PURIFICATION OF A THERMOSTABLE HIGH MOLECULAR WEIGHT FACTOR PROMOTING TUBULIN POLYMERIZATION

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1. Introduction

Tubulin preparations obtained from mammalian brain by an assembly–disassembly procedure are known to contain some proteins co-purifying with tubulin that stimulate its polymerization. Two groups of polymerization-promoting factors have been recognized up to now: the tau factor and high molecular weight (HMW) proteins.

Tau factor was first described in [1]. It was isolated from tubulin preparations [2,4] as well as directly from brain [2]. The purified tau preparations were shown to contain a group of closely related thermostable polypeptides with mol. wt ~60 000 [2,3].

HMW fractions capable of stimulating tubulin polymerization were obtained only from tubulin preparations [5–8]. They contained, as the main component, polypeptides with mol. wt ≧250 000, but a marked amount of other proteins, of lower molecular weight, appeared to be also present. Strictly speaking, it is still to be proved that it is HMW protein which is responsible for the stimulatory activity of HMW fractions.

We describe here a procedure for purifying an active HMW fraction from bovine brain and show also that the polymerization-promoting activity of HMW factor is thermostable. The procedure permits one to isolate tau factor as well.

Abbreviations: EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetate; SDS, sodium dodecyl sulphate

2. Materials and methods

Tubulin was isolated from bovine brain by a polymerization–depolymerization procedure [9] as modified in [10]. It was then purified from the associated proteins by column chromatography on phosphocellulose (Whatman P11, batch 2111/582) [1] in a buffer containing 50 mM imidazole adjusted to pH_{20} 6.7 with HCl, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A).

For estimation of the polymerization-stimulating activity of different fractions in the course of isolation of stimulatory factors, 0.35 ml tested fraction was mixed at 0°C with the same volume of purified tubulin to a final 2 mg tubulin/ml, then supplemented with 15 μl GTP and 15 μl EGTA solutions to a final 1 mM GTP and 1 mM EGTA. The fractions containing an excess of salt were pre-dialysed against buffer A. The mixture was warmed to 37°C, and tubulin polymerization was then monitored by the increase in $A_{330\ nm}$ [11]. After 20 min at 37°C the mixture was applied to grids coated with Formvar and carbon; the samples were negatively stained with 2% aqueous uranyl acetate and examined in an electron microscope.

Protein concentration was determined by measurement of A_{280} or by the Lowry method [12] calibrated with bovine serum albumin. The concentration of 1 mg/ml estimated by the Lowry method corresponded to $A_{280} = 0.51$ for the purified HMW factor and 0.44 for the tau factor.

For determination of ATPase activity, the samples

in buffer A were incubated with 1 mM ATP for 60 min at 37°C. The reaction was stopped by addition of an equal vol. 3% $HClO_4$, and the amount of inorganic phosphate was measured as in [13].

SDS–gel electrophoresis was performed on a linear 4–10% polyacrylamide gel gradient in the Weber and Osborn buffer system [14].

3. Results

3.1. *Purification of polymerization factors*

Tubulin polymerization factors were isolated directly from bovine brain. The sequence of the main fractionation steps is shown in fig.1. Brains were used within 3 h of slaughter. All operations, except thermoprecipitation, were carried out at 0–4°C; all the columns were equilibrated with buffer A.

About 4 kg brain was homogenized in 3.6 l buffer A. The homogenate was clarified by centrifugation at 11 000 × *g* for 30 min and subjected to thermoprecipitation in the presence of 1 M KCl, the high salt being included to avoid a co-precipitation of polymerization factors with the bulk of protein [4]. For

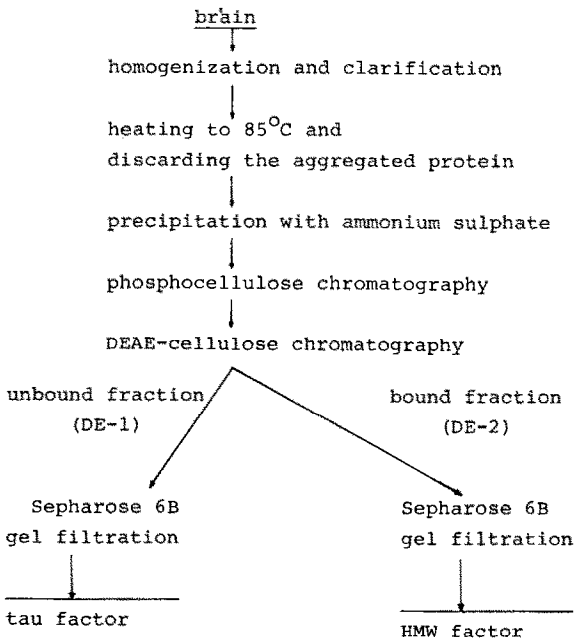

Fig.1. Sequence of the main purification steps.

