The interaction between subunits in the tubulin dimer

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Limited proteolysis and chemical cross-linking techniques have been used to study the interaction between α - and β -tubulin subunits. Trypsin digestion of tubulin dimer resulted in the cleavage of the α -subunit into two fragments, whereas chymotrypsin cleaved the β -subunit into two distinct fragments. All of these fragments have been mapped on the tubulin subunits by further proteolysis with formic acid. Cross-linking of trypsin- and chymotrypsin-cleaved subunits has been performed with two different cross-linker agents of different cross-linking distance. The addition of formaldehyde resulted in the cross-linking of the α -tubulin *N*-terminal fragment with β -tubulin *C*-terminal domain. The same result was obtained when methyl 4-mercaptobutyrimide was used.

Tubulin, the main component of microtubules, is a dimer of two strongly associated polypeptide chains (α and β), each with an M_r of 50 000 (Dustin, 1978). Recently, it has been indicated that limited trypsin proteolysis of the tubulin dimer cleaved the α -subunit at one single site (Brown & Erickson, 1983; Serrano *et al.*, 1984a), whereas chymotrypsin proteolysis resulted in cleavage at another single site of the β -subunit (Brown & Erickson, 1983; Maccioni & Seeds, 1983).

It was suggested that the tubulin subunits remained associated after tryptic or chymotryptic cleavage (Brown & Erickson, 1983; Serrano *et al.*, 1984a; Maccioni & Seeds, 1983). We have analysed this possibility by using a short-length lysine cross-linker, formaldehyde (Jackson, 1978), and a long-length lysine cross-linker, methyl 4-mercaptobutyrimide (Herin & Freda, 1977), to obtain a clearer picture of the interaction between tubulin subunits after limited proteolysis.

Our results suggested an interaction between the *N*-terminal fragment of the α -subunit (35 kDa) and the *C*-terminal fragment (20 kDa) of the β -subunit when formaldehyde or methyl 4-mercaptobutyrimide was used.

Materials and methods

Microtubule protein was obtained from porcine brain by two cycles of assembly–disassembly

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; MAPs, microtubule-associated proteins; PC-tubulin, tubulin purified by phosphocellulose chromatography

(Shelanski *et al.*, 1973). After the second cycle the depolymerized protein was chromatographed on a phosphocellulose column (Weingarten *et al.*, 1975) to obtain tubulin depleted of MAPs. This preparation (PC-tubulin) was used immediately. In some experiments tubulin was labelled in the α -subunit *C*-terminal residue with L-[U- 14 C]tyrosine as described by Arce *et al.* (1975). The protein was characterized by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the method of Laemmli (1970). The gels were stained with Coomassie Blue (Fairbanks *et al.*, 1971) or with 'Stains-All' dye (Campbell *et al.*, 1983). The M_r values for the tubulin fragments were calculated from their electrophoretic migration on gels, with serum albumin, tubulin, ovalbumin, tropomyosin and cytochrome *c* as standards.

Limited proteolysis of PC-tubulin (2 mg/ml) in 0.1 M-Mes (pH 6.4)/0.5 mM-MgCl₂/2 mM-EGTA (buffer A) with trypsin (1.25%, w/w), chymotrypsin (1.25%, w/w) or subtilisin (1%, w/w) (from Sigma) was allowed to proceed for 30 min at 30°C. The reaction was stopped by addition of 1 mM-PMSF or by adding sodium dodecyl sulphate to a final concentration of 1% (w/v) and boiling for 1 min.

Proteolysis with formic acid was performed as described by Sonderegger *et al.* (1982) with minor modifications. Briefly, gel slices containing the stained peptide were incubated with 75% formic acid in the dark at 37°C for 48 h. After the incubation the slices were washed with 50% (v/v) methanol/0.1 M-Tris/HCl (pH 6.8)/0.002% Bromophenol Blue until the mixture showed a blue colour

and then subjected to electrophoresis as described by Sonderegger *et al.* (1982).

