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Chemical characterisation of tubulin sites involved in the colchicine binding and polymerisation process

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Abstract. Tubulin, the basic structural component of microtubules requires nucleotides such as ATP and GTP for its assembly/disassembly process. This process is inhibited by colchicine which strongly interacts with this protein.

Goat brain tubulin was isolated by ammonium sulphate fractionation and DEAE cellulose chromatography. An electrophoretically homogeneous preparation was modified by chemical and enzymatic methods to examine involvement of specific amino acid residues at the polymerisation, the colchicine binding and the nucleotide binding sites. The modification of tubulin by acetylation and trypsinolysis suggests that lysine residues and the conformation of the protein molecule are important at the colchicine binding site.

The association constants of nucleotides with tubulin show that ATP binds tubulin less efficiently than GTP. The fact that acetylation of tubulin does not affect binding constants, indicates that lysine residues may not be involved at the binding sites of these nucleotides. However, the participation of trytophan residues at the nucleotide binding site is evident from fluorescence studies.

Keywords. Tubulin; lysine; polymerisation; colchicine binding.

1. Introduction

Microtubules play an important role in several aspects of cell structure and function such as mitosis, transport and maintenance of cellular shape and mobility. Microtubules are dynamic structures in equilibrium with the protein subunit tubulin, which is a heterodimer consisting of α and β subunits having a molecular weight of 55000, and which polymerises *in vivo* and *in vitro* (Weisenberg *et al* 1968; Bhattacharya and Wolf 1947; Snyder and Mackintosh 1976; Adelman *et al* 1968). Colchicine, an alkaloid also known to be an antimitotic drug, inhibits this assembly process of tubulin by strongly interacting with the protein at a specific site (Adelman *et al* 1968). It has been shown that tubulin contains 2 nucleotide binding sites. One (the N site) contains non exchangeable GTP (Weisenberg *et al* 1968; Snyder and Mackintosh 1976; Spiegelman *et al* 1977). The nucleotide bound to the second site (the E site) can exchange with an exogenous nucleotide (Weisenberg 1968; Bhattacharya and Wolf 1947; Snyder and Mackintosh 1976; Adelman *et al* 1968; Bryan and Wilson 1971).

Although the amino acid analysis of tubulin from several sources has been carried out and the sequence of tubulin from porcine brain determined (Krauhs *et al* 1981), the role of specific amino acid residues at the binding sites of tubulin is still unknown. Experiments were therefore undertaken to assess the role of specific amino acid residues, particularly lysine residues in the above processes, which was investigated by modifying tubulin chemically and enzymatically.

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2. Materials and methods

The following were obtained from Sigma Chemicals: MES (N-morpholino ethane sulphonic acid) (M-8250), EGTA [ethylene glycol bis (β amino ethylether) N,N',N' tetra-acetic acid] (E-4378), GTP (guanosine triphosphate) (G-5631), Tris (T-1503) glycine (G-7126), trypsin (T-8253). ^{3}H colchicine was purchased from the New England Nuclear Company.

2.1 *Purification of tubulin*

Tubulin was purified from goat brain according to Weisenberg's method (Weisenberg *et al* 1968), modified by Bhattacharya and Wolf (1947). In the modified method DEAE (diethyl aminoethyl) cellulose was used instead of DEAE Sephadex A-50 in PMG buffer (10 mM phosphate, pH 7·0, 10 mM $MgCl_2$ and 0·1 mM GTP). Tubulin adsorbed on the column was eluted with KCl gradient (0·2–0·8 M). Peak fractions were assayed for colchicine binding activity. The fractions having high tubulin content were pooled and concentrated by overnight dialysis at 0°C against 8 M glycerol and stored at -40°C until use.

Homogeneity of the tubulin preparation was checked by urea gel electrophoresis, by replacing water with urea by weight in acrylamide gels (Davis 1964) so that the gels contained 8 M urea. The running buffer was Tris-glycine system adjusted to pH 8·3. The samples were dialysed overnight in 8 M urea buffer.

Tubulin was polymerised at 37°C in MES buffer (100 mM MES, 0·5 mM $MgCl_2$, 1 mM GTP and 1 mM EGTA and 4 M glycerol) and turbidity measured at 350 nm. Tubulin was acetylated according to the method of Davies and Neuberger (1969) and the product was dialysed extensively against several changes of PMG buffer. Limited trypsinolysis of the protein was carried out at 37°C for 1 hr in PMG buffer at pH 7 with an enzyme-to-protein ratio of 1:120.

3. Results and discussion

The tubulin isolated from goat brain was a homogeneous preparation free from high molecular weight proteins, as shown by gel electrophoresis (figure 1a). Tubulin thus isolated has been characterised as the 6 S dimeric form with which all the experiments reported here were carried out.

