Binding of Tubulin to Substituted Sepharose

KARI HEMMINKI

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki 17, Finland

Tubulin is the main protein constituent of microtubules, which are involved in a number of physiological processes including cell division, cell motility, transport of proteins and support of extended structures. It is entirely unclear how these versatile functions are controlled at a molecular level. There are, however, a number of chemical interactions described for tubulin such as calcium sensitive polymerisation,[1] binding of guanosine nucleotides,[2] colchicine and vinca alkaloids,[3] which may contribute to the physiological functions of tubulin. In this paper another interaction of tubulin is described as it is shown to bind to substituted Sepharose containing aliphatic hydrocarbons. The binding is sensitive to a physiological salt concentration and can be used as a simple purification method for tubulin.

Materials and methods. Rat brains were homogenized in 30 mM sodium phosphate, pH 7.0, containing 10 % glycerol (=PG, about 10 ml/brain) using 20 strokes of a Teflon-glass homogenizer. The homogenate was centrifuged at 100 000 *g* for 60 min and the supernatant was used for further purification.

Brain supernatant was incubated with epoxy-activated Sepharose 6 B containing 12 atom hydrocarbon spacer groups (Pharmacia). Before use the epoxy groups of Sepharose were inactivated by incubating in 1 M Tris, pH 8.0 overnight followed by washes with 0.1 M borate pH 8.0, 0.1 M acetate pH 4.0 and 0.15 M NaCl. After the incubation the mixture of brain supernatant and Sepharose was centrifuged and the supernatant was decanted. Sepharose was taken up in PG and poured into a 2.5 ml syringe. The column was connected to an absorbance monitor (280 nm) and the column was eluted with PG and 75 mM NaCl until no more material came out. Tubulin was eluted with 0.15 M NaCl or with a salt gradient. Aliquots of the samples were taken for the colchicine binding assay,[4] performed at 3.7×10^{-5} M colchicine containing 3.4×10^{-6} M ^{3}H-colchicine (Amersham). All colchicine binding assays were carried out at 0.2 M NaCl. The remaining samples were dialysed against water and used for sodium dodecylsulfate (SDS) gel electrophoresis.[5] Protein was determined according to Lowry *et al.*[6]

Results. When brain supernatant was incubated with Sepharose 6 B containing hydrocarbon spacer groups about 15 % of the protein was observed to bind to it. The binding was ion sensitive and most of the bound protein was removed at 0.15 M NaCl (Fig. 1). The binding increased slightly with increasing incubation time; about 80 % of the maximal binding was observed when the supernatant was passed through the Sepharose column without any preincubation. No binding was observed with

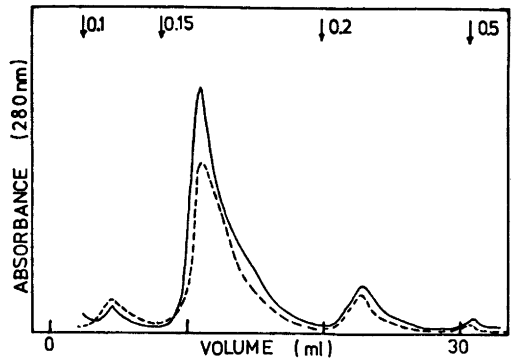

Fig. 1. Elution of protein bound to Sepharose 6 B containing spacer groups with increasing concentrations of NaCl. Rat brain supernatant was incubated with Sepharose at 37 °C for 1 h (———) or immediately passed through a Sepharose column (– – –). The figures on top refer to the molarity of NaCl.

