ISOLATION OF TUBULIN FROM PIG PLATELETS

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1. Introduction

Following the introduction of glutaraldehyde fixation [1], microtubules are now frequently seen in electron micrographs of blood platelets. They are present in the normal discoid-shaped cells as prominent circumferential rings lying subadjacent to the surface membrane. In the activated platelet, a condition which subsequently leads to increased adhesiveness and aggregation as part of the haemostatic process, the microtubules may then be seen extending into and throughout the length of the pseudopodia. Their morphology has been reviewed by Behnke [2], and by White [3]. Platelets also contain microfilaments which are smaller in diameter than the microtubules and these are now considered to be the components of an actomyosin-like contractile complex [4]. Whilst the microtubules are believed to have a cytoskeletal role and the microfilaments contractile potential [5], the exact functions and interrelationship of these two polymeric protein systems in platelet motile behaviour, such as shape change, aggregation and clot retraction, are by no means clear.

The best studied mammalian microtubular system is that of brain where the subunit protein of the tubules, known as tubulin, has been isolated [6–8], and may account for between 15 and 40% of the soluble proteins of brain [9]. This subunit is a dimer, has a mol. wt of 110 000–120 000 and a sedimentation coefficient of about 6S [10]. It can be split with denaturing agents to give two non-identical monomers with mol. wts of about 53 000 and 56 000 [11]. An important property of the tubulin dimer is its ability to bind the alkaloid colchicine [10,12] and this colchicine-binding activity is routinely used to monitor purification in the isolation of tubulins [10,13,14].

In this paper we describe the isolation of tubulin from pig platelets by an 'in vitro' polymerisation procedure. Its identification has been based upon a comparison of its properties with those of tubulins isolated from mammalian brains.

2. Materials and methods

[^{3}H]Colchicine (ring C-methoxyl-H3) of specific activity 2 Ci/mmol was obtained from the Radiochemical Centre, Amersham, England, and was stored in ethanol at −20°C until used. Unlabelled colchicine, GTP (Type II-S), MES*, EGTA and the protein standards for gel electrophoresis were all purchased from the Sigma Chemical Company. Vinblastine sulphate was a gift from Eli Lilly and Company. Most other chemicals used were of analytical grade. DEAE cellulose discs (DE 81) of diameter 2.3 cm were obtained from H. Reeve Angel and Co. Ltd., London.

2.1. *Isolation of platelet tubulin*

Platelets were isolated from freshly collected pig blood as described earlier by Harris and Crawford [15], with the exception that all procedures were carried out at room temperature.

The tubulin fraction was prepared from the soluble phase of platelet homogenates using a temperature-dependent polymerisation procedure based upon the Shelanski et al. [8] modification of the Weisenberg method [16], for brain tubulin polymerisation. The

Abbreviations: MES, (2[*N*-Morpholino]ethane sulphonic); EGTA, ethyleneglycol-bis-(β-amino-ethyl ether)*N*,*N*′-tetra-acetic acid; SDS, sodium dodecyl sulphate.

platelets were homogenised in 1.5 × volume of ice-cold polymerisation buffer using a MSE top-drive homogeniser. The polymerisation buffer consisted of 1 mM EGTA; 1 mM EDTA; 0.1 M MES; 0.3 M sucrose; 1 mM GTP, adjusted to pH 6.8 at 22°C with 8 M NaOH. The homogenate was centrifuged at 120 000 g_{av} for 50 min at 4°C. The soluble phase so obtained was made 4 M with respect to glycerol and incubated at 37°C for 30 min. After centrifugation (120 000 g_{av} 50 min at 25°C) the pellet was taken up in polymerisation buffer to a concentration of approximately 5 mg/ml.

