A basic protein from bovine brain that co-precipitates with tubulin in vitro

H. Ohmori, M. Kuba, S. Matsumura and A. Kumon[1]

Department of Biochemistry, Saga Medical School, Saga-shi, Saga-ken 840-01 (Japan)
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Summary. A 193 kDa protein consisting of 58 kDa subunits, which has pI values of 8.50 and 8.65, was purified from bovine brain cytosol. It formed heavy precipitates with tubulin, and the molar ratio of tubulin dimer to this protein in the precipitate was 3.2. In contrast to microtubules containing ordinary microtubule-associated proteins, these complexes remained stable against cold and 1 mM $CaCl_2$.
Key words. Basic protein; purification; turbidimetry; co-sedimentation with tubulin; bovine brain.

Microtubules are involved in a variety of cellular functions. Brain microtubules are composed of tubulin and microtubule-associated proteins (MAPs) that play pivotal roles in microtubule assembly. Two major groups of MAPs have been identified in neural tissues. These include high molecular mass components (350 and 270 kDa) termed MAPs 1 and 2, respectively[2,3], and tau proteins in the molecular mass range of 55–68 kDa[4,5]. However, it has been assumed that the brain contains other groups of tubulin-associated proteins distinct from the MAPs mentioned above, because more than 20 distinct polypeptides have been identified in brain microtubules by polyacrylamide gel electrophoresis (PAGE)[6]. In fact, three additional MAPs, that is, MAP 3 (180 kDa) in neuronal and glial cells[7], MAP 4 (215–240 kDa) in glias[8] and MAP 5 (320 kDa) in neonatal neurons[9], have recently been characterized. Furthermore, some basic proteins that interact with tubulin appear to be likely candidates for the moieties of both duplex microtubules in vivo[10], and cold-stable and double-walled microtubules isolated from brain homogenate[11,12].

The authors have made attempts to isolate proteins from bovine brain that interact with tubulin, and have found that a brain cytosol fraction which was retained on cation exchangers contained a basic protein designated tentatively as P 58 that consisted of 58 kDa subunits and co-sedimented with tubulin. The method for purification of P 58 and the nature of its interaction with tubulin are described in this paper.

Materials and methods. P 58 was purified at 4 °C as follows. Fresh bovine brains (500 g) were homogenized in 1500 ml of 20 mM imidazole-Cl (pH 7.0) containing 1 mM EGTA, 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM benzamidine and 10 mM 2-mercaptoethanol (Buffer A), and centrifuged at 22,000 × g for 1 h, after which the supernatant was applied to a DEAE cellulose column (5.0 × 10 cm). The effluent contained an activity which precipitated the polymerized tubulin upon ultracentrifugation on a discontinuous sucrose density gradient, as described below. PAGE of the precipitate in the presence of sodium dodecyl sulfate (SDS)[13] showed that tubulin co-sedimented with a 58 kDa protein (P 58). Therefore, fractions in the effluent were pooled for the purification of P 58, and were fractionated by ammonium sulfate (50–70%).