Peptide cross-linking of uncleaved or cleaved protein (2 mg/ml) with formaldehyde was achieved by incubating the sample in buffer A with 1% (w/v) formaldehyde, for 60 min at 4°C or for 15 min at 25°C. The cross-linking with methyl 4-mercapto-butyrimidate was also performed in buffer A, incubating the sample with the cross-linker agent at a final concentration of 15 mM for 15 min at 10°C. Then H₂O₂ was added to a final concentration of 10% (w/v) and the mixture incubated for 30 min at 10°C. Both reactions were stopped by addition of a molar excess of glycine, followed by the addition of sodium dodecyl sulphate to 1% (w/v) final concentration. Iodination of tubulin was performed as described by Bolton and Hunter (1973) who followed the method of Carlier *et al.* (1980).

Results

Limited proteolysis of tubulin and localization of the proteolytic fragments on the tubulin molecule

Trypsin digestion of tubulin resulted in the cleavage of the α -subunit into two fragments, α_N and α_C , with M_r values of 35000 and 17000 respectively, whereas the β -subunit remained uncleaved (Fig. 1b). However, limited proteolysis of tubulin with chymotrypsin resulted in the appearance of two fragments, β_N and β_C , with M_r values of 32000 and 20000 respectively, from the β -subunit, whereas the α -subunit remained uncleaved (Fig. 1c). To determine the localization of trypsin fragments on the tubulin molecule we labelled the C-terminal of the α -subunit with [¹⁴C]-tyrosine (Arce *et al.*, 1985) and found, after trypsin cleavage, that only the 17kDa fragment (α_C) was labelled. To confirm that the larger fragment (35kDa, α_N) was located at the N-terminal of the molecule, we performed a formic acid digestion.

Formic acid preferentially cleaves aspartic acid-proline bonds (Jauregui-Adell & Marti, 1975), and from the results obtained by Ponstingl *et al.* (1982) we know that such a bond is present at position 306–307 in the α -subunit. The incubation of the α -subunit with formic acid resulted in two fragments. One had an apparent M_r of 33000, which corresponds to the first 306 amino acids of α -tubulin, and the other was a smaller fragment with an apparent M_r of 17000 localized at the C-terminal region (Serrano *et al.*, 1984b; see also Fig. 7 below). Formic acid cleavage of the 35kDa trypsin fragment produced one fragment with an apparent M_r of 33000, indicating that the larger fragment of the α -subunit contained the N-terminus (α_N). Formic acid peptide analysis was also used to locate chymotrypsin fragments on the

Fig. 1. Limited proteolysis of tubulin

PC-tubulin (2 mg/ml) was digested with (b) trypsin (1.25%, w/v) or (c) chymotrypsin (1.25%, w/v) for 20 min at 30°C. The reaction was stopped with 1 mM-PMSF and 0.2 ml of each sample was subjected to electrophoresis in a 10%-polyacrylamide slab gel. The arrows indicate the position of the α - and β -tubulins and their corresponding proteolytic peptides. (a) shows the pattern for undigested tubulin.

β -tubulin subunit. From the results of Ponstingl *et al.* (1982), we know that there are two Asp-Pro bonds at positions 61–62 and 306–307 in the β -subunit. The formic acid proteolysis of the β -subunit yielded three fragments corresponding to the cleavage of those two bonds. The fragments are β_3 (M_r 3000) located at the N-terminus, β_2 (M_r 17500) located at the C-terminus and β_1 (M_r 30000) (Serrano *et al.*, 1984b; see also Fig. 7 below). Formic acid cleavage of the larger chymotryptic fragment of β -tubulin gives two fragments with apparent M_r values of 32000 and 30000, whereas formic acid cleavage of the smaller chymotryptic fragment of β -subunit gives another two fragments with apparent M_r values of 20000

and 17000. Taking these results together we suggest that the larger chymotryptic fragment of β -subunit is localized at the N -terminus and the smaller one at the C -terminus of β -tubulin.

Fig. 2. Schematic representation of the tubulin peptide map. The peptide map was obtained from the results of Fig. 1, those of Fig. 7 and those of Serrano *et al.* (1984a,c). Formic, tryptic and chymotryptic (Chymot) cleavages are indicated.