2.2. *Preparation of brain tubulins*

Rat, rabbit and guinea-pig brain tubulins were prepared by the procedure of Shelanski et al. [8], with the exception that Mg^{++} was not included in the polymerisation medium. The brain tissue was homogenised in a Potter-Elvehjem type homogeniser with a teflon pestle (specifications: 15.14 mm diameter; 0.11–0.15 mm clearance; Tri-R Instruments Inc). If necessary a second cycle of depolymerisation/polymerisation was carried out to completely remove actin.

2.3. *SDS-Polyacrylamide gel electrophoresis*

The protein samples were denatured by making a final concentration of 4% SDS, 8 M urea and 0.1 M β-mercaptoethanol and placing in a boiling water bath for 5 min.

SDS-Polyacrylamide slab gels (7.5% w/v and 20 × 6 × 0.2 cm) were prepared and run in a buffer system of 0.4 M boric acid adjusted with Tris (approx. 0.1 M) to pH 7.0, containing 0.1% SDS. Bromophenol Blue was used as tracking dye. Gels were run at 50 mA with between 5–8 samples per gel. They were stained, usually overnight, in 0.25% Coomassie Brilliant Blue R in methanol:acetic acid:water (45:10:45) and destained by diffusion in methanol: acetid acid: water (1:1:8).

SDS-Polyacrylamide stick gels (7.5% w/v and 9 × 0.5 cm) were used for molecular weight determinations. They were run at 3 mA/gel using the same buffer system and tracking dye as used for the slab gels. The gels were stained with Coomassie Blue as above but destained electrophoretically in methanol:acetic acid: water (1:1:8).

Densitometer traces of gels were obtained at 570 nm using a Gilford Model 2000 spectrophotometer with a Gilford gel scanning attachment.

2.4. *Colchicine binding assay*

Platelet fractions were assayed for colchicine-binding activity using the DEAE-cellulose filter disc assay of Borisy [17]. The final colchicine concentration in the assay mixture was 2 μM. Controls had bovine serum albumin substituted for the platelet fractions. Samples were incubated with colchicine for 2.0 h at 37°C. Radioactive material was eluted from the washed discs into a toluene–Triton X-100 based scintillation fluid and counted in a Philips liquid scintillation analyser with a counting efficiency for tritium of 25%.

2.5. *Electron microscopy*

A suspension of the polymerised protein was diluted tenfold in a buffer solution containing 0.17 mM EGTA; 0.17 mM EDTA; 17 mM MES; 50 mM sucrose, 0.17 mM GTP; 1 M hexylene glycol, pH 6.8. A drop of this diluted material was placed on a grid coated with 0.2% formvar and carbon and allowed to dry. The grids were then negatively stained with 1% uranyl acetate and examined in a Philips 301 electron microscope.

2.6. *Other procedures*

Rabbit muscle actin was prepared by a modification of the Carsten and Mommaerts procedure [18] applied to an acetone powder of rabbit back muscle prepared by the method of Bárány et al [19].

Protein was determined by the method of Lowry et al. [20], samples being diluted to overcome interference by sucrose [21]. The MES buffer does not significantly interfere in this assay [22]. Bovine serum albumin was used as the standard.

3. Results

Colchicine-binding activity was determined on the fractions obtained in the protein isolation procedure. Table 1 shows the results for a typical isolation experiment. The polymerised material accounted for 5.6% of the soluble phase recovered protein content. It has a [^{3}H]colchicine binding specific activity of 411.7 DPM/μg which was 35 times greater than that of the supernatant proteins after polymer removal.

