EFFECT OF LACTOPEROXIDASE-INDUCED α–β TUBULIN SPLITTING ON COLCHICINE BINDING AND POLYMERIZATION OF BRAIN TUBULIN

Bernard ROUSSET* and J. WOLFF

National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD 20205, USA

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1. Introduction

Lactoperoxidase binds to soluble tubulin in a specific manner with a stoichiometry of 2 enzyme molecules/heterodimer [1]. Molecular weight determinations revealed that this binding resulted in a splitting of the dimer such that a mixture of α-tubulin · lactoperoxidase and β-tubulin · lactoperoxidase complexes was produced. This splitting of tubulin into its presumably native monomers provided an opportunity to assess the role of dimerization on the two properties considered to be characteristic of tubulin: colchicine binding and polymerization. Here we describe the effect of lactoperoxidase and these two properties.

2. Materials and methods

Lactoperoxidase was obtained from Boehringer-Mannheim in 3.2 M ammonium sulfate; GTP type IIS, Mes, EGTA were obtained from Sigma and [^{3}H]colchicine from Amersham/Searle.

Tubulin was purified from rat or pig brain by two cycles of polymerization–depolymerization in 100 mM Mes, 1.0 mM EGTA, 0.5 mM $MgCl_2$ and 1.0 mM GTP (buffer A) according to [2]; the polymerization steps were carried out in buffer A supplemented with 4 M glycerol (buffer B). Depending on the source, tubulin represented 75–90% of the purified microtubule protein as judged by SDS–polyacrylamide gel electrophoresis. Solutions (6.0–15.0 mg/ml) were stored in liquid nitrogen in buffer A. Before use, the sample was rapidly thawed, maintained at 0°C for 15–30 min and centrifuged at 30 000 × *g* for 20 min at 2°C to remove aggregates. Depolymerized microtubule protein was used as the starting material. SDS–polyacrylamide gel electrophoresis was performed as in [1].

Abbreviations: Mes, 2-(*n*-morpholino) ethanesulfonic acid; EGTA, ethylene glycol-bis (α-aminoethyl ether)-*N*,*N*'-tetraacetic acid; microtubule protein, tubulin and associated proteins which are copurified by the temperature-dependent assembly–dissassembly procedure [2]

* Present address: Laboratoire de Médecine Expérimentale, FRA INSERM no. 30, Faculte de Médecine Alexis Carrel, 69372 Lyon Cedex 2, France

2.1. *Turbidity measurements*

Polymerization of microtubule protein was followed by the turbidity method in [3] at 417 or 350 nm on a Cary 219 or Beckman 25K recording spectrophotometer. Tubulin assembly was performed in buffer A or buffer B. Protein solutions were prepared at 0°C and reconstitution of microtubules was initiated by incubation at 26°C in a water-jacketed cuvette. The wavelength of 417 nm was chosen because it corresponds to an isosbestic point for the lactoperoxidase · microtubule protein mixture [1].

2.2. *Sucrose gradient centrifugation*

Linear sucrose gradients (5–17%) were prepared in buffer A. Microtubule protein was incubated with 1 μCi [^{3}H]colchicine (spec. act. 50 mCi/mmol) and unlabeled colchicine (2×10^{-5} M) for 90 min at 37°C and centrifuged in a Beckman SW 41 rotor at 40 000 rev./min for 14 h at 2°C. Fractions of 20 drops (0.35 ml) were collected from the bottom to the top of the gradients. Linearity of the gradient was checked by refractometry.

