The EMBO Journal vol.4 no.9 pp.2397–2402, 1985

Tubulin domains responsible for assembly of dimers and protofilaments

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Communicated by K.C.Holmes

The protein domains responsible for the dimerization and polymerization of tubulin have been determined using chemical cross-linking and limited proteolysis. The intra-dimer bond is formed by the N-terminal domain of α-tubulin and the C-terminal domain of β-tubulin. Conversely, the inter-dimer bond along protofilaments is formed by the N-terminal domain of β-tubulin (carrying the exchangeable GTP) and the C-terminal domain of α-tubulin. The domains of proteolytically cleaved tubulin remain tightly associated in solution. Apart from the monomer, tubulin shows three levels of assembly: the dimer, oligomer and polymer. Several oligomeric species can be visualized by electron microscopy of rotary shadowed phosphocellulose-tubulin, h.p.l.c. and non-denaturing gel electrophoresis. Tubulin's capacity to form the higher level aggregates is not destroyed by enzymatic nicking.

Key words: tubulin/domain structure/oligomers/cross-linking/limited proteolysis

Introduction

Microtubules are built from globular protein subunits, tubulin. The building block is a heterodimer consisting of α- and β-tubulin, each of mol. wt. 50 kd and of similar structure. Microtubules consist of 13 longitudinal protofilaments, each of which is built from an alternating sequence of α- and β-tubulin. All monomers have the same polarity within a microtubule. This means that the two monomers are combined in a head-to-tail fashion, when forming the dimer (for a review, see Dustin, 1984).

Each monomer binds one molecule of GTP. One of them does not exchange with GTP in solution and is therefore termed non-exchangeable (GTP_n). The other is exchangeable with depolymerized tubulin (GTP_e); it becomes non-exchangeable upon microtubule assembly and is hydrolyzed during this process (Jacobs *et al.*, 1974). Cross-linking studies have shown that GTP_e binds to β-tubulin, while GTP_n binds to α-tubulin (Geahlen and Haley, 1977; Hesse *et al.*, 1985; Nath *et al.*, 1985).

We have shown that both monomers may be subdivided into two major domains comprising roughly the N-terminal 3/5 and the C-terminal 2/5 of the molecule (Mandelkow *et al.*, 1985). The domains may be separated by limited proteolysis, using several proteases. In particular, trypsin cleaves α-tubulin at Arg 339 into a large and a small fragment (α_L, 36 kd, and α_S, 14 kd). Similarly chymotrypsin cleaves β-tubulin at Tyr 281 into two fragments (β_L, 30 kd, and β_S, 20 kd). In both cases the GTP binding sites are in the large N-terminal domains (i.e., GTP_e in β_L, GTP_n in α_L). There is another chymotryptic cleavage site in β-tubulin within 1 kd of the C terminus. Thus the 20 kd fragment of β-tubulin does not contain the C terminus itself.

The head-to-tail interaction between subunits along protofilaments suggests the possibility that the intra-dimer bond is formed by two complementary domains, i.e., α_S-β_L or α_L-β_S. By the same argument, the inter-dimer bond responsible for the elongation of protofilaments could also be formed by complementary domains. In this paper we address this question by chemical cross-linking studies and describe which of the domains are responsible for the interactions between monomers and dimers. Moreover, we show that there is a tight binding between domains that is not disrupted by enzymatic nicking. Thus, cleavage at the domain boundary does not destroy tubulin's capacity to form microtubules and different oligomeric species observed by several methods.

Results

Cross-linking of tubulin monomers and their domains

The idea of the experiment to be described is the following. We first cleave β-tubulin by limited digestion with chymotrypsin. Cleavage by itself does not separate the domains from each other (see below), but they can be separated by subsequent SDS-gel electrophoresis. After nicking the protein it is incubated with a cross-linker. The nicked and cross-linked protein is then separated in SDS-gels. If the cross-link occurs at the interface between α- and β-tubulin, the SDS-gel should show intact α-tubulin coupled to the interacting domain of β-tubulin.

After testing several cross-linkers the clearest results were obtained with the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). This water-soluble carbodiimide activates mainly carboxyl residues which can form amide linkages with amino groups of lysines (Mornet *et al.*, 1981; Wu-Chou *et al.*, 1984). Figure 1 shows an SDS-gel of phosphocellulose-tubulin (PC-tubulin) digested with chymotrypsin and then cross-linked with EDC for increasing times. Lane 1 shows the uncleaved and uncross-linked purified tubulin which is resolved into the two bands of α- and β-tubulin. Lane 2 shows the limited digestion of β-tubulin. Besides α- and β-tubulin, one observes the two cleavage products of β-tubulin, β_L (30 kd) and β_S (20 kd). Lanes 3–4 show the cross-linking after incubation with EDC for 60 and 210 min, respectively. Two new bands are seen. The top band at 100 kd corresponds to the cross-linked intact heterodimer. The new band at 70 kd increases with a concomitant decrease of the small 20-kd fragment. By contrast, the large fragment at 30 kd stays roughly constant. This suggest that the small C-terminal domain of β-tubulin is cross-linked to α-tubulin forming the 70 kd complex. (Note that the cross-linking occurs not only between monomers but also within them, leading to altered mobilities. The bands of α- and β-tubulin are less well resolved, and the band of the β_L domain is split.)

