ISOLATION OF NATIVE MICROTUBULES FROM PORCINE BRAIN AND CHARACTERIZATION OF SH GROUPS ESSENTIAL FOR POLYMERIZATION AT THE GTP BINDING SITES

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1. Introduction

In addition to tubulin, microtubules won by repolymerization contain additional associated proteins (MAPS). The composition of the complex apparently varies with the purification procedure applied [1–4]. At least one group of MAPS, the τ factor, probably has a regulatory function upon the process of tubulin polymerization [5,6], the biological role of the others is unknown. Indeed, their number and quantity does not seem to be very well determined. One of the reasons for this uncertainty lies in the danger of losses of the less essential members, or the adherence of additional proteins during the repolymerization steps of tubulin purification. We have therefore purified native microtubules, and identified several groups of proteins that copurify with tubulin through this process. Since 15–20% of the total microtubule protein consisted of these MAPS, we reinvestigated the position of SH groups, essential for polymerization, which we have described earlier [7,8]. It was found that these SH groups are located close to the GTP binding site of each tubulin subunit.

2. Materials and methods

Metrizamide was obtained from Nyegaard and Co., Oslo. *N*-ethyl-[^{14}C]maleimide (NEM) was a product of Amersham-Buchler, its specific radioactivity was 5 mCi/mmol. 2-Amino-6-(*S*-dinitrophenyl)-thiopurine riboside triphosphate was synthesized essentially as described for the corresponding ATP analogue [9]. Full details of this affinity label for tubulin will be published elsewhere. For electron microscopy, ultracentrifugation pellets, after fixation in buffered 2.5% or 5% glutaraldehyde solution overnight, were either subjected to negative staining with uranyl acetate in the usual manner, or treated with 1% OsO_4 solution and embedded in Spurr medium. Sections were stained with lead acetate and observed in an EM Philips 300 microscope. Gel electrophoreses were carried out in 7.5% polyacrylamide gels (acrylamide: bis = 19:1) containing 6 M urea and 0.1% SDS. The electrophoresis buffer was 0.1 M Tris–glycine, pH 8.3, containing 0.1% SDS.

The gels were stained with Coomassie brilliant blue R 250, and scanned in a Gilson model 2400 spectrophotometer. They were sliced, the single bands were dissolved in 1 ml of 30% hydrogen peroxide solution, and counted in Scintigel (Roth Co.), with the aid of internal standard corrections. Tryptic digestions, fingerprints, and autoradiographs were performed as described previously [10]. The determination of the GTP content of modified tubulin followed the fractional Dowex 1 adsorption procedure described

for the measurement of ATP content of muscle actin [11].

3. Results and discussion

3.1. *Purification of native microtubules*

The procedure was carried out at temperatures between 25°C and 35°C throughout, and on a single day. Porcine brain obtained fresh from slaughter was homogenized in an equal volume of 0.1 M MES buffer, pH 6.5, containing 1 mM EGTA, and 4 M glycerol. The mixture was centrifuged at 20 000 × *g* for 20 min. The pellets were discarded. The supernatants were centrifuged at 100 000 × *g* for 60 min. The turbid gelly pellets were resuspended in the same buffer, and the suspension was passed through a short, broad column of Sepharose S 2B, equilibrated with the same buffer at 32–35°C. This step served to remove a large part of membrane material and lipid, although the loss of approximately 40% of the tubules had to be incurred. The first and only slightly turbid protein fractions were centrifuged at 100 000 × *g* for 60 min again, and the pellets were resuspended in buffer free from glycerol. The suspension was layered upon 5-step metrizamide gradient (5, 10, 14, 17 and 20%), in the same buffer. The gradients were centrifuged for 2,5 h at 60 000 × *g*. The gradient isolated tubules in a band between 20 and 17% metrizamide, while the further bands at the top of the gradient, at the step to 10%, and to 14% concentration contained membrane material, microfilaments, and depolymerized actin, respectively. The compositions of the bands were investigated by electron microscopy (fig.1). This was supported by disc electrophoreses of all fractions of the purification steps. The densitometer scans of the gels (fig.3) were analyzed with the aid of a computer linked plotter [12]. The two tubulin bands represented 80–85% of the protein, while the bands, that distinctly copurified with tubulin (denoted as I–VII in fig.3) furnished 15–20% of the total. Impurities of approximately 5% remained. In a final control experiment, the tubules were depolymerized at 0°C in 0.1 M MES buffer, pH 6.5, containing 1 mM EGTA. The solution was then centrifuged at 100 000 × *g* at 0°C for one hour, whereupon a small pellet of membrane material was formed. Disc electrophoreses of the supernatant

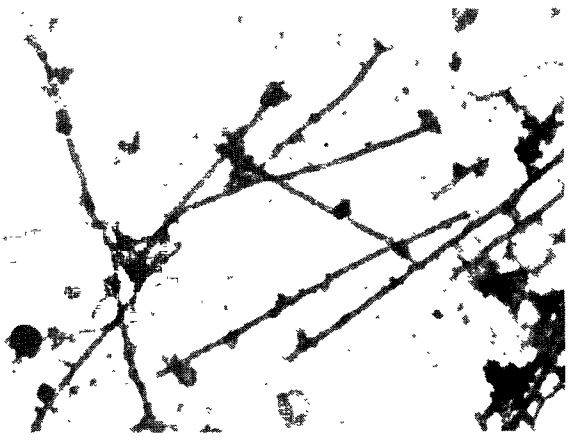

Fig.1 Negatively stained electron microscopic view of purified microtubules, 37 000 ×.