This tubulin was modified by acetylation and trypsinolysis. Acetylation of proteins at pH 8 predominantly leads to modification of lysine residues, although some tyrosine residues may also be modified (Davies and Neuberger 1969). Limited proteolysis by trypsin cleaves the peptide bonds formed by the carbonyl groups of lysine and arginine residues. Electrophoretic mobility of native, acetylated and trypsinated tubulin is shown in figure 1b. Acetylated tubulin moved as a doublet in a manner similar to native tubulin with no additional bands indicating the absence of heterogenous modifications. Its mobility was slightly increased as compared to native protein. This could be due to the masking of the amino groups of the lysine residues with acetyl groups which results in a net change of charge on the protein. Trypsinolysis of tubulin indicates fragmentation of the protein as evident from the increased number of bands. Those with mobility higher than the native tubulin are probably smaller size fragments, while

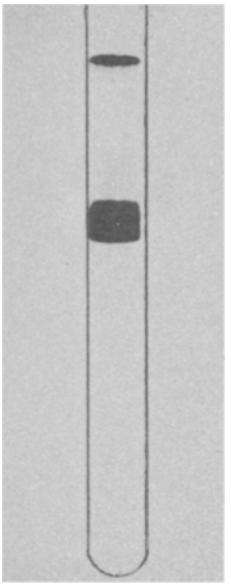

Figure 1a. Urea-gel electrophoresis of tubulin pooled from active fraction. Tubulin loaded, 120 mgs.

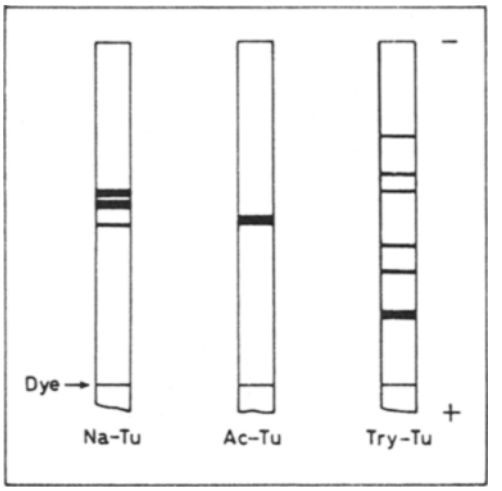

Figure 1b. Schematic diagram for gel electrophoresis of native tubulin (Na-Tu), acetylated tubulin (AC-Tu), trypsin-cleaved tubulin (Try-TU).

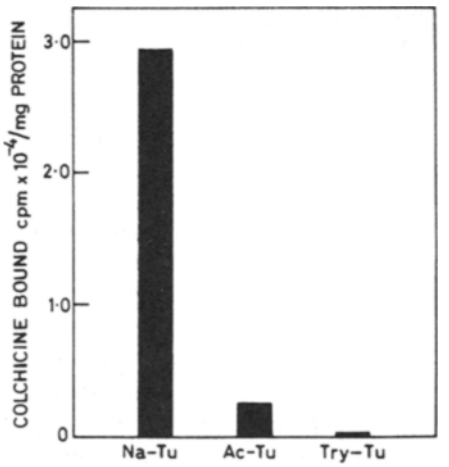

Figure 2. Colchicine binding activity of (i) native tubulin, (ii) acetylated tubulin, (iii) trypsinised tubulin.

the bands with lower mobility can be attributed to aggregation of trypsinised protein. Quantitation of the extent of acetylation and trypsin cleavage is in progress.

Binding of colchicine and the polymerisation process are important parameters to examine the biological activity of tubulin. The specific activity of colchicine binding to tubulin and its course of polymerisation have been shown in figures 2 and 3. Acetylated tubulin binds colchicine with only 10% efficiency and shows reduced polymerising

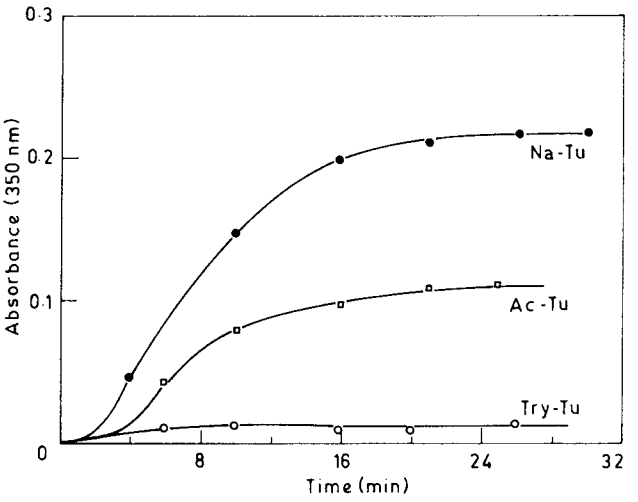

Figure 3. Polymerization tubulin (2·2 gms/ml) in MES buffer at pH 6·4 (0·1 M MES, 1 mM/EGTA, 1 mM GTP and 0·05 mM Mg^{++}) in 4 M glycerol; ●, native tubulin; □, acetylated tubulin; O, trypsinised tubulin.

capacity as compared to native tubulin. This suggests that amino acids modified by acetylation are in some way important at the drug binding site and in the assembly process. Colchicine-binding ability and the polymerising process are impaired due to limited trypsinolysis of tubulin. Fragmentation of tubulin by trypsin, as shown in figure 1b, failed to provide the intact tubulin structure with the native conformation which is essential for colchicine binding and also for polymerisation. It is therefore apparent that the peptide bonds involving basic amino acid residues are essential for the above processes.