Identification of cross-linked domains by domain-specific antibodies

The interacting domains may be identified directly by a domain-specific monoclonal antibody against β-tubulin. In Figure 2a we see SDS-gels of chymotryptically cleaved tubulin before (lane 1)

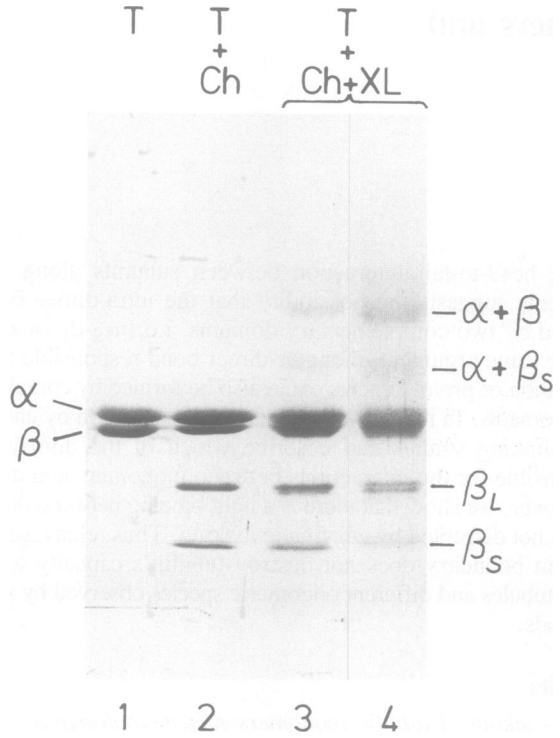

Fig. 1. SDS-urea gradient gel (5–20% acrylamide, 1–8 M urea) of PC-tubulin (**lane 1**), PC-tubulin nicked by chymotrypsin (**lane 2**), and PC-tubulin nicked by chymotrypsin and cross-linked by 10 mM EDC at 15°C for 60 min (**lane 3**) and 210 min (**lane 4**). **Lane 1** shows α- and β-tubulin, **lanes 2–4** show the fragments due to digestion (30 kd = β_L, 20 kd = β_S), **lanes 3–4** show higher mol. wt. products of cross-linking (70 kd = α + β_S, 100 kd = α + β). T = tubulin, Ch = chymotrypsin, XL = crosslinker.

and after cross-linking (lane 2) and, as a control, the cross-linked PC-tubulin dimer (lane 3). Figure 2b and c shows the corresponding immunoblots with monoclonal antibodies against α- and β-tubulin. The β-monoclonal antibody recognizes specifically the small domain of β-tubulin (Figure 2c, lane 7) since it labels β-tubulin and the small domain, but not the large one. (In this respect the antibody is similar to the polyclonal β-specific antibody used in our earlier study.) In the cross-linked protein the antibody labels β-tubulin (50 kd), the intact α-β-heterodimer (100 kd, top band) and the intermediate band at 70 kd (lane 8). This confirms the previous conclusion that this band is composed of α-tubulin and the C-terminal domain of β-tubulin. Similarly, the α-specific antibody stains intact α-tubulin (and no fragments since α-tubulin is fairly resistant to chymotrypsin under these conditions). It also stains the intact heterodimer and the 70-kd complex of α-tubulin and the C-terminal fragment of β-tubulin (lane 5).

An analogous experiment was performed with limited tryptic digestion. This mainly results in the cleavage of α-tubulin. One observes cross-linking of β-tubulin with the large N-terminal domain of α-tubulin, with the concomitant disappearance of the large fragment in the SDS gel (data not shown). However, the interpretation is not as straightforward as in the previous case. The new cross-linked species has a mol. wt. of 50 + 36 = 86 kd that is not well resolved from the cross-linked intact dimer. Moreover, the small fragments tend to produce unspecific aggregates which overlap with the digestion pattern (see Mandelkow *et al.*, 1985); this is particularly pronounced with α-tubulin.

Association of domains in tubulin monomers and dimers

The fragments produced by limited proteolysis remain tightly associated in most buffer conditions. This can be shown in several