The ammonium sulfate fraction was dissolved in 100 ml of 20 mM 2-(N-morpholino)-ethanesulfonic acid (MES) (pH 6.0) containing 1 mM EGTA, 1 mM EDTA, 0.25 mM PMSF, 0.5 mM benzamidine and 10 mM 2-mercaptoethanol (Buffer B), and was dialyzed against Buffer B. The dialysate was applied to a CM cellulose column (5.0 × 7.0 cm), and P 58 was eluted with 450 ml of 0.15 M NaCl in Buffer B. After dialyzing the P 58 fraction against Buffer A, the dialysate was chromatographed on a phosphocellulose column (1.6 × 15 cm) with a 500-ml linear gradient of NaCl (0.2–0.5 M) in Buffer A. P 58 was eluted at 0.30–0.45 M NaCl. After dialyzing this fraction against Buffer B, it was chromatographed again on a CM cellulose column (1.6 × 15 cm) with a 300 ml-linear gradient of NaCl (0.05–0.20 M). P 58 was eluted with minor contaminants as a single peak around 0.1 M NaCl. Fractions containing P 58 were concentrated and dialyzed against 1 M NaCl in Buffer A, followed by gel filtration on a Sephacryl S-300 column (2.8 × 90 cm). P 58 was eluted at the position of catalase, which has a Stokes radius of 5.2 nm. The P 58 fractions were concentrated and dialyzed against 20 mM Tris-Cl (pH 8.0), 1 mM EGTA, 10 mM 2-mercaptoethanol and 1 M NaCl (Buffer C). The dialysate was layered on a linear sucrose density gradient (5–20%) in Buffer C and centrifuged at 170,000 × g for 10 h. Fractions of P 58 were concentrated and dialyzed against 20 mM MES buffer (pH 6.7). The final preparation contained 15 mg of protein.

The activity of P 58 as a precipitant of tubulin was assayed as follows. Tubulin employed for this assay was purified from bovine brain by phosphocellulose column chromatography[4]. Initially, the purified tubulin (4.5 mg/ml) was polymerized by incubation at 37 °C for 1 h in 0.1 M MES (pH 6.7), 1 mM $MgCl_2$, 2 mM EGTA, 1 mM GTP and 10 mM 2-mercaptoethanol (Buffer D), and the polymerized tubulin (0.5–2 mg) was then layered on a 10-ml discontinuous sucrose density gradient with an upper layer of 25% sucrose containing P 58 and Buffer D (6 ml) and a lower layer of 50% sucrose in Buffer D (4 ml). After centrifugation at 170,000 × g for 2 h at 25 °C, the supernatant was divided into 23 drop fractions (0.6 ml) which were drawn from the bottom of the tube. The precipitate was suspended in 0.6 ml of Buffer D. A 4-µl aliquot of each fraction was analyzed by SDS-PAGE in the absence or presence of 8 M urea. The contents of Coomassie blue associated with protein bands in the gel were measured[14], if necessary. In the presence but not the absence of P 58, the polymerized tubulin entered the lower layer under these centrifugation conditions.

Turbidimetry at 350 nm was carried out at 4 °C or 30 °C in 20 mM MES (pH 6.7), 1 mM EGTA and 10 mM 2-mercaptoethanol (Buffer E). EGTA was replaced with 1 mM $CaCl_2$, 1 mM $MgCl_2$ or 1 mM MgGTP, in some experiments.

Isoelectric focusing (IEF) of the P 58 preparation was carried out with a 5% polyacrylamide slab gel containing 13% glycerol and 6.3% Pharmalyte (pH 3–10) in a flat bed apparatus (FBE 3000) from Pharmacia, according to the instruction manual.

Results and discussion. The results of SDS-PAGE of the final P 58 preparation are shown in figure 1 A. Based on the contents of the dye associated with protein bands, the P 58 preparation consisted of 93% of the 58 kDa protein (indicated with an arrow head) and 7% of two co-purified polypeptides just below the 58 kDa band. The Stokes radius of P 58 estimated by gel filtration with Sephacryl S-300[15], and its sedimentation coefficient determined by centrifugation on a linear sucrose density gradient[16] were 5.2 nm and 8.5×10^{-13} s, respectively. Accordingly, the native molecular mass of P 58 was calculated to be 193 kDa, based on the assumption that its partial specific volume was 0.74 ml/g[17], indicating that P 58 consists of three or four subunits of 58 kDa. Figure 1 B also shows that P 58 had two isoforms of different pIs, 8.50 and 8.65. P 58 was denatured by boiling