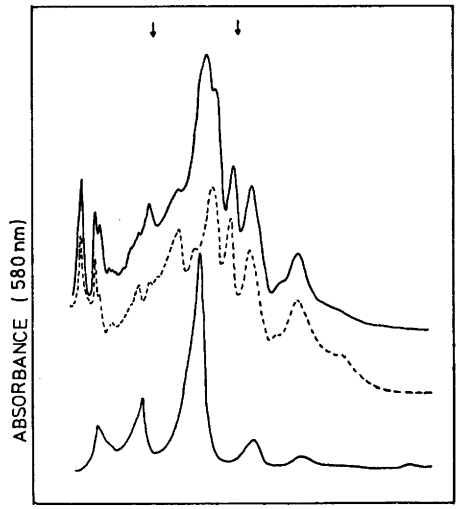

Fig. 2. SDS-polyacrylamide gel electrophoresis of rat brain supernatant protein (top), protein unbound to Sepharose 6 B containing spacer groups and protein bound to Sepharose (bottom). The gels were stained with Coomassie blue. The arrows on top refer to the migration of bovine serum albumin (68 000 daltons) and ovalbumin (43 000 daltons) used as molecular weight standards.

Table 1. Protein content and ^{3}H-colchicine binding of the rat brain fractions bound to Sepharose 6 B containing spacer groups. Means ± SD of 5 determinations.

	Protein content %		Colchicine binding cmp/mg protein × 10^{-3}
	− octanoate	+ octanoate	− octanoate
Supernatant	100	100	23 ± 4
Bound fraction	16 ± 3	0.6 ± 0.3	159 ± 8
Unbound fraction	84 ± 6	99 ± 2	3 ± 2

Sepharose 6 B containing no spacer groups indicating that the substitution of Sepharose was responsible for the binding.

The bound protein was characterized on SDS-polyacrylamide gels (Fig. 2). The binding was selective and efficient: the major component bound displayed a molecular weight of about 55 000 dalton and it was almost completely removed from the supernatant. One of the large molecular weight polypeptides could be a dimer of the 55 000 dalton polypeptide as it had a calculated molecular weight of 110 000 dalton.

Tubulin is a major protein constituent of brain soluble protein.[7] Its molecular weight [8] is similar to that of the bound polypeptide suggesting that this may be tubulin. For this reason colchicine binding assays were performed as this ligand specifically binds to tubulin (Table 1). The colchicine binding activity of the bound protein was enriched about 7-fold over the total homogenate which is almost maximal considering the large amount of tubulin in the supernatant. A corresponding reduction in the colchicine binding activity was noted in the unbound supernatant. The data indicate that the bound protein was markedly purified tubulin. The role of the hydrocarbon chain as the binding component was further suggested by incubating brain supernatant and Sephadex 6 B containing the spacer groups in the presence of an excess (5 %) of octanoic acid (caprylic acid). The added hydrocarbon chains effectively inhibited the binding (Table 1).

Another control experiment was performed to confirm that the bound material was tubulin. Brain soluble protein (100 000 *g* supernatant) was labelled with ^{3}H-colchicine and tubulin was prepared by vinblastine precipitation.[7] A substantial portion of the protein and radioactivity was bound to substituted Sepharose (results not shown).

Discussion. It is shown in this paper that tubulin has affinity to substituted Sepharose containing hydrocarbon chains and a terminal Tris molecule. In order to describe the type of interaction between tubulin and substituted Sepharose experiments have recently been carried out with Octyl-Sepharose CL-4B. As tubulin has no affinity to this preparation (Hemminki, unpublished observation), it is likely that the terminal Tris molecule is responsible for the binding observed in the present article. The sensitivity of this interaction to physiological ion concentrations may be related to cellular functions of tubulin including its association with membranes.[9,10]

The described interaction of tubulin with Tris-Sepharose may be applied for a convenient purification of the protein. About 7-fold purification is obtained. The degree of purity is at least as high as that obtained with any of the conventional one-step purification methods used for tubulin.[4,11] The procedure using affinity chromatography [12] has not replaced the conventional methods due to its cumbersome ligand requirements and other difficulties.

Acknowledgements. The skilled technical assistance of Mrs. Kirsti Salmela and Mrs. Ulla Riihivaara is appreciated. The study was supported by the National Research Council for Medical Sciences, Finland and the Sigrid Jusélius Foundation.

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Received June 16, 1976.