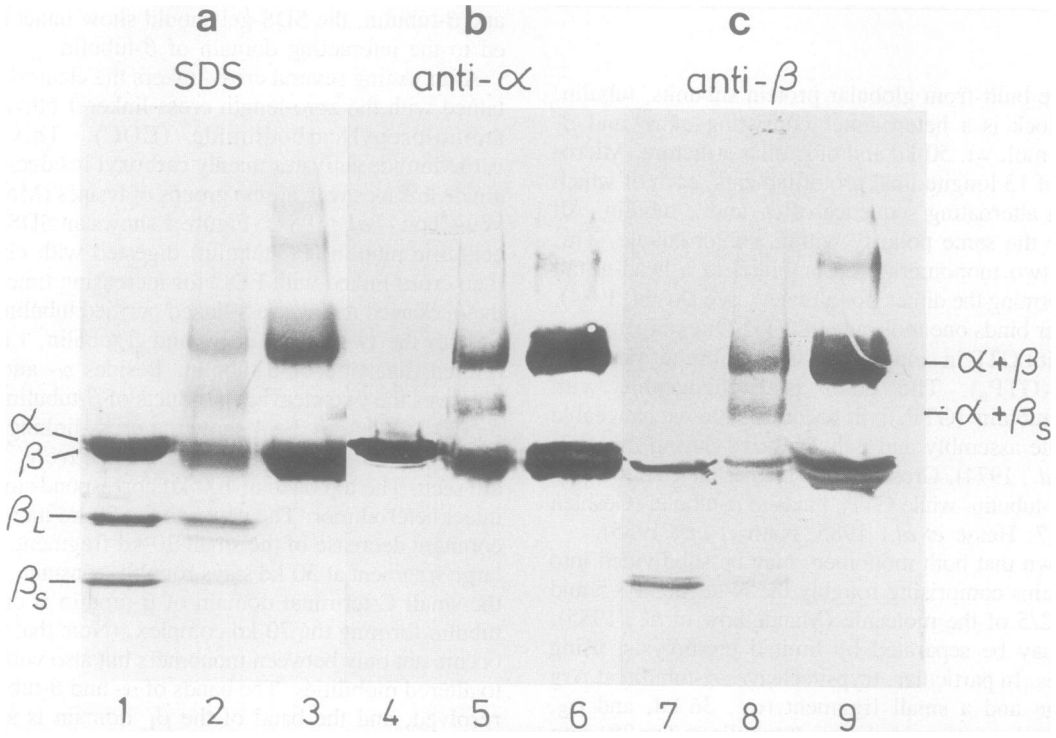

Fig. 2. SDS gradient gel (4–20% acrylamide) of nicked and cross-linked PC-tubulin, and immunoblots with monoclonal antibodies against α- and β-tubulin. **(a) Lane 1**, PC-tubulin briefly digested by chymotrypsin; **lane 2**, digested protein cross-linked with EDC; **lane 3**, cross-linking of undigested protein. **(b)** Same samples as in **(a)**, blotted with α-monoclonal antibody (**lanes 4–6**). No fragments are labeled since chymotrypsin does not attack α-tubulin under the conditions used. The bands labeled in **lane 5** correspond to α-tubulin, α + β_S (70 kd), and α + β (cross-linked heterodimer, 100 kd). **(c)** Same samples as in **(a)**, blotted with β-monoclonal antibody (**lanes 7–9**). **Lane 7** shows that the antibody is specific for the β_S domain at 20 kd. This band disappears with cross-linking, and a new one at 70 kd appears (**lane 8**). It contains the β_S domain since it is labeled by the antibody, indicating that the intra-dimer bond is formed by α-tubulin and the small domain of β-tubulin.

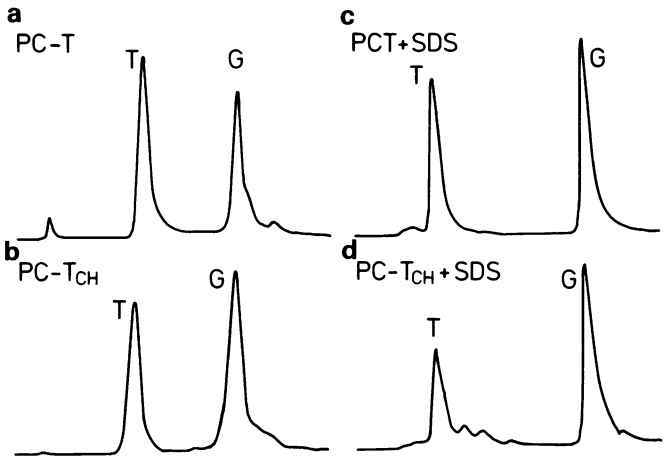

Fig. 3. H.p.l.c. chromatograms of PC-tubulin and its chymotryptic digests with or without SDS. Column TSK 3000 SV (0.75 × 50 cm), running buffer 0.1 M Pipes pH 6.9, 0.4 M NaCl, 5% acetonitrile, 25% glycerol (**a** and **b**) or 0.1% SDS (**c** and **d**), 1 mM each of $MgSO_4$, EGTA and DTT, 20°C, injection volume 10 μl. Retention times are given in brackets. (**a**) PC-tubulin, 12 mg/ml. The two major peaks are tubulin dimers (26.97, left) and GTP (37.31, right). The small peak on the left (16.76) is due to higher aggregates in the breakthrough volume. (**b**) PC-tubulin, 12 mg/ml, digested with 20 μg/ml of chymotrypsin for 60 min at 15°C and chromatographed immediately. The major peaks of tubulin dimers (26.68) and GTP (37.18) are the same as in (**a**). No aggregates in the breakthrough are observed. (**c**) PC-tubulin as in (**a**), with 0.1% SDS. Major peaks are tubulin monomers (20.55, left) and GTP (37.02, right), with aggregates just before the monomer peak (17.80). (**d**) PC-tubulin digested as in (**b**), with 0.1% SDS. One observes the main peaks of monomers (20.34) and GTP (36.84), as well as the two chymotryptic fragments of β-tubulin (23.17, 25.14). The figure shows that the enzymatic fragments are dissociated only in denaturing conditions (compare **b** and **d**).