2.3. *Assays*

Colchicine binding was determined by the DEAE

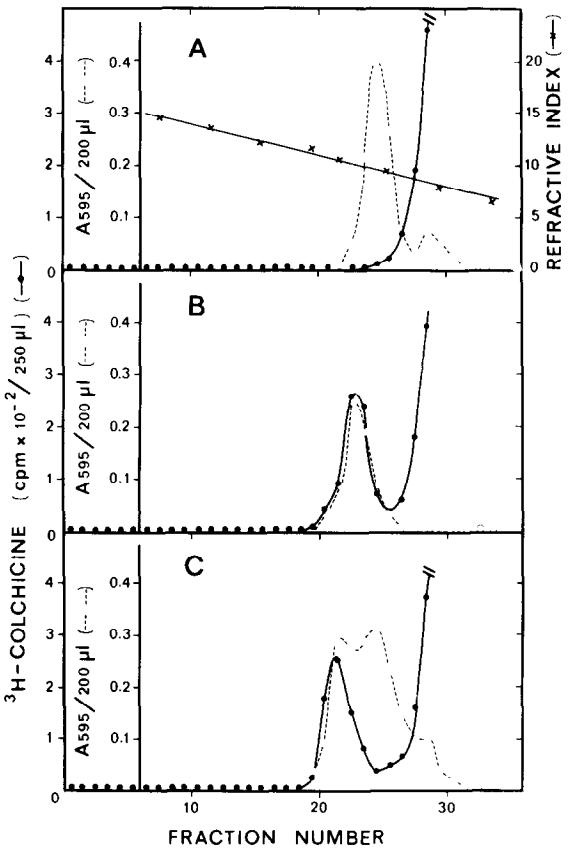

Fig.1. Sucrose gradient centrifugation of [^{3}H]colchicine · tubulin complex in the presence of lactoperoxidase. Soluble rat brain microtubule protein (165 µg) was incubated in 10 mM phosphate buffer (pH 6.8) containing 0.1 mM GTP and 10 mM $MgCl_2$ with [^{3}H]colchicine (final conc. 2.0×10^{-5} M) (B) and lactoperoxidase (446 µg) (C) for 10 min at 0°C and 90 min at 37°C. In a third type of incubation, lactoperoxidase was incubated with [^{3}H]colchicine in the absence of microtubule protein (A). The mixtures were layered on 5–17% sucrose gradient in buffer A and centrifuged at 200 000 × *g* for 14 h at 2°C. Fractions collected from the bottom to the top of the gradients were counted for [^{3}H]colchicine (•) and assayed for protein (○) and sucrose content (x): refractive index expressed in arbitrary units.

filter disc method [4]. Proteins were assayed by the method in [5] using bovine serum albumin as standard. The protein content of sucrose gradients was determined as in [6] using the Bio-Rad protein reagent. Absorbance was measured at 595 nm. Lactoperoxidase was also assayed spectrophotometrically using $\epsilon_{410\ nm}$ 0.78 ml/mg. Lactoperoxidase in 3.2 M ammonium sulfate had an $A_{412}/A_{280} = 0.8$.

3. Results and discussion

3.1. *Colchicine binding*

When [^{3}H]colchicine was added to two cycle microtubule protein, incubated for 90 min and fractionated on a sucrose gradient, the position of the label coincided with the tubulin peak (fractions 23–24, fig.1B). On the other hand, in the presence of lactoperoxidase at a lactoperoxidase/tubulin molar ratio of 3.5, conditions under which there is a complete dissociation of the tubulin heterodimer [1], the bound [^{3}H]colchicine was displaced toward a large species (fraction 22), characteristic of the lactoperoxidase · α-tubulin and lactoperoxidase · β-tubulin complexes (fig.1C) (app. mol. wt ≃ 140 000). That this is not due to colchicine binding to lactoperoxidase is shown in fig.1A.

To determine the stoichiometry of colchicine bound to tubulin at different degrees of dissociation of the tubulin subunits, [^{3}H]colchicine binding was measured at different lactoperoxidase tubulin molar ratios. It is apparent from fig.2 that colchicine bound as well to the tubulin dimer as to the mixture of α-tubulin · lactoperoxidase and β-tubulin · lactoperoxidase. These findings strongly suggested that the

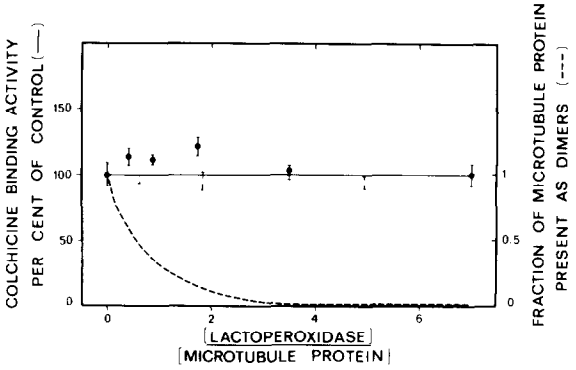

Fig.2. Colchicine binding activity of the lactoperoxidase · tubulin complex. Brain microtubule protein from rat (216 µg/ml) (○) or pig (193 µg/ml) (•) was incubated with 1 µCi [^{3}H]colchicine and 2.0×10^{-5} M colchicine in 10 mM sodium phosphate (pH 6.8) 10 mM $MgCl_2$ and 0.1 mM GTP for 10 min at 0°C and 90 min at 37°C. Lactoperoxidase was added at the beginning of the incubation period, bound [^{3}H]-colchicine was determined according to [4]. The dashed line indicates the fraction of microtuble protein (tubulin) present as dimers. The values were calculated from results obtained by spectrophotometric titration of the lactoperoxidase · tubulin complex formation [1].