thermoprecipitation, the clarified homogenate was diluted with an equal vol. 2 M KCl in buffer A and divided into 500 ml portions which were then heated with occasional stirring in a waterbath at 95°C for 8–10 min. The temperature of the portions reached 82–87°C. After the homogenate had been cooled, the aggregated material was pelleted (11 000 × *g*, 10 min) and discarded.

Solid ammonium sulphate was gradually added to the resulting supernatant to a 41% saturation, and the mixture was left for 30 min. The precipitate formed was collected by centrifugation (3000 × *g*, 5 min) and resuspended in 80 ml buffer A, the suspension was dialysed (3 × 12 h) against the same buffer and finally clarified (31 000 × *g*, 30 min). At this point it was already possible to detect the polymerization-promoting activity of the preparation by turbidity measurement or electron microscopy.

The material was then applied to a phosphocellulose column (bed vol., 100 ml; flow rate, 36 ml/h); the column was washed overnight with buffer A and eluted with 0.5 M KCl in the same buffer. Only the eluate was active. After dialysis against buffer A, it was applied to a DEAE-cellulose (Whatman DE-52) column (bed vol., 25 ml, flow rate, 30 ml/h). Both the unbound fraction (DE-1 fraction) and that eluted from DEAE-cellulose with 0.5 M KCl in buffer A (DE-2 fraction) possessed the stimulatory activity and were further purified by gel filtration on Sepharose 6B. The DE-2 fraction was $A_{280} \simeq 2$; vol., 12 ml. The DE-1 fraction was concentrated before gel filtration to the same volume and to $A_{280} \simeq 3.5$ by being applied to a 20 ml phosphocellulose column and eluted from it with buffer A containing 0.5 M KCl.

Peaks resulting from gel filtration were concentrated to $A_{280} > 0.3$ on phosphocellulose columns of appropriate volumes (of a few milliliters), as above, and assayed for the assembly-promoting activity. The profile of elution of DE-1 fraction from a Sepharose 6B column is shown in fig.2a. Added to tubulin, the material of each of peaks I, II and III induced an increase in A_{330} at 37°C, but only in the case of peak I was this increase GTP-dependent and the structures formed were microtubules. Peak I contained a group of polypeptides migrating during SDS–electrophoresis slightly slower than tubulin subunits (fig.3a) and apparently identical with tau factor [2]. No HMW polypeptides were present in this tau preparation.

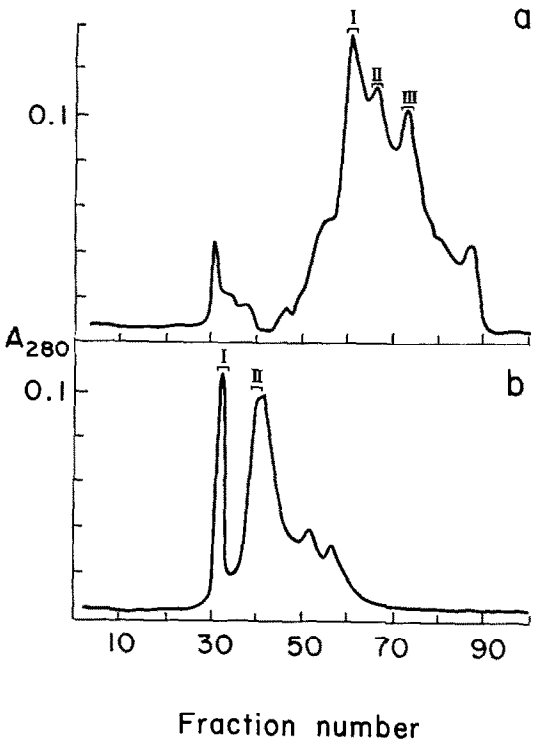

Fig.2. Gel filtration of DE-1 (a) and DE-2 (b) fractions on Sepharose 6B. A 6 ml sample was applied to 2.3 × 120 cm column and eluted with buffer A at a 12 ml/h flow rate; 6 ml fractions were collected.