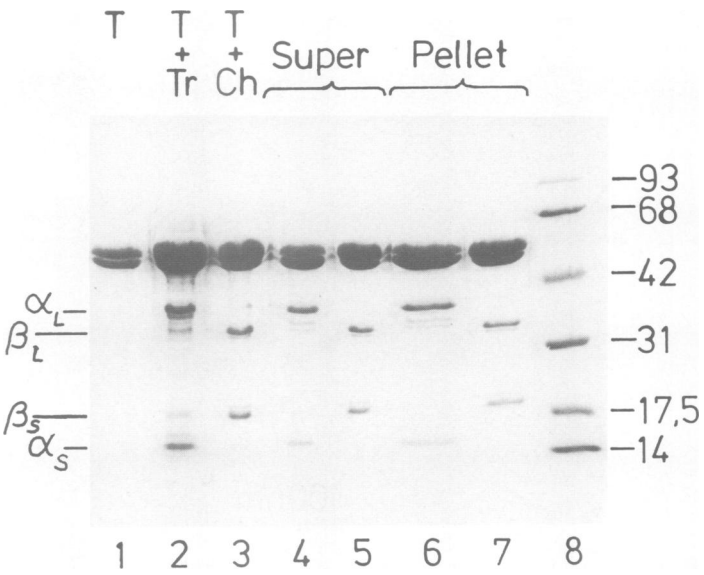

Fig. 4. SDS gel (12.5%) showing assembly of digested tubulin. **Lane 1**, PC-tubulin, **lanes 2** and **3**, PC-tubulin digested by trypsin and chymotrypsin. This protein was subjected to a cycle of temperature-dependent assembly and disassembly. **Lanes 4, 5**, supernatant of tryptic and chymotryptic digests after assembly corresponding to **lanes 2** and **3**. **Lanes 6, 7**, pellets of digested and assembled protein. Comparison between **lanes 4, 5** and **6, 7** shows that about half of the protein co-assembled through one cycle, and that the fractions of digested and undigested protein is roughly the same in the pellet and in the supernatant. **Lane 8**, marker proteins.

ways. When the nicked protein is run over a molecular sieve column (Sepharose 4B) in standard Pipes reassembly buffer it elutes as the tubulin dimer (or higher aggregates, see below). The same is observed with ion-exchange chromatography (DEAE-Sepharose) and elution with a salt gradient (NaCl or KCl). When the chymotryptic digest is applied to a DEAE column and eluted with a gradient of 0−3 M guanidinium hydrochloride (GuHCl) in Pipes reassembly buffer part of the N-terminal fragment of β-tubulin runs slightly ahead of the rest of the protein, as shown by subsequent SDS-electrophoresis of the fractions (not shown). This is in agreement with the cross-linking studies (Figure 2) showing that the β_L domain can be separated from the remainder of the dimer. However, because of aggregation, this method is not suitable for the purification of fragments.

The association of fragments was further investigated by h.p.l.c. on a TSK 3000 molecular sieve column. A series of conditions was tested, including Pipes, Tris, or MES buffers, pH 6.7−6.9, up to 0.5 M NaCl or KCl, up to 2 mM Ca^{2+}.

The fragments could be separated only in denaturing conditions such as 0.1% SDS. As an example, Figure 3a shows the elution profile of PC-tubulin as a control (with main peaks corresponding to the tubulin dimer and GTP), and Figure 3b shows the same protein after limited chymotryptic cleavage (again showing only the dimer and GTP peaks because no separation of fragments occurs). Figure 3c and d shows corresponding samples denatured in 0.1% SDS. In the case of PC-tubulin the major peaks are those of the tubulin monomer and GTP (Figure 3c); whereas after chymotryptic digestion one also observes intermediate bands corresponding to the fragments of β-tubulin (Figure 3d). Some higher aggregates eluting before the tubulin dimer or monomer fraction are seen as well, particularly with undigested protein (Figure 3a, c). These experiments show that the nicked tubulin retains its capability of forming monomers, dimers, and some oligomers.

Co-assembly of nicked tubulin into microtubules and oligomers

Having established that the nicked protein remains associated we asked whether it is still able to assemble. This was checked by the following experiment. PC-tubulin was nicked by trypsin or chymotrypsin (~50−70%), the protease was inhibited by phenylmethylsulfonyl fluoride (PMSF), and the protein was carried through one cycle of temperature-induced assembly and disassembly. Similar fractions of nicked protein were observed before and after assembly (Figure 4). This agrees with the results of Brown and Erickson (1983) and Maccioni and Seeds (1983) obtained in different buffer systems.

We then asked whether co-assembly of nicked tubulin occurs also with assembly forms smaller than microtubules. In particular, a fraction of tubulin occurs in the form of oligomers smaller than the well-known rings present in cold microtubule protein. The oligomers seem to be important in assembly and have been seen by X-ray scattering both in microtubule protein with microtubule-associated proteins (MAPs) and with purified PC-tubulin (Mandelkow *et al.*, 1980; Bordas *et al.*, 1983).

Oligomers formed from intact or nicked PC-tubulin can be demonstrated directly by electron microscopy. The sample shown in Figure 5 was obtained by carrying the protein through one assembly and disassembly in 25% glycerol-reassembly buffer, then spraying it onto freshly cleaved mica, vacuum-drying, and rotary shadowing.

