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Changes in the relative electrophoretic mobility of higher plant tubulin subunits in SDS-polyacrylamide gels

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The relative electrophoretic mobility of higher plant tubulin subunits in SDS-polyacrylamide gels varies depending upon the electrophoretic methods used to separate them. When reduced and alkylated rat and carrot tubulin heterodimers were separated by one method, the α-tubulin subunits, identified by means of a highly specific antibody, migrated more slowly than the β-tubulin subunits. However, when separated by another method, the carrot α-subunit migrated more rapidly than its β-subunit, while the relative mobility of rat brain tubulin subunits was unchanged. The two gel systems differ principally in the pH of the separating gel during electrophoresis, suggesting that pH markedly influences the interaction of SDS with the plant, but not the vertebrate α-tubulin.

Introduction

Tubulin is a heterodimeric protein that is composed of subunits, designated α- and β-, which can be separated by acrylamide gel electrophoresis under denaturing conditions [1–3]. The amino acid sequence of the tubulins has been conserved during evolution to a remarkable degree [4]. Nevertheless, some important biochemical differences have been noted in the tubulins isolated from higher plants and vertebrates. Plant microtubules in vivo are less sensitive to the tubulin-binding drug, colchicine, than are animal microtubules [5]. This is due to the reduced affinity of plant tubulins for colchicine [6,7]. Plant and animal tubulins also differ in the number and size of the peptides produced upon proteolytic cleavage, with the α-tubulins showing more differences than the β-tubulins [8,9].

Of particular importance to this work reported here is the observation that plant and vertebrate tubulins differ in the rate of migration of their α-tubulin subunits in denaturing gel systems. Independent studies have shown that the α-subunit from a fern, the alga *Chlamydomonas* and several species of higher plants migrates more rapidly than vertebrate α-tubulin, and therefore exhibits a lower apparent molecular weight. However, these studies found that the plant α-tubulin continues to migrate more slowly than the β-tubulin, as is the case with vertebrate tubulins [9]. In contrast, other studies have reported that the plant α-tubulin not only migrates faster than the vertebrate α-tubulin, but also faster than the plant β-tubulin

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[10–2]. An α-tubulin subunit that migrates faster than the β-tubulin subunit also has been observed after the electrophoretic separation of the tubulins of a number of microorganisms [13–15].

This so-called 'flip' in the migration of tubulin subunits in denaturing gels has resulteld in some confusion as to the identity of α- and β-tubulins in higher plants. Clearly their identity cannot be ascertained on the basis of relative electrophoretic migration characteristics alone. More importantly, these studies suggest that plant α-tubulins may have a substantially lower molecular weight than the α-tubulins of vertebrates and actually maybe smaller than plant β-tubulins.

The present study, however, demonstrates that, depending upon electrophoretic conditions, plant tubulins show a shift in the relative migration of their α- and β-subunits. The relative migration of rat brain tubulin subunits was the same under all conditions examined. The ability to manipulate the relative migration rates of higher plant α-tubulins demonstrates that the separation of the tubulin subunits is not due solely to a divergence in their molecular weight, and it is a further indication that there may be an important difference in the structure of plant and animal α-tubulins.

Materials and Methods

Tubulin isolation. Suspension cultures of carrot cells (*Daucus carota*, line W001C) were cultured as described [16]. Tubulin heterodimers were isolated from the cultured cells using three somewhat different procedures [6,11,17]. Each of these methods is based on DEAE-cellulose ion-exchange chromatography to bind selectively and separate the acidic tubulin dimer from other soluble proteins. They differ mainly in the number and amounts of proteinase inhibitors added to the homogenization buffer. The α- and β-tubulin monomers were isolated from the purified dimers by means of preparative gel electrophoresis [18,19]. The subunits were visualized with Coomassie Blue, electroeluted and concentrated using Centricon units (Amicon Corp, Danvers, MA). Tubulins were reduced with dithiothreitol and alkylated with either iodoacetamide or iodoacetate according to the method of Lane [20].