Fig. 3. Double digestion of tubulin with subtilisin and chymotrypsin

PC-tubulin (2 mg/ml) was digested with subtilisin (1%, w/v) for 30 min at 30°C. The reaction was stopped with 1 mM-PMSF and, to remove the proteinase, the digested tubulin was subjected to an additional polymerization cycle (Serrano *et al.*, 1984b). Undigested (a) and subtilisin-digested (b) tubulin were digested with chymotrypsin and subjected to electrophoresis as indicated in Fig. 1. In the Figure are indicated the mobilities of PC-tubulin (PC-Tb), subtilisin-digested tubulin (S-Tb) and the two chymotryptic peptides of them (β_N and β_C).

Combining the above results, a map of tubulin peptides has been constructed (Fig. 2).

The result shown in Fig. 2 was further confirmed when a double digestion with subtilisin (Serrano *et al.*, 1984b) and chymotrypsin was done (Fig. 3). Since subtilisin cleaves a 4 kDa fragment from the C -terminus of both subunits (Serrano *et al.*, 1984b), we have isolated subtilisin-digested tubulin (Serrano *et al.*, 1984c) and cleaved it with chymotrypsin (Fig. 3). The results of double digestions indicated a faster electrophoretic mobility of the β_C fragment of double-digested tubulin, whereas that of the β_N fragment of chymotrypsin or subtilisin/chymotrypsin-digested tubulin did not change.

Cross-linking of tubulin

The cleaved α - or β -subunit could interact with the remaining intact subunit. Some of the possible interactions between the cleaved fragments and the intact subunits are indicated in Fig. 4. To test whether any of the tryptic fragments of the cleaved α -subunit or the chymotryptic fragments of cleaved β -subunit could interact with the remaining intact tubulin subunit we carried out the following experiments.

When tubulin dimer was incubated with formaldehyde (Fig. 5), a peptide that corresponded to the α - β dimer (Fig. 5a) was found. However, when tubulin was first partially proteolysed with chymotrypsin (Fig. 5b) or trypsin (Fig. 5c), and afterwards incubated with formaldehyde, new cross-linked peptides were observed. For trypsin-cleaved tubulin, a peptide with an apparent M_r 95000 ($\alpha_N + \beta$) was found, whereas for chymotrypsin-cleaved tubulin, the apparent M_r for the new peptide was 80000 ($\alpha + \beta_C$). These results seem consistent with possibility I for trypsin-cleaved tubulin and possibility II for chymotrypsin-cleaved tubulin (see Fig. 4).

Fig. 4. Cross-linking between fragments from tubulin subunits

(a) Theoretical possibilities of cross-linking between tryptic α -fragments and β -subunit. (b) Theoretical possibilities of cross-linking between chymotryptic β -fragments and α -subunit.

Fig. 5. Cross-linking of proteolytic fragments of tubulin (Tub) with formaldehyde

PC-tubulin (2mg/ml, 0.2ml), digested with trypsin or chymotrypsin as indicated in Fig. 1, was cross-linked with 1% (w/w) formaldehyde for 15 min at 25°C. The samples were then subjected to electrophoresis on a 7.5–12% polyacrylamide gradient slab and stained. The results for cross-linked (even-numbered lanes) and non-cross-linked peptide (odd-numbered lanes) are shown. (a) Undigested tubulin; (b) chymotrypsin-digested tubulin; (c) trypsin-digested tubulin. The position of the cross-linked peptides (dimer, 95 kDa and 80 kDa) is indicated by arrows. Also indicated is the position of a 68 kDa peptide used as an internal marker of M_r (arrowhead). Sometimes, in trypsin-digested tubulin, a doublet (35–32 kDa) was found, as indicated in the Figure; however, similar results were found with preparations in which only the 35 kDa was present (results not shown).