Oligomeric species are also detected when running PC-tubulin on non-denaturing gels (Lee *et al.*, 1973; Kravit *et al.*, 1984; Correia and Williams, 1985). In our native gel system we observe several bands corresponding to the monomer, dimer, trimer, etc.

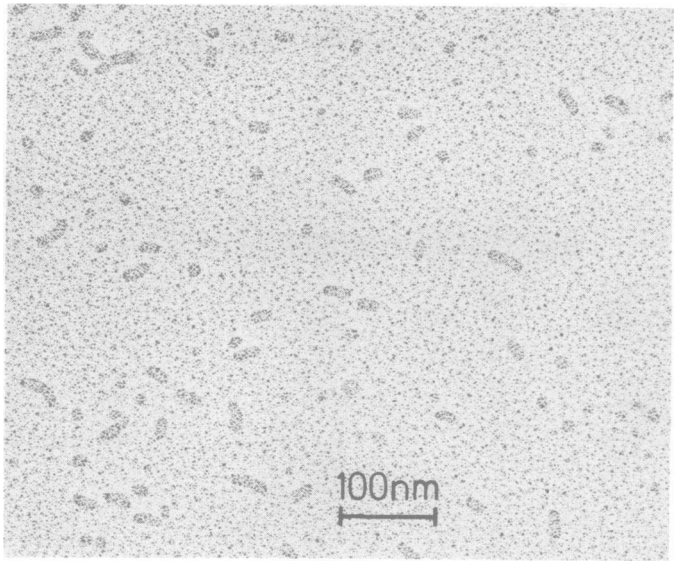

Fig. 5. Oligomers of PC-tubulin visualized by glycerol spray and rotary shadowing. The protein was polymerized at 10 mg/ml in reassembly buffer with 25% glycerol, then depolymerized for 10 min on ice, diluted 1:9 in 0.1 M NH_4-acetate reassembly buffer, 50% glycerol, and sprayed on freshly cleaved mica at 4°C. Similar images are obtained after partial proteolysis (>50%) of the protein.

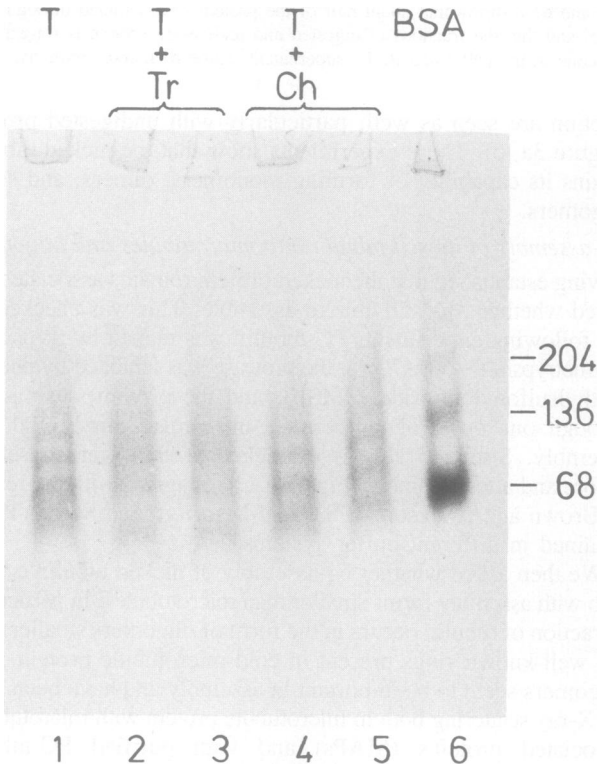

Fig. 6. Non-denaturing gradient gel (2 – 10% polyacrylamide, gel buffer Tris-HCl, pH 7.5; reservoir buffer diethylbarbituric acid, pH 7.0). **Lane 1**, control of undigested PC-tubulin; **lanes 2** and **3**, limited digestion with trypsin (about 60% of α-tubulin nicked); **lanes 4** and **5**, limited digestion with chymotrypsin (about 70% of β-tubulin nicked); **lane 6**, marker of bovine serum albumin containing monomers, dimers and trimers. Enzymatic nicking does not disrupt the interactions between the domains so that fragments smaller than tubulin monomers are not observed.

of tubulin (Figure 6, lane 1). A similar pattern is seen after limited tryptic (lanes 2, 3) or chymotryptic digestion (lanes 4, 5). The capacity of forming the oligomers is preserved even though a when more than half of the protein is nicked. This means that the cleaved protein is still capable of forming oligomers. It appears that the bonds which stabilize the tubulin monomers, dimers, oligomers or polymers (microtubules) are strong enough to compensate for the cleavage of the polypeptide chain at one site.

Discussion

Domains forming the intra-dimer and inter-dimer bonds

The results described above represent a step towards a molecular understanding of tubulin and microtubules. Earlier studies by several authors have shown that microtubules consist of polar dimers, and that the two monomers have the same polarities. The non-exchangeable GTP_n is on α-tubulin, the exchangeable one, GTP_e, on β-tubulin (Geahlen and Haley, 1977; Nath *et al.*, 1985; Hesse *et al.*, 1985); the latter becomes non-exchangeable and is hydrolysed during microtubule assembly. Thus it would be plausible to conclude that the GTP_e site is part of the bond(s) that is responsible for microtubule assembly. Alternatively, GTP binding and hydrolysis might induce a conformational change which affects the bonds between subunits over some distance. This question has been addressed in the present study.