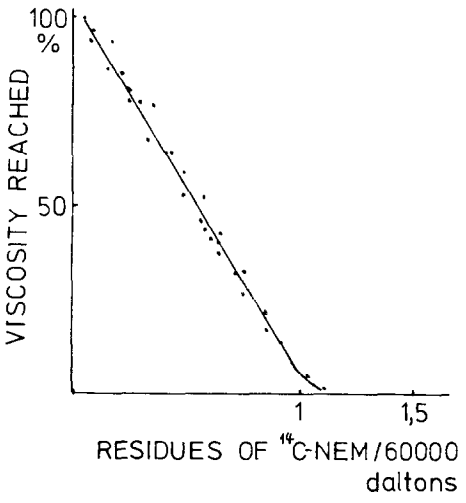

Fig.2. Polymerization inhibition after reaction of tubulin with *N*-ethylmaleimide (NEM). Depolymerized tubulin (final concentration 4–6 mg/ml in GTP containing MES buffer) was treated with 0.01 vol. of NEM solution in 50% ethanol, containing between 0.05 and 1.2 μmol of NEM per μmol of tubulin at 0°C. After 10 min at this temperature, the solution was introduced into an Ostwald type viscosimeter and kept at 37°C. The rise in viscosity was measured at 3- to 5-min intervals for 25 min. Polymerized tubulin, treated at 37°C with NEM in the same manner, will depolymerize during 20 min to the same extent as the polymerization inhibition shown here

showed the bands I–VII, besides the two tubulin subunit bands. We therefore conclude that these proteins belong to, and are attached to the physiological system of microtubules.

3.2. *Characterization of SH groups, essential for polymerization*

We have first reported [7] that the polymerization of tubulin is blocked by reaction with *N*-ethylmaleimide. The inhibition was now found to depend on the alkylation of 1 SH group per 60 000 daltons (fig.2). A tryptic fingerprint of tubulin modified in this manner with *N*-ethyl-[^{14}C]maleimide showed only two radioactive spots in autoradiography, corresponding to 85% of the total radioactivity applied. These results imply the existence of one SH group essential for polymerization in the tubulin monomer. In view of the relatively high content of MAPS, however, it appeared necessary to reinvestigate the labeling of the protein complex. Purified tubuli were therefore depolymerized in the usual manner in the presence of the stoichiometric amount of one mol