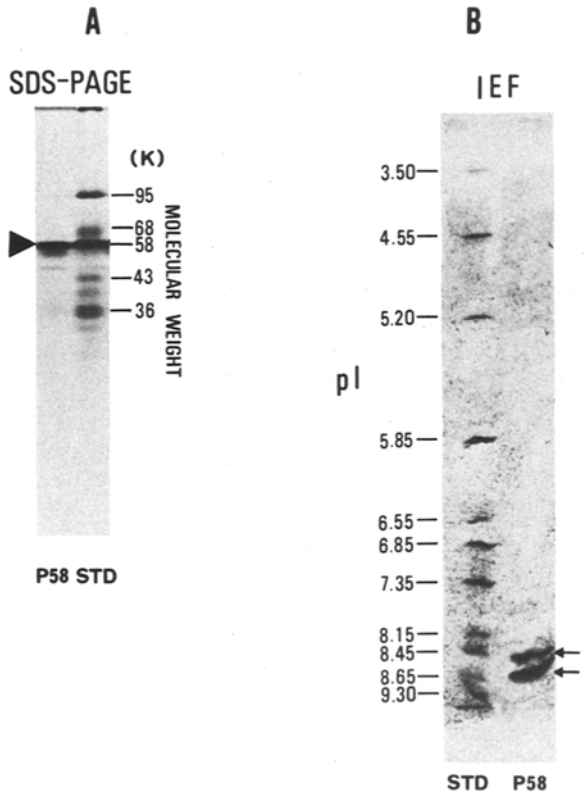

Figure 1. SDS-PAGE and IEF of the purified P58. *A* SDS-15% PAGE. Lane P58, purified P58 (5 µg); lane STD, standard proteins. *B* IEF. Lane P58, purified P58 (20 µg); lane STD, standard proteins from Pharmacia for pI calibration.

for 5 min and formed insoluble aggregates, in contrast to tau proteins which are heat-stable[4].

When P58 was added to the tubulin solution at 30 °C, the mixture became turbid only under the conditions indicated by the stippled area in figure 2. The turbidity reached a maximal level within 2 min and turbid complexes were precipitable by centrifugation for 20 min at 10,000 × g. The turbidity of mixtures containing fixed concentrations of tubulin increased with increments in P58 and then plateaued at a different point for each concentration of tubulin. On the other hand, increments in tubulin concentration resulted in an initial increase in turbidity of mixtures containing more than 0.1 mg/ml of P58, followed by a decrease which occurred at a different point for each concentration of P58. The turbidity peak at a given concentration of P58 was observable when the molar concentration ratio of tubulin dimer (110 kDa) to P58 (193 kDa) was 1.75. The reduction of turbidity by addition of tubulin at concentrations above this ratio suggested that additional tubulin molecules changed the structure of the tubulin-P58 complex. Furthermore, the presence of 1 mM $MgCl_2$ or 1 mM $CaCl_2$ in the reaction mixture increased the turbidity by about 50% with no other effects on the entire profile of figure 2, while 1 mM GTP had no effect on the turbidity of the solution containing 1 mM $MgCl_2$ (data not shown). Similar results were obtained at 4 °C (data not shown). These properties of the tubulin-P58 complex were different from those of microtubules polymerized by high molecular mass MAPs, because tubulin polymerization by the MAPs is induced at 37 °C by MgGTP and inhibited either by 2 mM $CaCl_2$ or by lowering the incubation temperature to 0 °C[18]. The tubulin-P58 complex appeared to have properties similar to those of cold/Ca^{2+}-stable microtubules from rat sympathetic neurons which were stable at 4 °C against 5 mM $CaCl_2$[19]. In this context, it is noteworthy that the cold/Ca^{2+}-stable microtubules from rat brain contain several accessory proteins including a 56 kDa polypeptide on SDS-PAGE[20], whose molecular mass is very close to that of P58.