By using the dye Stains-All, which stains Ca^{2+} -binding proteins (Campbell *et al.*, 1983), we found (L. Serrano, F. Wandosell, J. C. Oier & J. Avila, unpublished work) that β -tubulin stains blue whereas α -tubulin stains pink. However, when the α -tubulin subunit was cleaved by trypsin digestion, the 17 kDa fragment (α_c) became blue-stained whereas the 35 kDa fragment (α_N) remained pink. When β -tubulin subunit was cleaved by chymotrypsin digestion the 20 kDa fragment (β_c) stained blue, whereas the 32 kDa fragment (β_N) was stained pink (results not shown). The fact that the 80 kDa cross-linked peptide stained blue with Stains-All could be due to the presence of two C-terminal regions ($\alpha_c + \beta_c$) plus one N-terminal region (α_N). Moreover, the pink colour of the 95 kDa cross-linked peptide would be consistent with the presence of two N-terminal regions ($\alpha_N + \beta_N$) plus one C-terminal region (α_c). These results also suggest that the 95 kDa peptide was composed of a β -tubulin subunit plus the N-

Fig. 6. Cross-linking of chymotrypsin-digested tubulin (Tub) with methyl 4-mercaptobutyrimidate

(a) Undigested tubulin (2mg/ml, 0.2ml) was cross-linked with methyl 4-mercaptobutyrimidate (15 mM) as indicated in the Materials and methods section. (b) Chymotrypsin-digested tubulin (2mg/ml, 0.2ml) was cross-linked as indicated above. (c) Tubulin (2mg/ml, 0.2ml) was cross-linked as indicated in (a). Once the cross-linking reaction was stopped, the sample was digested with chymotrypsin as indicated in Fig. 1.

terminus of an α -subunit, whereas the 80 kDa peptide contained the α -subunit plus the C-terminal fragment of β -tubulin. Similar results to those of Fig. 5 were found when the cross-linking was done at different temperatures (4 and 25°C) or for different incubation times (from 15 to 60 min at 4°C). Similar results were also obtained with methyl 4-mercaptobutyrimidate. Furthermore, when this cross-linker was used, the same peptides as found in Fig. 5 were obtained when the tubulin molecule was first cross-linked and afterwards proteolysed with chymotrypsin or when the cross-linking was performed after the proteolysis (Fig. 6), suggesting that the cleavage did not affect the interaction between tubulin subunits.

A further experiment to test the interaction between tubulin subunits was performed. Fig. 7 shows the formic acid digestion of ^{125}I -labelled tubulin subunits without cross-linking and that of the cross-linked peptide corresponding to α - β dimer. Since the formic fragments of both subunits showed a different electrophoretic mobility (see

Fig. 7. Determination of the cross-linked regions from tubulin (Tub) subunits by mapping with formic acid (a) ^{125}I -PC-tubulin (2mg/ml) was cross-linked with formaldehyde as indicated in the Materials and methods section. The sample was applied on a 7.5%-polyacrylamide slab gel. The cross-linked dimer and the mixture of α - and β -subunits were cut out separately from the gel and subjected to cleavage with formic acid. The cleaved protein was then applied to a 10%-polyacrylamide slab gel. The gel was cut in pieces and the radioactivity present in each piece of the cross-linked dimer fraction (●) or in the un-cross-linked subunits (○) measured. The inset on the right shows the pattern of digestion with formic acid of a mixture of α - and β -tubulin subunits run in parallel in the same gel and stained with Coomassie Blue. The other inset (in the centre, top) shows a model for the localization of the interaction between tubulin subunits. It shows the formic acid peptide fragments of α - and β -subunits and a possible interaction between α_1 and β_2 (see the text).

Fig. 7, inset) and formic acid hydrolysis does not cleave the bond caused by formaldehyde cross-linking, it is possible to analyse which one of the formic acid fragments remained cross-linked. From Fig. 7 it can be seen that the broad peak corresponding to α_1 and β_1 was displaced in the cross-linked-peptide fragments to the position of β_1 , suggesting a decrease in the proportion of α_1 . Also whereas α_2 is present in the formic acid digestion products of the cross-linked tubulin dimer, β_2 was absent. Finally, a peptide (probably $\beta_2 + \alpha_1$) with a slightly higher M_r than that of undigested tubulin subunits appears among the fragments of the cross-linked tubulin dimer. The inset to Fig. 7 shows a possible explanation of the above results, in which we suggest that the interaction between subunits in the tubulin dimer is between β_2 and α_1 .