The subunits of microtubules have longitudinal bonds along the protofilaments and lateral ones between protofilaments. Structural studies have shown that the tubulin dimer is oriented along the protofilaments, i.e., the intra-dimer bond between α- and β-tubulin is longitudinal (for a review of the structure, see Amos, 1982). This implies that there is also a longitudinal inter-dimer bond between α- and β-monomers of axially adjacent dimers. These two types of longitudinal bonds appear to dominate the assembly properties of tubulin. This is evidenced by the fact that protofilaments form the basic feature of all polymorphic tubulin assembly forms, independently of how variable the lateral bonds are (compare, for example, microtubules with parallel protofilaments, duplex tubules with two sets of protofilaments crossing roughly at right angles, and zinc-induced sheets with pairs of anti-parallel protofilaments). Moreover, even smaller assembly forms such as tubulin rings and oligomers are composed of protofilaments (Mandelkow *et al.*, 1983). Thus it is very probable that the predominant intra- and inter-dimer bonds are both oriented along the protofilaments in an alternating fashion (although other possibilities are not strictly excluded).

We have shown (Mandelkow *et al.*, 1985) that both tubulin monomers consist of two major domains. They are cleavable by various proteases around residue 300. Thus the larger domain is N-terminal, the smaller one is C-terminal. Evidence that the domains are units in the functional sense comes from the observation that the sequence homologies related to nucleotide binding are all in the N-terminal domain. Since GTP_e is on β-tubulin this means that the exchangeable nucleotide is on the β_L domain. Conversely, the GTP_n is bound to the α_L domain.

There is very little information on the structure and arrangement of the domains within a tubulin monomer. They could lie side-by-side, on top of one another, inside-outside, etc. Thus, in principle, the intra-dimer bond could be formed by any combination of domains (e.g., α_L-β_S, α_S-β_L or bonds involving more than two domains). The experiments reported here (Figure 2) show that the intradimer bond is formed between the N-terminal domain of α-tubulin and the C-terminal domain of β-tubulin, i.e., α_L-β_S. This implies that the non-exchangeable GTP_n site is internal to the dimer. Using the structural arguments summarized above, the longitudinal inter-dimer bond is of the form α_S-β_L.

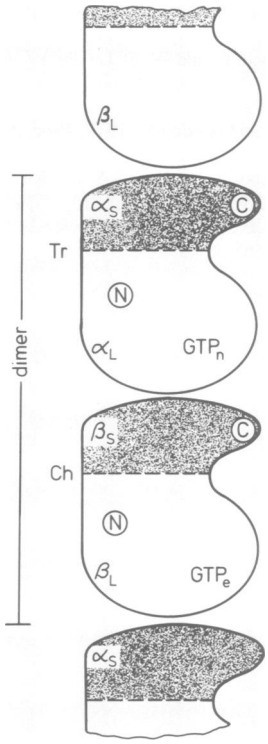

Fig. 7. Model of interactions between domains along a protofilament. The intra-dimer bond is formed by the domains α_L (containing the non-exchangeable GTP) and β_S. The inter-dimer bond is formed by the domains β_L (containing the exchangeable GTP or GDP) and α_S. The domains exposed at the two ends of a protofilament are α_S and β_L.

This shows that the exchangeable nucleotide site is indeed near the interface responsible for protofilament growth. These features are diagrammed in Figure 7. Growth is possible in both directions, either by addition of an α_S domain to the exposed β_L end of a protofilament carrying a GTP_e 'cap' (bottom of dimer model), or by addition of a GTP_e-carrying β_L domain to an exposed α_S end of a protofilament (top of dimer model). At this stage we do not know which of the two ends is the fast-growing one (plus-end).

Assembly of nicked tubulin into microtubules and oligomers

Although the boundary between the domains around residue 300 is susceptible to proteases, the integrity of the polypeptide backbone seems to be of secondary importance for the functional properties of tubulin. Thus, the nicked monomers and dimers remain associated in reassembly buffer and can be separated only in denaturing conditions. Similarly, the nicked protein can be carried through a cycle of assembly and disassembly in Pipes reassembly buffer, in agreement with observations by Brown and Erickson (1983) obtained in glutamate buffer. The nicked protein tends to form more microtubules with 14 rather than 13 protofilaments, as well as polymorphic forms (e.g., hooks, see Mandelkow and Mandelkow, 1979). This is particularly obvious after treatment with chymotrypsin. This enzyme cleaves not only at Tyr 281 but also very near the C terminus, thereby removing or modifying its negative charge. This may be responsible for the increase in polymorphism (Mandelkow *et al.*, 1985), a behavior that is analogous to that observed after enzymatic cleavage with subtilisin (Serrano *et al.*, 1984; Sackett *et al.*, 1985).

An interesting feature is the occurrence of oligomers of purified PC-tubulin. In the case of MAP-containing microtubule protein the importance of oligomers for microtubule assembly had been deduced previously from time-resolved X-ray studies. These structures may be regarded as short stretches of protofilaments, probably complexed with some MAP(s) (Mandelkow *et al.*, 1980, 1983). Subsequent scattering experiments showed that purified PC-tubulin contained oligomers as well (Bordas *et al.*, 1983), so that MAPs are not required for oligomer formation.