dimeric state of tubulin was not necessary for colchicine binding and that the colchicine binding site is located on either the α or β subunit. Moreover, these data suggest that the separated subunits exist in the undenatured state when complexed with lactoperoxidase, since the binding of colchicine, unlike that of 1-anilino-8-naphthalene sulfonate, decays rapidly in mild denaturing conditions such as aging [7]. Additional evidence that the subunits persisted in the native state was provided by experiments that show that the separated subunits can polymerize upon removal of the lactoperoxidase (see below).

3.2. *Polymerization*

Lactoperoxidase binding to intact microtubules was demonstrated using labeled lactoperoxidase in the presence of 4 M glycerol [1]. Turbidity measurements showed that polymerization of tubulin in the presence of lactoperoxidase (molar ratio $\leqslant 0.45$ mol lactoperoxidase/mol tubulin) was identical to that seen with tubulin alone (compare curves 1 and 2 of fig.3A). When the molar ratio of lactoperoxidase to tubulin was doubled (curve 3), there was a 50% decrease in the extent of polymerization. In contrast, in the absence of glycerol, no polymerization could be measured at this molar ratio (curve 5). Thus, glycerol partially prevents the inhibitory effect of lactoperoxidase on tubulin polymerization. In the absence of glycerol and a molar ratio of >0.1, lactoperoxidase inhibited tubulin polymerization as a linear function of the concentration (fig.3B). It should be noted that the stoichiometry for this inhibitory effect of lactoperoxidase approaches 1.0 when corrected for contaminating proteins in the tubulin preparation. The maximum blocking effect of lactoperoxidase is reached when the concentration of intact dimers is below the critical concentration. Starting with pig brain microtubule protein at 2.0 mg/ml (containing ~80% tubulin), one can calculate that, at lactoperoxidase/tubulin molar ratio = 1.0 which was completely inhibitory for tubulin assembly, ~40% of tubulin, i.e., 0.64 mg/ml would remain as dimers. The critical concentration of the starting microtubule protein solution was 0.62 mg/ml.

The inhibition of polymerization does not result from irreversible denaturation of the α and β subunits resulting from their contact with lactoperoxidase. Lactoperoxidase was completely removed from a mixture of lactoperoxidase and rat brain tubulin (fig.4A). When the tubulin recovered from such a procedure was added to limiting quantities of 2 cycle microtubule protein, it retained its ability to polymerize (fig.4B). This tubulin does not polymerize by itself at 0.5 mg/ml (critical conc. 0.2 mg/ml) since the phosphocellulose chromatography used to remove the lactoperoxidase led also to the removal of the microtubule associated proteins.

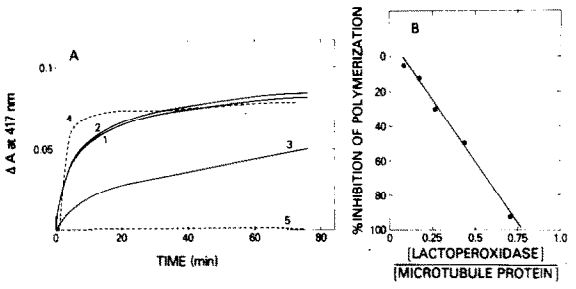

Fig.3. Effect of lactoperoxidase on tubulin assembly. (A) Pig brain microtubule protein (1.26 mg/ml) was polymerized at 26°C in buffer B (4 M glycerol) (curves 1–3) or in buffer A (dashed curves 4,5). Lactoperoxidase concentrations were (μg/ml): 0 (curves 1,4), 450 μg/ml (curve 2), 900 (curves 3,5). (B) Pig brain microtubule protein (2.0 mg/ml) was polymerized in the presence of increasing concentrations of lactoperoxidase in buffer A at 26°C. Lactoperoxidase concentrations were (μg/ml): 0, 136, 272, 409, 681 and 1090 for curves 1–6, respectively. The % inhibition of polymerization was calculated form plateau values. The molar concentration of microtubule protein was calculated using mol. wt 110 000.