Electrophoresis and immunoblot analysis of tubulins. Two sodium dodecyl sulfate (SDS)-polyacrylamide slab gel systems were used. One was a modification of the Laemmli [19] procedure, while the other was a modification of the Studier [18] system. The stacking gels contained 0.1% SDS, 2 mM EDTA and pH 6.8 Tris buffer in either case, but the Studier stacking gels contained 64 mM Tris, while the Laemmli stacking gels contained 62 mM Tris. The Laemmli separating gels contained 375 mM Tris, pH 8.6, with 0.1% SDS, and 2 mM EDTA, while the Studier separating gels contained 150 mM Tris, pH 8.4, with 0.1% SDS and 2 mM EDTA. The running buffer employed in the Studier system contained 50 mM Tris, pH 8.3, 380 mM glycine, 0.1% SDS and 2 mM EDTA, while the Laemmli running buffer contained 24 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS and 2 mM EDTA. All gels contained 0.065% *N,N,N′,N′*-tetramethylethylenediamine, 0.14% ammonium persulfate, and acrylamide/bisacrylamide in the ratio 30:0.8. With either gel system, the sample buffer contained 2% SDS, 5 mM EDTA, 5 mM dithiothreitol, 10% glycerol and 0.001% bromphenol blue, but the Studier sample buffer contained 50 mM Tris, pH 6.8, while the Laemmli sample buffer contained 62 mM Tris, pH 6.8. The separating and stacking gels were poured on the same day and placed at 4°C overnight. They were used the following morning for electrophoretic separations conducted at room temperature without cooling. The samples separated on gels prepared by the Laemmli procedure were run at constant current (20 mA), while samples separated on gels prepared by the method of Studier were electrophoresed at constant voltage (90 volts). Following electrophoresis, the proteins were silver stained [21]. For immunodetection, the proteins, separated by electrophoresis, were transferred to nitrocellulose membranes [22]. The membranes with adsorbed tubulins were air-dried, blocked with 3% bovine serum albumin in phosphate-buffered saline (10 mM sodium phosphate and 0.9% NaCl, pH 7.2) and reacted either with a polyclonal antibody, raised against taxol-assembled soybean microtubules, which reacts to the α-subunit of carrot and rat brain tubulins, or to a commercially available monoclonal antibody which is β-tubulin specific (Amersham, Arlington

Heights, IL). The polyclonal antibody was localized using iodinated protein A, while the binding of the monoclonal antibody was detected with secondary antibody linked to alkaline phosphatase [23]. Unless otherwise noted, reagents were purchased from Bio-Rad (Richmond, CA).

Results

Electrophoretic migration of plant tubulin subunits was not influenced by the tubulin heterodimer isolation method

Carrot tubulin heterodimers were isolated by one of three different methods and electrophoretically separated with the Studier gel system [18]. The method of isolation did not affect the electrophoretic mobility of the carrot tubulin subunits. In each case the tubulin subunits nearly comigrated with those of rat brain tubulin (Fig. 1, panel I). Immunoblot analysis of these proteins, using a specific anti-α-tubulin polyclonal antibody, visualized only one reactive band in each of the three tubulin preparations (Fig. 1, panel II). The immunoreactive protein exhibited an M_r of 56 000, suggesting that proteolysis of the α-tubulin was negligible in the three preparations. No immunoreactive lower molecular weight bands were observed in any of the samples after the blots were reacted with the anti-α-tubulin antibodies.