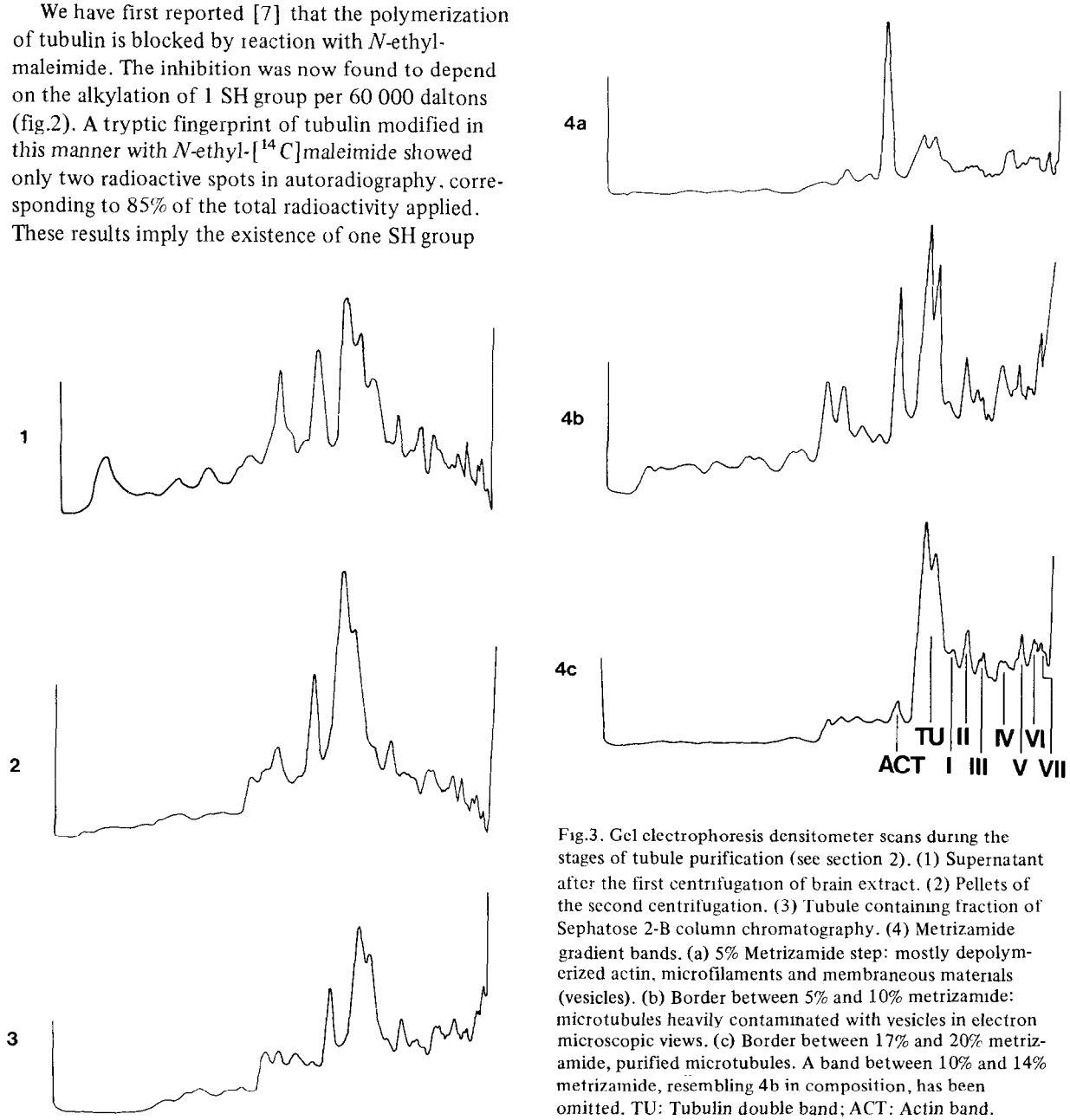

Fig.3. Gel electrophoresis densitometer scans during the stages of tubule purification (see section 2). (1) Supernatant after the first centrifugation of brain extract. (2) Pellets of the second centrifugation. (3) Tubule containing fraction of Sephatose 2-B column chromatography. (4) Metrizamide gradient bands. (a) 5% Metrizamide step: mostly depolymerized actin, microfilaments and membraneous materials (vesicles). (b) Border between 5% and 10% metrizamide: microtubules heavily contaminated with vesicles in electron microscopic views. (c) Border between 17% and 20% metrizamide, purified microtubules. A band between 10% and 14% metrizamide, resembling 4b in composition, has been omitted. TU: Tubulin double band; ACT: Actin band.

of *N*-ethyl-[^{14}C]maleimide per 60 000 daltons, and samples of the modified protein were run on polyacrylamide gel electrophoreses. The distribution of radioactive label in the gel was determined as described in section 2. The two tubulin subunit bands were labeled to even amounts, while none of the other bands contained any of the radioactivity. The radioactivity regained in this manner amounted to 78% of the amount applied to the gels.

Further identification of these SH groups became possible by reaction of tubules with the covalently labeling GTP analogue 2-amino-6-(*S*-dinitrophenyl)-thiopurine riboside triphosphate. Tubules were depolymerized with the aid of the γ,β-^{32}P-labeled nucleotide, and 0.8 mol of the analogue were coupled to 60 000 daltons of the protein in the repolymerized state, after concomitant hydrolysis of the triphosphate to the diphosphate. The evaluation of the corresponding gel electrophoreses revealed a label exclusively in the two tubulin bands to approximately even amounts. In the tryptic fingerprints of this modified tubulin, a single radioactive spot was found in autoradiography.

The two modifying reagents compete for the same binding site on tubulin. When the protein had first reacted with *N*-ethylmaleimide as described above, only one-third of the stoichiometric amount of the [^{32}P]GTP analogue became covalently bound, and the tryptic fingerprint now produced a series of 6–8 labeled peptides in autoradiography. When, on the other hand, the protein was first labeled covalently with cold GTP analogue, the amount of *N*-ethyl-[^{14}C]maleimide bound under the same experimental conditions as in the previous alkylations, was reduced by 50%. Again the tryptic fingerprints now showed several new peptides labeled in autoradiographs. Thus, both reagents evidently become attached to the two SH groups at the GTP binding site with a much higher reaction rate than to other binding sites; if these two are blocked, the slower side reactions with other sulfhydryls become predominant.

N-Ethylmaleimide does not inhibit polymerization of tubulin simply by blocking GTP binding, however. This was shown by determinations of the GTP content of tubulin preparations after stoichiometric alkylation of the two SH groups. In all experiments, the full amount of 1 GTP per tubulin subunit was still bound by the protein. However, under the conditions inducing polymerization (addition of glycerol, warming to 37°C), no hydrolysis to the diphosphate occured.

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