Synthesis of 2,4,4′,5,5′-Pentachloro-2′-hydroxydiphenyl Ether, a Potential Precursor to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

ROLF GÖTHE and CARL AXEL WACHTMEISTER

Environmental Toxicology Unit, Wallenberg Laboratory, University of Stockholm, S-104 05 Stockholm, Sweden

The toxicity of certain chlorinated dibenzo-*p*-dioxins has initiated intensified research on their formation in the production and use of chlorinated phenols and compounds derived therefrom.[1] In certain cases intermediates have been observed, *e.g.* nonachloro-2-hydroxydiphenyl ether, which undergoes ring closure to octachlorodibenzo-*p*-dioxin under different conditions.[2–5] This paper describes the synthesis of 2,4,4′,5,5′-pentachloro-2′-hydroxydiphenyl ether, an analogue precursor to the particularily toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and therefore of obvious interest for chemical and toxicological investigations. The compound is prepared by demethylation of the corresponding methyl ether, which is obtained from the potassium salt of 3,4-dichloro-6-methoxyphenol on reaction with 1,2,4,5-tetrachlorobenzene in dimethyl sulfoxide. A similar procedure has been used in the direct synthesis of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin from 4,5-dichlorocatechol.[6] The present method seems more suitable than the alternative Ullmann ether synthesis which gave somewhat complicated product mixtures containing, *e.g.*, dechlorination products.

Experimental. 2,4,4′,5,5′-Pentachloro-2′-methoxydiphenyl ether. The potassium salt of 3,4-dichloro-6-methoxyphenol[7] (0.25 g; 1.3 mmol) and 1,2,4,5-tetrachlorobenzene (0.5 g; 2.3 mmol) in DMSO (13 ml) were heated to 100 °C for 60 h. The mixture was cooled to 20 °C and unreacted tetrachlorobenzene was partly removed by filtration. Sodium hydroxide (0.5 M, 50 ml) was added to the solution. The neutral components were isolated after extraction with hexane (3 × 10 ml; 0.24 g). 2,4,4′,5,5′-Pentachloro-2′-methoxydiphenyl ether was isolated by preparative TLC (Silica gel HF, ethyl ether – hexane, 35 – 65, R_F 0.7) and was crystallized from ethanol, m.p. 111 – 112 °C. Anal. $C_{13}H_7Cl_5O_2$: C, H. MS (IP 70 eV; *m/e*): pertinent peaks: 370 (M) and 320 (M – CH_3Cl).[2,4] ^{1}H NMR (100 MHz, $CDCl_3$): δ 3.81 (3 H, s), 6.76 (1 H, s), 7.04 (2 H, two singlets 1.5 Hz apart.), 7.50 (1 H, s). UV [abs. ethanol (log ε)]: 289 (4.10), 296 (sh, 4.05) nm.

2,4,4′,5,5′-Pentachloro-2′-hydroxydiphenyl ether. The crude product mixture mentioned above (100 mg), was dissolved in dichloromethane (2 ml). The solution was cooled in a dry ice-acetone mixture, and boron tribromide (100 mg) in dichloromethane (0.2 ml) was added. The mixture was allowed to reach 20 °C and was kept overnight. Hexane (4 ml) was added and 2,4,4′,5,5′-pentachloro-2′-hydroxydiphenyl ether was isolated by means of sodium hydroxide extraction (2 M, 2 × 10 ml) and acidification. Yield 30 mg, needles from hexane, m.p. 125 – 127 °C, MS [IP 10 eV; *m/e*; (% rel. int.)]: 356 (90, M), 321 (5, M – Cl), 320 (5, M – HCl), 286 (42, M – 2Cl), 180 (100, ether cleavage) 177 (20, ether cleavage). ^{1}H NMR (100 MHz, CCl_4): δ 5.40 (1 H, s), 6.70 (1 H, s), 8.00 (1 H, s), 8.05 (1 H, s), 8.48 (1 H, s) UV [abs. ethanol (log ε)]: 290 (4.04), 297 (sh. 4.00) nm.

Acknowledgement. Financial support was given by the Product Control Board.

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Received June 11, 1976.