For analysis of the turbid complex in figure 2, tubulin was centrifuged together with P58 on a discontinuous sucrose density gradient, and their distribution in the gradient was examined (fig. 3). In the case of either tubulin alone (fig. 3A) or P58 alone (fig. 3B), each protein was distributed only in the upper layer and neither entered the lower layer nor the precipitate. On the other hand, centrifugation of the two proteins together gave a distribution profile distinct from that of each protein alone (fig. 3C). About 50% of the loaded protein was precipitated and about 15% was detected in the lower layer. Since both proteins had the same mobility on SDS-PAGE, the exact ratio of the two proteins in the precipitate and lower layer could not be determined. As the authors found that P58 migrated more slowly than tubulin on SDS-PAGE containing 8 M urea (lanes P58 alone and tub alone, respectively, fig. 3D), the same preparations employed in figure 3C were analyzed again on 8 M urea/SDS-PAGE (fig. 3C). The dye concentrations in the tubulin bands in the lower layer and precipitate were 1.8-fold higher than those in P58 bands, and the molar ratio of the tubulin dimer (110 kDa) to P58 (193 kDa) was calculated to be 3.2. This ratio did not change for any precipitate produced by varying the amount of tubulin applied from 0.5 to 2 mg (data not shown).

P58 is a basic protein, as shown in this paper, and its role in the brain is not known at present. Some basic proteins including histones, protamine, encephalitogenic protein, polylysine, polyarginine and spermine are known to interact in vitro with tubulin, resulting in the formation of double-walled microtubules[10,12,21]. These complexes are composed of an inner microtubule that is wrapped in an electron-dense layer and an outer tubulin wall[12], and do not lose their integrity when the incubation temperature is decreased[10]. On the other hand, it has been reported that lactoperoxidase (pI = 9) binds to tubulin and forms a soluble complex[22], while glyceraldehyde-3-phosphate dehydrogenase (pI = 8.5)

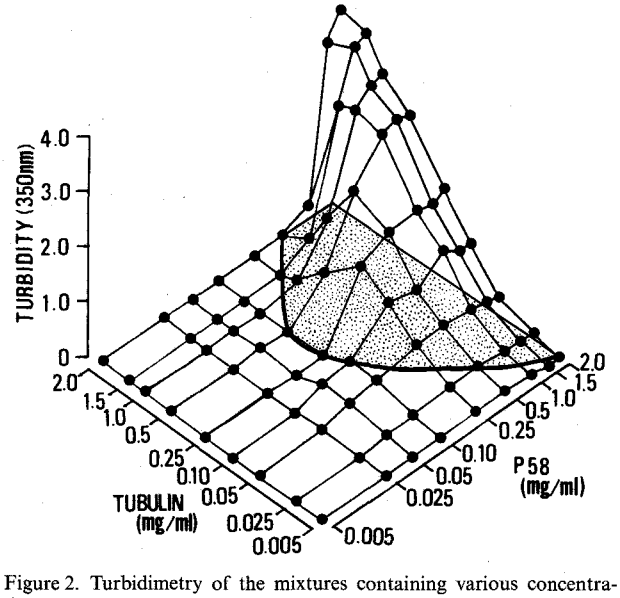

Figure 2. Turbidimetry of the mixtures containing various concentrations of tubulin and P58. The reaction mixture contained various concentrations (0.005–2.0 mg/ml) of tubulin and P58 in Buffer E, and turbidity was measured after the incubation for 5 min at 30 °C.