The mechanism of stabilization of microtubles by glycerol is not understood. We have observed that 70 μM colchicine which completely blocked tubulin polymerization in the absence of glycerol, inhibited tubulin assembly by only 50% in the presence of 4 M glycerol (B.R., J.W., unpublished). That glycerol interferes with colchicine binding to tubulin was reported [8]. Moreover, glycerol reduces the sensitivity to calcium [9]. Glycerol also protects microtubules against lactoperoxidase. This was seen [1] in microtubules polymerized in the presence of 4 M glycerol which binds 0.2–0.3 mol lactoperoxidase/tubulin dimer, and here where glycerol prevents the inhibitory effect of lactoperoxidase on tubulin assembly when the lactoperoxidase/microtubule protein molar ratio is <0.45.

The mechanism of the inhibition of polymerization of tubulin by lactoperoxidase is not entirely clear at present. In addition to the effect on the critical concentration mentioned above, it is possible that various substoichiometric effects of lactoperoxidase may play a role. These are:

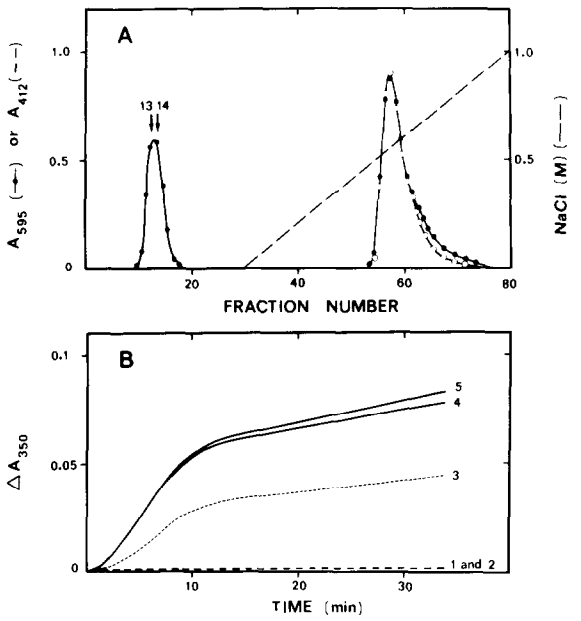

Fig.4. Polymerization of rat brain tubulin after interaction with lactoperoxidase. (A) Chromatographic fractionation of lactoperoxidase · tubulin mixture. Rat brain microtubule protein (7.15 mg) was incubated with lactoperoxidase (17.5 mg) in buffer A without GTP for 90 min at 25°C (conditions for complete lactoperoxidase · tubulin complex formation [1]). The mixture was then chromatographed on a phosphocellulose column (1.5 × 30 cm) equilibrated with the same buffer. After the elution of a first protein fraction, a NaCl gradient was applied. Fractions (2.5 ml) were assayed for protein (A_{595}) and lactoperoxidase (A_{412}). (B) Tubulin polymerization. Fractions 13,14 (top of the peak of excluded material on phosphocellulose) were assayed for their ability to polymerize in buffer A at 26°C in absence (curves 1,2) or in the presence of small amounts of rat brain microtubule protein (curves 4,5). Protein concentration was: fraction 13, 0.5 mg/ml (curve 1); fraction 14, 0.52 mg/ml (curve 2); 2 cycle rat brain microtubule protein, 0.42 mg/ml (curve 3); as 1 + 3 (curve 4); as 2 + 3 (curve 5).

(i) The capping of the growing end of the microtuble by a α-tubulin · lactoperoxidase β-tubulin · lactoperoxidase complex in a manner analogous to that for the colchicine · tubulin complex [10];
(ii) Incorporation of such a complex into the microtubule with a consequent change in the association or dissociation rate constants [11];
(iii) Binding of lactoperoxidase to 36 S ring oligomers [1] in a manner again analogous to colchicine [12], and consequent inhibition of polymerization.

We conclude that the colchicine binding activity and the polymerization capacity of tubulin are differently altered by the dissociation of the α and β subunits. Lactoperoxidase does not affect colchicine binding. Since a maximum of 1 colchicine is bound/dimer, the colchicine binding site is probably located on only one of the two tubulin subunits and the presence of the other subunit is not obligatory. On the other hand, binding of lactoperoxidase interferes with the ability of tubulin to polymerize by a mechanism that appears to involve the dissociation of the subunits and possibly other effects as well.

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