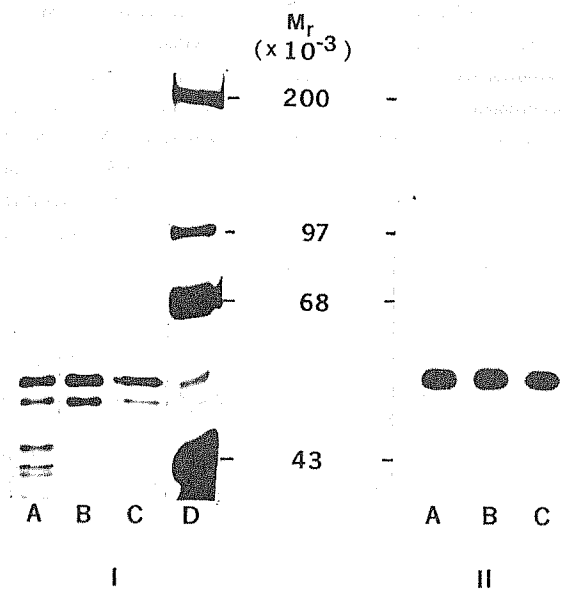

Fig. 1. The electrophoretic mobility of carrot tubulins subunits. Carrot tubulin heterodimers were isolated by one of three different methods and subjected to electrophoresis using a modified Studier gel system. Lane A, tubulins isolated by the method of Cyr et al. [17] lane B, tubulins isolated according to the method of Dawson and Lloyd [11]; lane C, tubulins isolated according to the method of Morejohn et al. [6]; lane D, molecular weight markers, including rat brain tubulin. Panel I illustrates the silver stained gel. Panel II illustrates the same samples which were transferred to nitrocellulose after electrophoretic separation and reacted with a polyclonal antibody which recognizes only α-tubulins. Iodinated protein A was used to detect antibody binding.

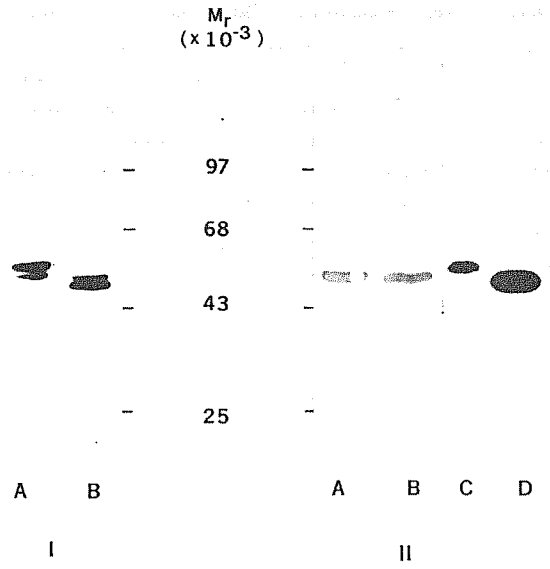

Fig. 2. The relative electrophoretic mobility of carrot α- and β-tubulins can be experimentally manipulated. Carrot tubulins (lane B), isolated according to the method of Dawson and Lloyd [11], and rat brain tubulins (lane A) were electrophoretically separated on gels prepared by the method of Laemmli [19]. In panel I, the proteins have been silver stained. In panel II, the proteins were transferred to nitrocellulose filters after electrophoresis and either reacted to a β-tubulin-specific monoclonal antibody, where antibody binding was detected with an alkaline phosphatase-linked secondary antibody (lanes A and B), or the blot was reacted with a polyclonal antibody which recognizes rat and carrot α-tubulin, and binding was detected with iodinated protein A (lanes C and D). The same filter was used for both immunolocalizations to assure accurate alignment. Lanes A and C contained rat brain tubulin, while lanes B and D contained carrot tubulin.

The electrophoretic migration of tubulin subunits as a function of the gel system

Purified tubulin heterodimers, isolated by the method of Dawson and Lloyd [11], were separated with the Laemmli gel system. The Laemmli gel system did not produce as much separation of the α- and β-tubulin subunits of either carrot or rat as did the Studier gel system. Under these conditions, the carrot tubulin monomers migrated with apparent $M_r = 55\,000$ and 54000, while the rat brain tubulin subunits migrated with apparent M_r 57000 and 55000 (Fig. 2).