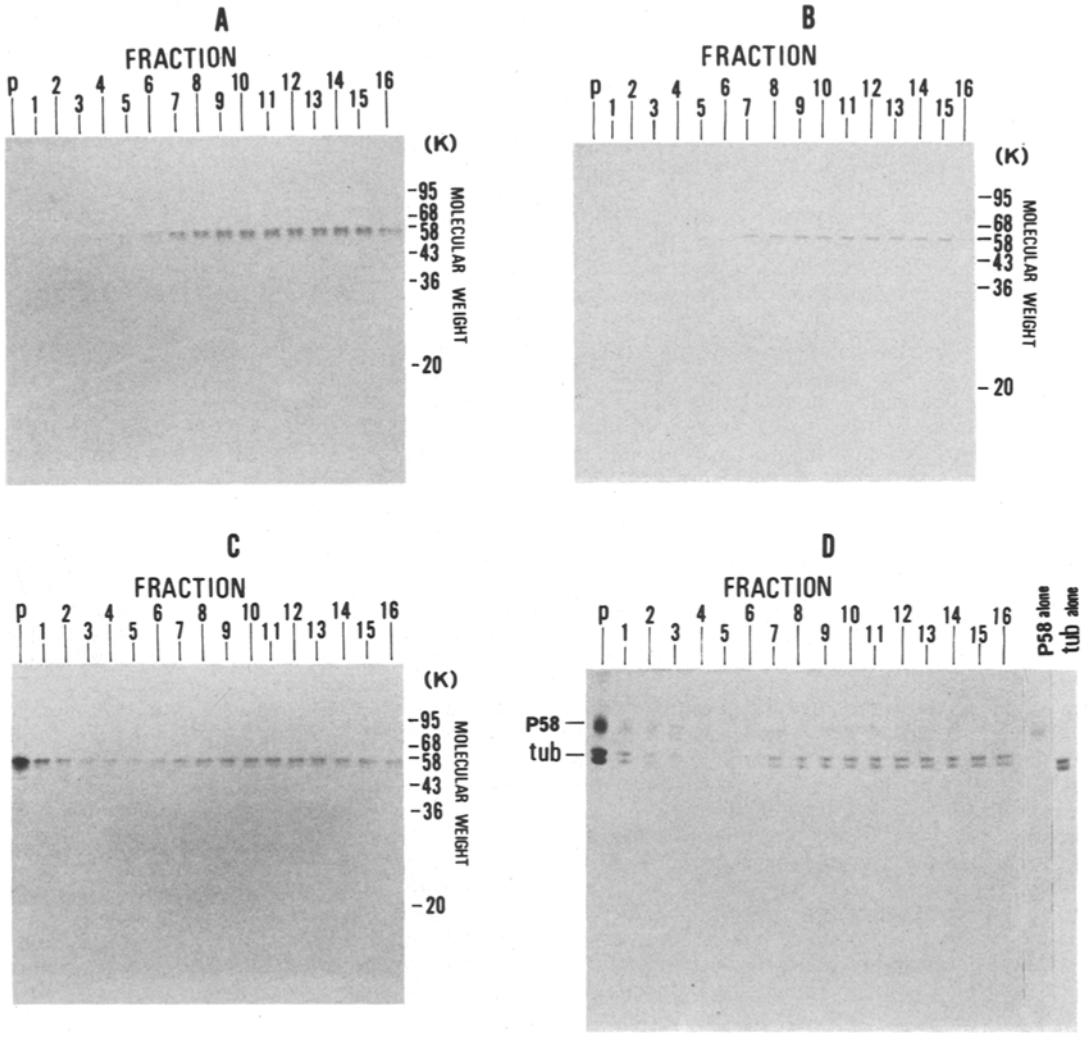

Figure 3. Ultracentrifugation of tubulin, or/and P 58 on a discontinuous sucrose density gradient. Proteins employed for ultracentrifugation: *A* tubulin (2 mg); *B* P 58 (1 mg); *C* and *D* tubulin (2 mg) and P 58 (1 mg). Electrophoretic conditions: *A*, *B* and *C* SDS-15% PAGE; *D* 8 M urea/SDS-10% PAGE. Samples for electrophoresis: lane P, precipitate; lanes 1–16 represent fractions from the bottom to the top of the gradient; lane P 58 alone, P 58; lane tub alone, tubulin.

has a bundling effect on microtubules [23]. These findings indicate that basic proteins interact with tubulin in various manners. Moreover, some basic proteins are also believed to participate in the formation of special microtubule clusters in vivo [24] and cold-stable [11, 19] and double-walled [11] microtubules in brain homogenate. Therefore, there is a possibility that P 58 is a component for microtubules with a special structure.

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