Discussion

Tubulin interacts with different molecules (proteins, nucleotides etc.) through domains already mapped on the molecule (Brown & Erickson, 1983;

Serrano *et al.*, 1984a,b,c; Maccioni & Seeds, 1983). These maps were obtained after proteinase (trypsin, chymotrypsin and subtilisin) cleavage of the tubulin molecule followed by chemical proteolysis. We have located the positions of tryptic and chymotryptic fragments in the molecule. Our results for the latter case were identical with those reported by other laboratories with a different technique (Mandelkow *et al.*, 1985). This map has allowed us to localize the region involved in the interaction between tubulin subunits. Here we present some data about the localization of the region of interaction between both tubulin subunits. This region is located in the tryptic fragment that includes the *N*-terminus of the α -subunit and in the chymotryptic fragment from the β -subunit, which contains the *C*-terminus. However, this interaction is not localized at the end of the molecule, since the 4kDa *C*-terminal segment of the β -subunit is not directly involved in the interaction between tubulin subunits (Serrano *et al.*, 1984b). Our results agree also with previous data (Serrano *et al.*, 1984a) indicating that trypsin digestion releases fragments (with M_r values equal

to, or lower than, 20000) that contain the α -subunit C-terminal, which are unable to remain associated with the tubulin dimer.

Tubulin dimers can interact in an intramolecular or intermolecular fashion. The latter type of association was not likely to occur in the present work, since we have used purified tubulin depleted of associated proteins and at a relatively low concentration of protein. Under such conditions, practically all of the tubulin remains in a dimeric form and oligomers are not formed (results not shown). A similar interaction between tubulin subunits was found when the proteolysis was done before or after cross-linking, and by using either formaldehyde or methyl 4-mercaptobutyrimidate. These results suggest that the interaction between the α - and β -fragments was not an artefact that arose as a consequence of the procedure used.

The α -region that interacts with the β -subunit shows a high homology (70%) with the corresponding N-terminal region of the β -subunit, as indicated by Ponstingl *et al.* (1982).

However, the β -region that interacts with the α -subunit has only a 30% homology with its corresponding α -fragment located at the C-terminus (30%). Since it has been suggested by some authors (Gozes & Barnstable, 1982) that homodimers may exist, their low extent of formation, if any, might be due to this low degree of homology between subunits in the region between the bonds cleaved by formic acid and subtilisin (Serrano *et al.*, 1984b; Ponstingl *et al.*, 1982).

Our results are in agreement with the suggestion of Ponstingl *et al.* (1983) that hydrophobic areas present in regions corresponding to the proteolytic α - and β -fragments are likely to be involved in tubulin-tubulin interactions. These regions appeared to be localized near the tryptic and chymotryptic cleavage points, probably about position 260 in both the α - and the β -subunit. Moreover, on the basis of the hydrophilic pattern, calculated from the sequence determined by Ponstingl *et al.* (1982) by the method of Hopp & Woods (1981), we found that the proteolytic cleavages produced on the α -subunit by trypsin or subtilisin, and on the β -subunit by chymotrypsin or subtilisin, are probably located in hydrophilic regions. We can also see that, in β -tubulin, there are two different hydrophobic 'pockets' in the region in which this subunit interacts with the α -subunit. One of them is located close to the region suggested by Ponstingl *et al.* (1983) for the interaction between subunits. The fragment of α -tubulin that binds to the β -subunit is too large for us to speculate about the possible hydrophobic region involved in such an interaction. The region around amino acid-260 of the tubulin subunits, suggested

by Ponstingl *et al.* (1983) to be the one responsible for the interaction between subunits, is compatible with our results, since a lysine residue is located at position 280 of α -subunit. However, we cannot rule out the possibility that other regions of the α -tubulin proteolytic fragment could be also involved in the interaction with β -tubulin.

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