The isolation procedure was further monitored using SDS-polyacrylamide gel electrophoresis. Fig.1 shows a slab gel of the platelet fractions obtained during a typical preparation and illustrates the high degree of

Table 1
[^{3}H]Colchicine-binding activity of tubulin isolated from the platelet soluble phase

Fraction	Protein mg	Specific activity DPM/μg
Platelet tubulin	14.2	411.7
Supernatant after tubulin removal	238.0	11.7

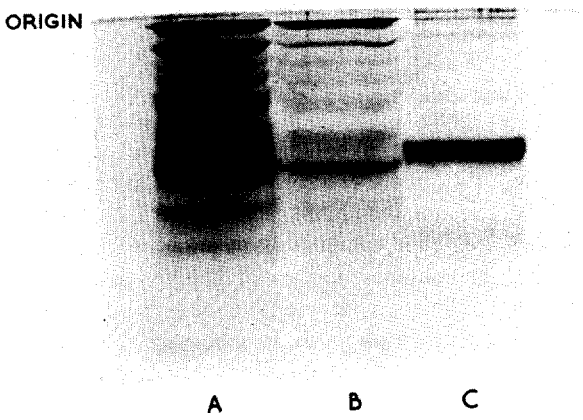

Fig.1. Electrophoresis of platelet fractions in SDS-polyacrylamide gel. A) Platelet soluble phase. B) Supernatant after removal of the tubulin. C) Platelet tubulin polymerised from soluble phase.

purification that can be obtained with the one-step polymerisation procedure applied to the platelet soluble phase.

The molecular weight of the platelet tubulin was determined using SDS-polyacrylamide gel electrophoresis, (fig.2) which gave a value of 55 000 daltons (S.D. 1500, n = 6). Further electrophoresis experiments with purified tubulins isolated from rat, rabbit and guinea pig brains showed co-migration of these proteins with the tubulin band in the purified platelet preparations. An example of these co-electrophoresis experiments is shown in fig.3. The platelet tubulin migrated as a closely spaced doublet similar to that seen in brain tubulin. Also present in both brain and platelet tubulin preparations were 2 and 3 minor high molecular weight polypeptides (figs.1,3,4).

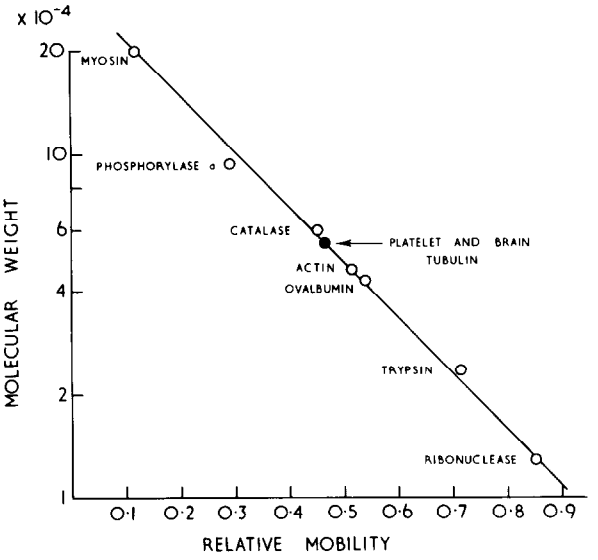

Fig.2. Graph showing the linear relationship between protein, molecular weight and relative mobility on SDS-polyacrylamide gels. Relative mobility was calculated as the ratio of the distance travelled by the protein compared with that travelled by the marker dye.

In the early part of this work polymerisation was performed without the inclusion of EDTA in the buffer system. This gave preparations containing a high proportion of actin as identified by co-electrophoresis with rabbit skeletal muscle actin and with platelet actin.

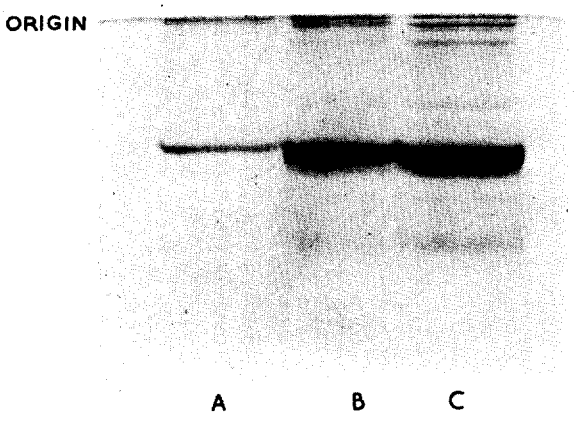

Fig.3. Co-electrophoresis of platelet tubulin with rabbit brain tubulin in SDS-polyacrylamide gel, A = Brain tubulin, B = Platelet tubulin, C = Platelet tubulin and brain tubulin.