Binding of GTP and ATP (adenosine triphosphate) to native, acetylated and trypsinised tubulin was studied fluorimetrically (figures 4a and b) GTP binds tubulin much more efficiently than ATP as evident by the association constants. However, GTP and ATP also bind acetylated and trypsinised tubulin as efficiently as the native form. It therefore shows that blocking of the basic residues by acetylation and cleavage of tubulin by trypsin does not affect binding of the nucleotides. Since trypsin fragments tubulin, it is likely that the nucleotide-binding site is preserved in one of the fragments which binds GTP and ATP as efficiently as the native protein. The integrity of tubulin is therefore not absolutely essential for binding of the nucleotide. Since in these experiments interaction of nucleotides was examined by monitoring the fluorescence of tubulin arising from tryptophan residues, our results indicate involvement of this residue at the nucleotide binding site.

From the above results it is evident that acetylation and limited proteolysis affect both the colchicine-binding capacity of tubulin and its polymerisation process but trypsinolysis does not affect the nucleotide-binding site. It implies that amino acid residues involved in colchicine binding are also important in the polymerisation process. It therefore follows that colchicine inhibits the polymerisation process by blocking or masking the amino acid residues which are essential for the polymerisation reaction and that the integrity of tubulin involving peptide bonds with lysine residues is essential for microtubule formation.

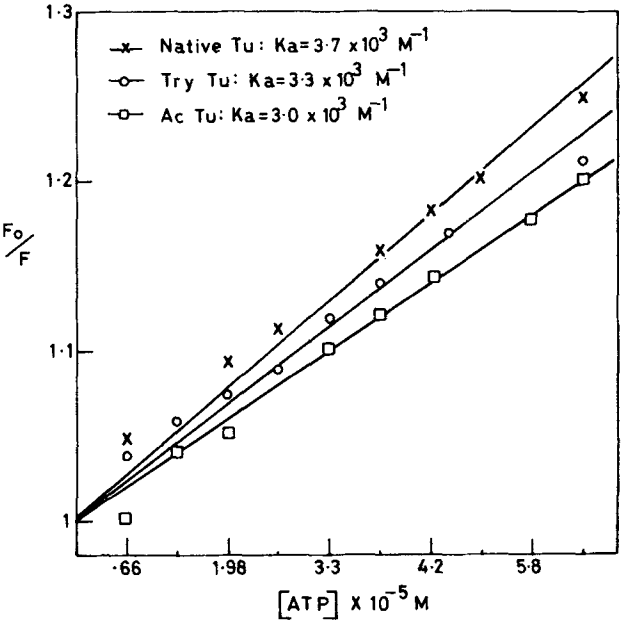

Figure 4a. Stern-Volmer plot for the association of ATP with tubulin. Tubulin taken 10^{-6} M in all three cases; – × – native tubulin; □, acetylated tubulin; ○, trypsinised tubulin.

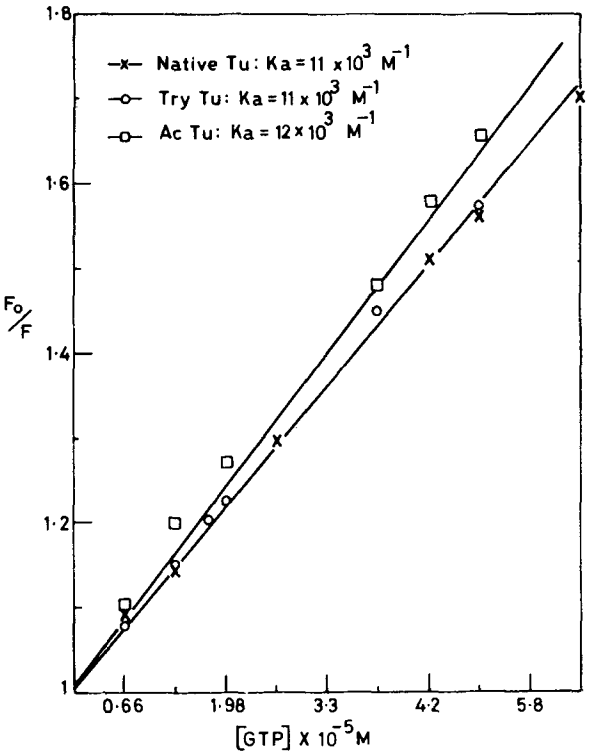

Figure 4b. Stern-Volmer plot for the association of GTP with tubulin. Tubulin taken 10^{-6} M in all cases; – × –, native tubulin; □, acetylated tubulin; ○, trypsinised tubulin.

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