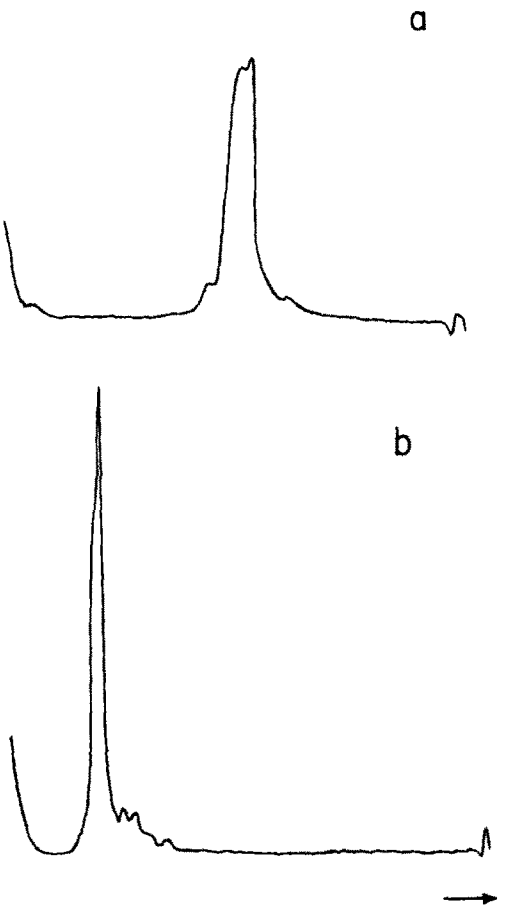

Fig.3. SDS–gradient gel electrophoresis of tau (a) and HMW (b) preparations. The gels loaded with ~20 μg protein were stained with Coomassie brilliant blue R-250 and scanned at 630 nm.

Figure 2b shows the elution profile of the DE-2 fraction. The concentrated material of peak II, which consisted of HMW polypeptides (see below), was the only one possessing the assembly-promoting activity.

From 4 kg brain, we usually obtained ~5 mg of each factor.

3.2. *Characteristics of the HMW preparation*

Tubulin polymerization induced by the HMW preparation (peak II, fig.2b) was GTP-dependent, did not occur in the cold and, as judged by electron microscopy, resulted in the assembly of normal microtubules. At final 0.5 mg HMW factor/ml and 2 mg tubulin/ml the polymerization was accompanied with an ~0.6 A_{330} increase. The HMW preparation had a typical protein ultraviolet A_{277} max.

Protein composition of the preparation was analysed by SDS electrophoresis on gradient gels. The main band, which consisted in fact of two closely spaced ones, clearly distinguished at lower loadings, and some minor bands, were revealed (fig.3b). The molecular weight of the main component was higher than that of the myosin heavy chain, i.e., >200 000; the minor components migrated somewhat faster than the main one. No tau proteins were found, even when overloaded gels were examined.

As our preliminary data show, antisera to the HMW preparation react (by double immunodiffusion) with the brain tubulin preparation obtained by the assembly–disassembly procedure; besides, during SDS–electrophoresis the main component of the

HMW preparation comigrates with the HMW doublet that is contained in the tubulin preparation. It is likely that we have isolated directly from brain the same HMW factor as that which co-polymerizes with tubulin.

The HMW preparation in buffer A had no detectable ATPase activity (<0.2 nmol × mg^{-1} × min^{-1}) under conditions similar to those where the activity of tubulin preparations obtained by assembly–disassembly had been revealed [15].

HMW factor was thermostable in brain homogenate (as seen from the isolation procedure itself) and also in the purified form. Pre-incubation of the HMW preparation in buffer A at 85°C for 5 min did not affect its ability to promote microtubule assembly as judged both by turbidity measurement and electron microscopy.

4. Discussion

A procedure has been developed to isolate tubulin polymerization factors in large amounts directly from bovine brain homogenate, and both HMW and tau factors were obtained. Two main features of the procedure should be noted:

1. The use of thermoprecipitation in the presence of a high salt concentration at the very beginning of purification, before standard biochemical fractionation methods.
2. The absence of the tubulin assembly–disassembly steps generally used to co-purify polymerization factors.

The main component of our HMW preparation is a doublet with mol. wt >200 000. Only minor components have mol. wt <200 000, but even these are much heavier than tau polypeptides. Thus, one may reasonably believe now that HMW proteins themselves are able to promote tubulin polymerization, as can the tau factor [2,4].

Like the tau factor [2], the HMW factor is thermostable. The retention of activity after incubation at 85°C seems to be rather unusual for a protein of mol. wt >200 000. It is yet unclear whether the heated HMW factor can retain (or restore) its native conformation or that this conformation is not necessary for stimulation of microtubule assembly.

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