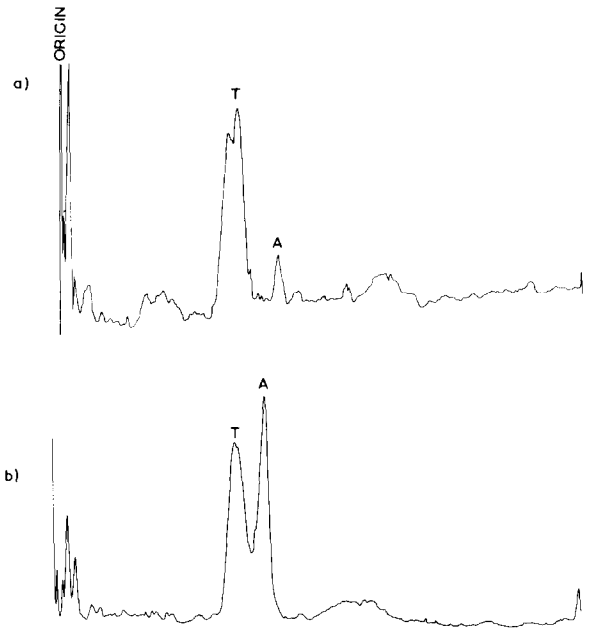

Fig.4. Densitometer traces of SDS-polyacrylamide gels of platelet tubulin prepared by polymerisation a) in the presence of EDTA and b) in the absence of EDTA. The scans show tubulin (T), actin (A) and the high molecular weight components.

Fig.4 shows densitometer scans of SDS-polyacrylamide gels of preparations performed in the presence and absence of EDTA. The contamination by actin is substantially reduced by the inclusion of EDTA in the polymerisation medium.

Electron microscopy of the tubulin pellet dispersed in diluted polymerisation buffer and negatively stained with uranyl acetate revealed large numbers of filamentous strands ranging between 5 nm and 40 nm in thickness [fig.5].

4. Discussion

Although microtubules are an established cytoplasmic feature of almost all eukaryotic cells [23], detailed chemical studies of the subunit protein, tubulin, have been to date largely confined to material isolated from two sources, viz. mammalian brain tissue [10,24,25], and sea urchin sperm [26–28]. These sources are rich in tubulin, but in other cells where the tubulin content is lower and relatively large amounts of actomyosin-like proteins are also present, tubulin isolation and purification is more difficult.