The electrophoretically separated subunits were blotted to nitrocellulose and reacted first with the β-tubulin-specific monoclonal antibody, and then with the α-tubulin-specific polyclonal antiserum. The rat brain subunit with an apparent M_r of 55000 and the carrot subunit with an apparent M_r of 55000 reacted with the anti-β-tubulin antibody (Fig. 2, panel II, lanes A and B, respectively), while the α-tubulin-specific antiserum reacted with the M_r 57000 rat subunit and the M_r 54000 carrot subunit (Fig. 2, panel II, lanes C and D, respectively)

Carrot α- and β-tubulin subunits were separated by Studier preparative gels and re-electrophoresed in either a Laemmli (Fig. 3) or a Studier (Fig. 4) analytical gel to see whether the gel system alone was responsible for the shift in carrot α-tubulin migration. The relative position of the carrot α- and β-tubulins again were found to shift depending upon the gel system used. In both gel systems the carrot β-tubulin nearly co-migrated with the rat brain β-subunit. However, in the Laemmli gel system the carrot α-tubulin migrated faster than either the carrot or rat β-tubulin (Fig. 3), while in the Studier system the carrot α-tubulin migrated more slowly than the carrot β-tubulin,

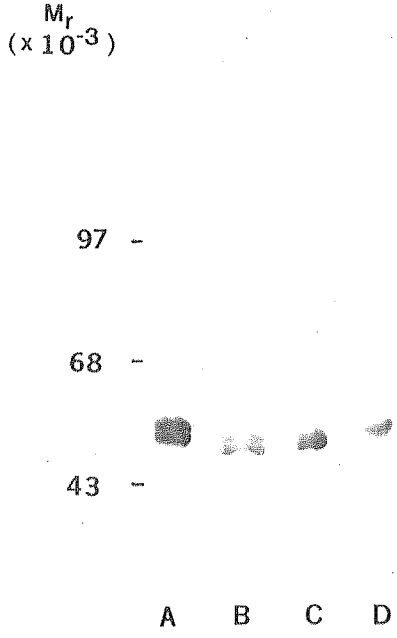

Fig. 3. The relative mobility of carrot α- and β-tubulins, isolated in the Studier gel system, after re-electrophoresis in Laemmli gels. Carrot tubulins, isolated according to Dawson and Lloyd [11], were electrophoresed on a preparative Studier gel and the monomers were excised and electroeluted. The monomers were then re-electrophoresed on a Laemmli gel. Lane A, rat brain tubulin dimers; lane B, carrot tubulin dimers; lane C, carrot α-tubulin; lane D, carrot β-tubulin. The gel was silver stained.

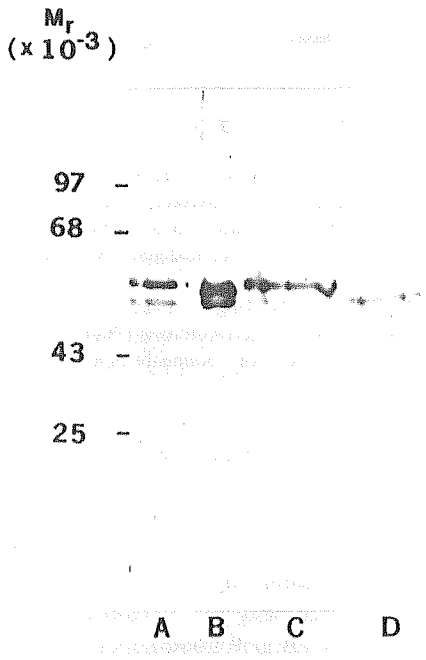

Fig. 4. Carrot tubulin monomers were isolated by a Studier preparative gel and re-electrophoresed in another Studier gel. Carrot tubulins, isolated according to Dawson and Lloyd [11], were electrophoresed on a preparative Studier gel and the monomers were excised and electroeluted. The monomers were then re-electrophoresed on a Studier gel. Lane A, rat brain tubulin dimers; lane B, carrot tubulin dimers; lane C, carrot α-tubulin; lane D, carrot β-tubulin. The gel was silver stained.