The presence of oligomeric species has also been detected by non-denaturing gel electrophoresis (Lee *et al.*, 1973; Kravit *et al.*, 1984; Correia and Williams, 1985). There is a debate as to whether the oligomers seen in native gels exist in solution or whether they are artefacts (Correia and Williams, 1985). Moreover, we do not know how the oligomers observed in native gels are related to those seen by X-ray scattering or electron microscopy. The question of oligomers is significant because of their potential involvement in microtubule nucleation and/or elongation. There may be different types of oligomes: some of them are in dynamic equilibrium with dimers and/or monomers and are thus assembly-competent, others may be artefactual aggregation products, induced, for example, by oxidation, ageing, etc. (Detrich and Williams, 1978).

In summary, enzymatic cleavage and cross-linking was used in this study to identify the domains responsible for the formation of dimers and protofilaments. The nicked protein appeared to be able to form the same assembly products as the intact protein, i.e., oligomers and polymers. Thus the integrity of the polypeptide chain near the domain boundary is only of secondary importance. This is probably due to extended bonding surfaces between the subunits, in analogy with many other oligomeric proteins.

Materials and methods

Protein preparation

Microtubule protein from porcine brain was prepared by two cycles of temperature-dependent assembly and disassembly in the presence of 25% glycerol, modified after Borisy *et al.* (1975). The reassembly buffer was 0.1 M Pipes pH 6.9, with 1 mM each of $MgSO_4$, EGTA, GTP and dithiothreitol (DTT). PC-tubulin was purified by phosphocellulose chromatography following Weingarten *et al.* (1975), with several modifications as described (Mandelkow *et al.*, 1985). This procedure yields up to 40 mg/ml of PC-tubulin from the column which was stored in liquid nitrogen.

Limited proteolysis

PC-tubulin in 0.1 M Pipes pH 6.9 or 0.1 M MES pH 6.5, 1 mM each of $MgSO_4$, EGTA and GTP, was digested at protein concentrations between 1 and 15 mg/ml, using trypsin (Sigma) or chymotrypsin (Sigma) at 15°C and an enzyme:protein molar ratio of 1:50 – 1:100. Proteolysis was stopped after 30 – 60 min by 2 mM PMSF. For analysis on native gels the digestion was performed at 4°C and at an enzyme:protein ratio of 1:10. Digestion was stopped after 5 – 10 min.

Cross-linking

Cross-linking with the zero-length cross-linker EDC (Serva) was performed according to Wu-Chou *et al.* (1984), with the following modifications: 1 mg/ml of enzymatically cleaved or uncleaved PC-tubulin in Pipes reassembly buffer was incubated with 10 mM EDC at 15°C (stock solution 100 mM in the same buffer, freshly prepared prior to use). The reaction was quenched at various times (10 – 240 min) by addition of an equal volume of SDS sample buffer, (0.063 M Tris/HCl pH 6.8, 5% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.001% bromophenol blue).

SDS-PAGE

We used 0.5 mm thick microslab polyacrylamide gels according to Matsudaira and Burgess (1978). SDS-PAGE was performed following Laemmli (1970), with modifications (Mandelkow *et al.*, 1985). Gels were either 12.5% acrylamide or had a linear gradient of 4 – 20% in the resolving gel and 4% acrylamide in the stacking gel. In some cases a co-linear urea gradient from 1 to 8 M was used.

PAGE under non-denaturing conditions

The gels were made of a linear 2 – 10% acrylamide gradient without stacking gel according to Blackshear (1984). Native conditions were maintained by using a resolving gel buffer of 0.57 M Tris/HCl, pH 7.5, and a reservoir buffer of

0.03 M diethylbarbituric acid/0.008 M Tris, pH 7. All assays and sample preparations for native gel electrophoresis were performed in the cold (4°C) immediately before the gel was run.

Immunoblotting

After PAGE, the proteins were electrophoretically transferred onto nitrocellulose sheets following Bittner *et al.* (1980) and Towbin *et al.* (1979), with modifications as follows. Electrophoresis was performed for 2 h at 4°C and 150–200 mA. The nitrocellulose sheets (Schleicher and Schüll BA 85, 45 μm) were soaked in 2% BSA/PBS for 1 h at 37°C and then incubated overnight at 4°C with monoclonal antibodies against α- or β-tubulin (Amersham, mouse ascites fluid) in 1:1000 to 1:2000 dilution in 2% BSA/PBS (1 ml antibody solution per 10 cm^2 sheet). After washing, the bound antibody was detected with a second antibody, conjugated to horse radish peroxidase (DAKO, rabbit IgG against mouse IgG). Incubation time of the antibody in 1:50 dilution in 2% BSA/PBS was 4 h at room temperature. The color reaction with chloronaphthol-containing solution was stopped after 1–2 min by rinsing the sheets in water.

H.p.l.c.

