simplest explanation of these results is to suppose that the breakdown of tubulin involves the cleavage of one disulfide bond and the formation of another. GSH and DTT would catalyse the first reaction (cleavage of a disulfide bond) but inhibit the second (formation of a disulfide bond) while the oxidized glutathione would have the reverse effect. catalysing the second reaction and inhibiting the first. One possible scheme illustrating this is shown in fig.1. The effect of reduced and oxidized glutathione added together cannot be predicted, of course, but will depend on the relative ability of the two compounds to catalyse and inhibit the various reactions. From the data in table 1, it can be seen that the ability of the two compounds to catalyse the reactions leading to breakdown is greater than their inhibitory effects. While not essential to the conclusions reached, it is perhaps of interest that neither GSH nor GSSG

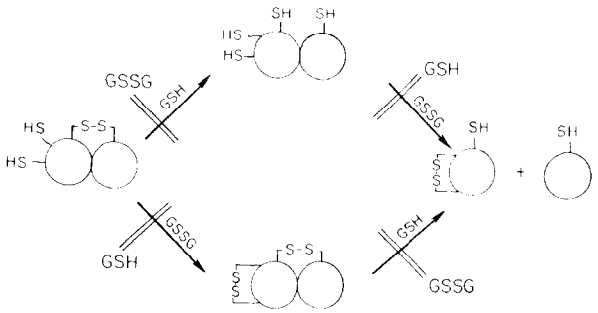

Fig.1. One possible mechanism for the breakdown of tubulin.

altered the kinetic characteristics of colchicine binding. The measured extent of breakdown of tubulin in the presence of these two compounds added separately was independent of the [^{3}H] colchicine concentration over the range $1-5 \times 10^{-6}$ M and was also independent of the time of incubation with colchicine (15 vs 30 min).

The data in table 1 provide no information as to the number of sulfhydryl groups involved in the breakdown process nor, assuming that 4 or more groups are involved, do they provide any information as to the possible order of the two (or more) reactions. Experiments designed to distinguish between these various possibilities involved incubating tubulin with either reduced or oxidized glutathione, passing it over Sephadex columns and reincubating portions with either the same or the other compound. This approach was unsuccessful. It was not possible to obtain complete recovery of tubulin (in the absence of colchicine) over either Sephadex or Bio-Gel columns. Recovery was particularly poor after incubation with GSSG. The studies of Stevens and his colleagues [5], however, provide good evidence that four (or more) rather than three sulfhydryl groups are involved and that, as depicted in fig.1, the two (or more) reactions can proceed in either order. These investigators studied the effects of mercaptoethanol and dithiodiglycol (oxidized mercaptoethanol) on the birefringence of spindles of *Pectinaria gouldi* oocytes. They found that both compounds, when added separately, produced a rapid decrease in birefringence but, when the two compounds were added together, the rate of loss of birefringence was decreased.

The concentration of reduced glutathione within cells is known to be high, ranging from 0.1 to 5 mM depending on the tissue [6,7]. In contrast, levels of oxidized glutathione are considerably lower. It seems probable therefore, that tubulin can exist within cells in the reduced, but not the oxidized, form. This latter form is apparently an in vitro phenomenon as, indeed, is the breakdown of tubulin. If reduced tubulin does exist in vivo, then presumably it is oxidized during assembly. In this connection, it has been known for many years that the TCA soluble sulfhydryl content of many marine eggs fluctuates during the cell cycle, being highest at metaphase [8]. While initially it was thought that the sulfhydryl compound involved was glutathione, the studies of Sakai and Dan [9] implicate a small protein or polypeptide other than GSH. It seems possible that this compound forms as tubulin is oxidized either prior to or during spindle assembly. Whether or not this last speculation proves correct, it will be of interest to study the assembly in vitro, not only of freshly prepared tubulin, but also of material pretreated with reduced glutathione.

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