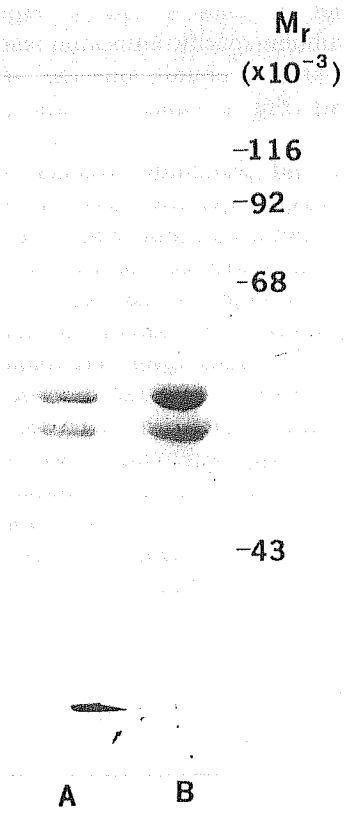

Fig. 5. The relative mobilities of carrot α- and β-tubulins in the Studier gel system are not altered by previous electrophoresis in the Laemmli gel system. Carrot tubulins were electrophoresed on a preparative Laemmli gel. The heterodimer was excised after Coomassie Blue staining and re-electrophoresed on a Studier gel (lane B) along with carrot tubulin which had never been subjected to electrophoresis (lane A). The gel was stained with Coomassie Blue.

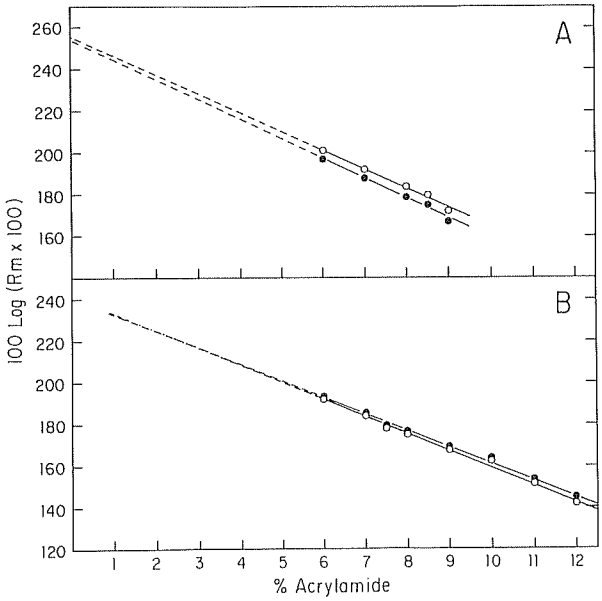

Fig. 6. The electrophoretic mobility of carrot tubulins as a function of the percentage acrylamide in the separating gel. Electrophoresis was performed in either the Studier (A) or Laemmli (B) gel systems. The migration of the α- (●) and β- (○) subunits were determined relative to the bromphenol blue dye front. The data were plotted according to Hedrick and Smith [23] and the lines were fitted to the data points using a least-squares method.

although somewhat faster than the rat α-tubulin (Fig. 4).

Conversely, carrot tubulin dimers were separated by preparative gel electrophoresis using the Laemmli [19] system and then re-electrophoresed in a Studier analytical gel. Because the Laemmli gel system does not give good separation of the α- and β-tubulin, both subunits were excised together from the Laemmli gel and re-electrophoresed in a Studier gel, along with freshly isolated carrot tubulin. As shown by the data in Fig. 5, electrophoresis in the Laemmli gel system did not alter irreversibly the relative electrophoretic behavior of the carrot α-tubulin. The carrot tubulin previously subjected to electrophoresis in the Laemmli gel system migrated as did freshly isolated carrot tubulin in the Studier gels.