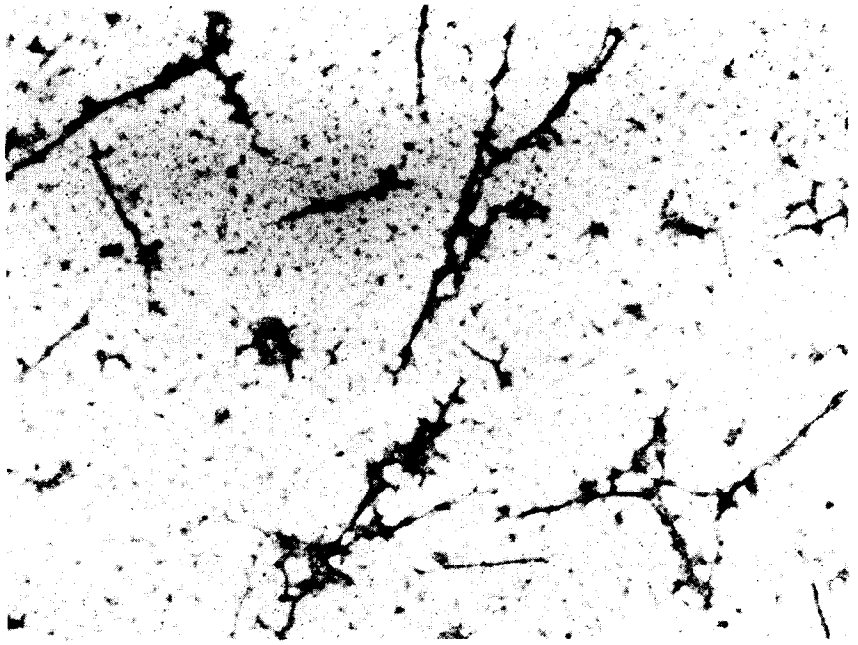

Fig.5. Electron micrograph of polymerised platelet protein, negatively stained with uranyl acetate (Magnification × 45 000).

In our studies with platelets we have found that the more conventional procedures for tubulin isolation based upon ammonium sulphate fractionation and DEAE-ion exchange chromatography [10,13] were inapplicable and gave poor yields of tubulin with large amounts of actin. Similarly, the use of the Vinca alkaloid, Vinblastine sulphate to precipitate tubulin from the platelet soluble phase, (a technique sometimes used to isolate tubulin from other sources [29]) resulted in very impure preparations in which actin again predominated.

Our most satisfactory procedure for tubulin isolation from the platelet-soluble phase has been based upon the temperature-dependent assembly of the polymer in a medium containing glycerol, GTP and both EGTA and EDTA. Platelets contain endogenous Mg^{2+} and are rich in actin, and polymerisation of the tubulin in the absence of EDTA results in actin contamination. The proportion of actin present in the isolated tubulin is even higher if 0.5 mM Mg^{2+} is included in the polymerisation medium as recommended in the Shelanski et al. [8] procedure for brain tubulin isolation. It has recently been reported that in the presence of Mg^{2+}, plasmodium actin polymerises into a linear and more flexible structure, different from that of the usual two stranded helical polymer of F-actin [30,31]. If platelet actin can form similar Mg^{2+} polymers this could explain a requirement for EDTA in the platelet tubulin purification by polymerisation.

The present procedure produces an actin-free platelet tubulin which has a subunit mol. wt of 55 000 on SDS-polyacrylamide gel electrophoresis and which co-electrophoreses with brain tubulins from three different mammalian species. The purified material has a colchicine-binding specific activity 35 times greater than the remaining proteins in the soluble phase after tubulin removal. The preparation also contains 2 or 3 minor polypeptides of high molecular weight, which may represent the dynein-like components seen in preparation of cilia and flagella tubulins and recently reported present in brain tubulin preparations isolated by in vitro polymerisation [32,33].

The isolation from human platelets of a colchicine-binding protein, presumed to be microtubular protein, has been previously reported using ammonium sulphate fractionation and ion-exchange chromatography [34]. This protein enhanced the Mg^{2+} ATPase activity of both skeletal muscle myosin and the platelet myosin-like protein 'Thrombosthenin M', but its identification as tubulin was not firmly established and the presence of actin not entirely excluded.

The platelet tubulin polymer seen in our electron micrographs appears to form by linear aggregation giving filaments of varying length and width. Lateral association of the filaments may account for some of this heterogeneity but structures comparable in size and character to the 18 nm to 30 nm microtubules present as a circumferential bundle in whole cell preparations [35] do not appear to be formed in our procedure. Since a filamentous substructure with a centre to centre spacing of about 6 nm has been identified in microtubules present in platelet cytoplasm [35] the linear tubulin polymer produced in the present experiments may represent an intermediate stage in the microtubule assembly process.

The morphological similarity of microtubule subfilaments and the contractile microfilaments seen in platelets has led to some speculation that both these structures may be composed of the same basic subunit. Our present biochemical identification of tubulin, coexisting with actin, in pig platelets suggest that these earlier electron microscopic observations may now warrant a different interpretation.

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