PC-tubulin and its proteolytic digests were analysed on a h.p.l.c. unit (LKB) fitted with a TSK 3000 SW column (0.75 cm × 50 cm) and a TSK-GSWP precolumn (0.75 cm × 7.5 cm). The running buffer was 0.1 M Pipes pH 6.9, 0.4 M NaCl, 5% acetonitrile, 25% glycerol or 0.1% SDS), 1 mM each of $MgSO_4$, EGTA and DTT, 20°C, injection volume 10 μl, flow-rate 0.7 ml/min. U.v. absorption was measured at 280 nm and recorded on a c-R1A Chromatopac plotter (Latek) with automatic recording of retention times. The column was calibrated with the marker proteins glutamate dehydrogenase (350 kd), lactate dehydrogenase (140 kd), enolase (85 kd) and adenylate kinase (22 kd).

Electron microscopy

PC-tubulin at 10 mg/ml in 0.1 M Pipes reassembly buffer, with 25% glycerol, was polymerized for 10 min at 37°C, then depolymerized for 10 min on ice, and diluted to 1 mg/ml in cold 0.1 M NH_4-acetate buffer (with 1 mM GTP, EGTA, DTT), 50% glycerol. The solution was then sprayed onto freshly cleaved mica following Elliott and Offer (1978), vacuum-dried in a Balzers BAE 080T recipient, rotary-shadowed with 2 nm Pt at an angle of 7° and backed with 20 nm carbon at 90°C. The replica was floated off on distilled water, picked up on 400 mesh grids, and observed in a Philips 400 T electron microscope at 46 000 magnification.

Acknowledgements

We thank Ursula Rühl for skilful technical assistance with the h.p.l.c. experiments and protein preparations, and Gabi Eulefeld for drawings and photography. We are especially indebted to Eckhard Mandelkow for stimulating discussions throughout this work. This work was supported in part by the Deutsche Forschungsgemeinschaft.

References

Amos,L.A. (1982) in Harris,J.R. (ed.), *Electron Microscopy of Proteins*, Vol. **3**, Academic Press, London, pp. 207-250.
Bittner,M., Kupferer,T. and Morris,C.F. (1980) *Anal. Biochem.*, **102**, 459-471.
Blackshear,P.J. (1984) *Methods Enzymol.*, **104**, 237-255.
Bordas,J., Mandelkow,E.-M. and Mandelkow,E. (1983) *J. Mol. Biol.*, **164**, 89-135.
Borisy,G.G., Marcum,J.M., Olmsted,J.B., Murphy,D.B. and Johnson,K.H. (1975) *Ann. N.Y. Acad. Sci.*, **253**, 107-132.
Brown,H.R. and Erickson,H.P. (1983) *Arch. Biochem. Biophys.*, **220**, 46-51.
Correia,J.J. and Williams,R.C. (1985) *Arch. Biochem. Biophys.*, **239**, 120-129.
Detrich,H.W. and Williams,R.C.,Jr. (1978) *Biochemistry (Wash.)*, **17**, 3900-3907.
Dustin,P. (1984) *Microtubules*, published by Springer Verlag, Heidelberg.
Elliott,A. and Offer,G. (1978) *J. Mol. Biol.*, **123**, 505-519.
Geahlen,R.L. and Haley,B.E. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4375-4377.
Hesse,J., Maruta,H. and Isenberg,G. (1985) *FEBS Lett.*, **179**, 91-95.
Jacobs,M., Smith,H. and Taylor,E.W. (1974) *J. Mol. Biol.*, **89**, 455-468.
Kravit,N.G., Regula,C.S. and Berlin,R.D. (1984) *J. Cell Biol.*, **99**, 188-198.
Laemmli,U.K. (1970), *Nature*, **227**, 680-685.
Lee,J.C., Frigon,R.P. and Timasheff,S.N. (1973) *J. Biol. Chem.*, **248**, 7253-7262.
Maccioni,R.B. and Seeds,N.W. (1983) *Biochemistry (Wash.)*, **22**, 1567-1572.
Mandelkow,E.-M. and Mandelkow,E. (1979) *J. Mol. Biol.*, **129**, 135-148.
Mandelkow,E., Harmsen,A., Mandelkow,E.-M. and Bordas,J. (1980) *Nature*, **287**, 595-599.
Mandelkow,E., Mandelkow,E.M. and Bordas,J. (1983) *J. Mol. Biol.*, **167**, 179-196.
Mandelkow,E.-M., Herrmann,M. and Rühl,U (1985) *J. Mol. Biol.*, **185**, 311-328.
Matsudaira,P.T. and Burgess,D.R. (1978) *Anal. Biochem.*, **87**, 386-396.
Mornet,D., Bertrand,R., Pantel,P., Audemard,E. and Kassab,R. (1981) *Nature*, **292**, 301-306.
Nath,J.P., Eagle,G.R. and Himes,R.H. (1985) *Biochemistry (Wash.)*, **24**, 1555-1560.
Sackett,D.L., Bhattacharyya,B. and Wolff,J. (1985) *J. Biol. Chem.*, **260**, 43-45.
Serrano,L., de la Torre,J., Maccioni,R.B. and Avila,J. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5989-5993.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.
Weingarten,M.D., Lockwood,A.H., Hwo,S.Y. and Kirschner,M.W. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 1858-1862.
Wu-Chou,S., Robinson,A.E., Hrabeta,E. and Packer,L. (1984) *Biochem. Biophys. Res. Commun.*, **124**, 565-571.

Received on 18 June 1985