The pH of the Studier and Laemmli separating gels initially was different, and this difference became magnified during electrophoresis. The initial pH of the stacking gels prepared according to Studier [18] and Laemmli [19] were identical (pH = 6.8), but the separating gels differed by 0.2 pH units (pH = 8.6 for the Laemmli gels and pH = 8.4 for the Studier gels). The Studier system also was more heavily buffered than the Laemmli gel (the electrode buffers are 50 mM Tris vs. 24 mM Tris, respectively). After electrophoretic separation, the pH of the two separatory gels became more divergent. The Laemmli separatory gel had a pH of 9.2 at the completion of electrophoresis while the Studier separating gel had a pH of 8.6.

Carrot tubulins also were separated in different percentage acrylamide gels, prepared according to Studier [18] or Laemmli [19]. The relative mobili-

ties of the carrot tubulin subunits were determined under these conditions and the data were plotted according to Hedrick and Smith [23]. The plots of the log of the subunit mobility against acrylamide concentration gave two nonparallel lines in either case. However, with the Studier gel system, the lines met near 0% gel concentration, while with the Laemmli gel system they intersected well before 0% acrylamide (Fig. 6). The Laemmli gel system did not give good separation of the carrot α- and β-tubulins at any percentage acrylamide.

Discussion

Electrophoresis in SDS-polyacrylamide gels has been shown to yield a reliable estimate of the molecular weight of many proteins [25–27]. However, tubulin subunit migration in SDS-polyacrylamide gels is somewhat anomalous. Although direct protein sequencing has shown that vertebrate brain α- and β-tubulins both have molecular weights near 50 000, they migrate in SDS gels with apparent molecular weights of approximately 57 000 and 55 000, respectively [28,29]. In fact, Bryan and Wilson [1] demonstrated by means of Ferguson plots that sea urchin α- and β-tubulin were separated electrophoretically because of a charge, and not a molecular weight difference.

Tubulin subunit separation is affected by the type of SDS used to denature the proteins [13]. Presumably SDS varies in purity and these impurities alter the ability of the detergent to interact with the hydrophobic domains within the tubulin molecules. The detergent interacts with proteins to convert their native secondary structures to homogeneous rod-like configurations. Incomplete SDS-induced denaturation would therefore affect the average molecular cross-sectional presentation of the tubulins as they are sieved through the acryamide matrix. Differences in cross-sectional presentations of two proteins, even those with the same molecular weight, would result in different relative migration distances. Although we found that heating the carrot tubulins in sample buffer for 2, 5 or 10 min, or increasing the SDS concentration in the sample buffer to 4%, made no difference in their relative mobility, this does not rule out incomplete SDS interaction with the tubulins as the cause of this anomaly.

There are two differences between the Laemmli [19] and the Studier [18] gel systems. First, the Studier gels are run at constant voltage while the Laemmli gels are run at constant current. Secondly, the pH of the separating gels initially is different and this difference becomes magnified during electrophoresis, in part because the Studier system is more heavily buffered. A Studier gel run at constant voltage experiences a decrease in current flow (and therefore a decrease in the net ionic flux) as ions leave the gel over the course of an electrophoretic run. Conversely, a Laemmli gel, run at constant current, experiences no net change in ionic flux during electrophoresis. The combination of a lower buffering capacity and a greater amount of ionic movement in the Laemmli gel system act together to bring about a more drastic change in pH than that experienced by the Studier gel system. Suprenant et al. [30] demonstrated that the pH of the separating gel affects the separation of tubulin proteins.

These results suggest that the pH of the Laemmli gel favors a more complete interaction of SDS with the plant α-tubulin so that it migrates faster in this system. The observation that the relative mobility of plant α-tubulins can be experimentally manipulated, while animal tubulins cannot, suggests that the structure of these two related proteins has diverged. Furthermore, this interpretation implies that the Laemmli gel system may give a more accurate estimate of the molecular weight of the plant α-tubulin. It will be necessary to determine the molecular weight of the plant tubulins by an independent means before any conclusions can be made about this. However, it is interesting to note that in *Chlamydomonas*, where the α- and β-tubulin molecular weights have been deduced by sequencing full length cDNAs encoding the proteins, the α-tubulin has a slightly smaller molecular weight than the β-subunit [31,32].

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