Involvement of colchicine binding site of tubulin in the polymerisation process

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Received 14 December 1982

Role of lysine residues in the colchicine binding site and in the assembly–disassembly process was examined. It was observed that at 4°C (pH 7.5–8, 8 ± 1) lysine residues and the N-terminal methionine residue of tubulin were all buried within the molecule. Evidence indicates that ϵ-amino groups of lysine residues of tubulin are shared by both the colchicine binding site and the polymerisation process.

Tubulin *Acetylation* *Lysine* *Colchicine binding* *Polymerisation* *Conformation*

1. INTRODUCTION

Microtubules are dynamic structures in equilibrium with their protein subunit tubulin, a heterodimer of α- and β-subunits, which polymerises in vivo and in vitro [1]. The polymerisation process is inhibited by several antimitotic drugs such as colchicine, colcemid, podophyllotoxin and vinblastine [2]. Further, it has been shown that polymerised tubulin does not bind these drugs as the binding sites are probably masked and are not available for the ligand binding [3]. It is not known whether the colchicine binding site is masked because of its participation in the assembly process. This suggests the possibility of polymerisation and colchicine binding sites being linked together, in that modification of the former leads to an impairment of the latter or vice versa. In this report we have assessed the role of the colchicine binding site in the assembly process by chemical modification of the lysine residues. Evidence indicates that the amino groups of lysine residues of tubulin are shared by both the colchicine binding site and the site involved in polymerisation process.

Abbreviations: MES, 2-(*N*-morpholinoethanesulfonic acid); EGTA, ethyleneglycol-bis-(β-amino-ethyl-ether) *N,N*-tetraacetic acid; GTP, guanosine-5′-triphosphate; PMG buffer, phosphate, magnesium chloride and GTP buffer

2. MATERIALS AND METHODS

The following were obtained from Sigma Chemicals: MES (M-8250), EGTA (E-4378), GTP (G-5631), Tris (T-1503), glycine (G-7126), Trypsin (T-8253). [^{3}H]Colchicine was purchased from New England Nuclear Company.

Tubulin was purified from goat brain according to Weisenberg's method modified by Bhattacharya et al. [4]. It was eluted with a KCl gradient. 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 tubulin preparation was checked by urea gel electrophoresis. This was done by replacing water with urea by weight in acrylamide gels [5], so that the gels contained 8 M urea. The samples were dialysed overnight in 8 M urea buffer. Percent purity of the protein was calculated from densitometric scanning and was found to be about 95% pure tubulin.

Tubulin was polymerised at 37°C in MES buffer (100 mM MES, 0.5 mM $MgCl_2$, 1 mM GTP and 1 mM EGTA, 4 M glycerol), and turbidity measured at 350 nm. Colchicine binding was done by standard filter assay method using Whatman DE 81 filter discs. The filter discs were thoroughly

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

dried and extracted overnight in scintillation fluid prior to counting.

Acetylation of tubulin was carried out according to the method of Davies and Neuberger [6]. 10 λ acetic anhydride was used to acetylate a sample of 3.3 mg/ml of tubulin. The acetylated product was dialysed extensively against several changes of PMG buffer. Time course of acetylation was studied by the assessment of the number of amino groups, by Moore and Stein's method [7], by taking aliquots at different time intervals. The number of lysine residues acetylated at the end of the experiment was determined by FDNB analysis [6]. The N-terminal amino acid was determined by the method of Fraenkel Conrat [8] after separating the ether soluble and water soluble DNP amino acid derivatives. Thin-layer chromatography of the DNP samples was done by using authentic DNP amino acids as reference samples.

3. RESULTS AND DISCUSSION

Very little is known about the chemical aspects of goat brain tubulin. Information on the amino acid composition and N-terminal amino acid was not found in the literature. Hence, it was necessary to determine the N-terminal amino acid and also the total number of ϵ-amino groups of lysine residues present in this protein. Our analysis showed that native tubulin contains about 43 ± 1 lysine residues. The values are quite close to those from calf brain tubulin having 50 lysines and porcine brain tubulin having 36 lysine residues [9,10]. Methionine was identified as the N-terminal amino acid as has been observed in tubulins from other sources [10].

Acetylation of tubulin was carried out between pH 7.5–8.0. On urea gel electrophoresis acetylated (90 min) tubulin, moved as a doublet similar to native tubulin without any additional bands indicating that it was a homogenous preparation. However, modified tubulin moved with a slightly greater mobility (0.639) compared to that of native tubulin (0.552), which could be due to the decrease in the net positive charge on the protein as a result of acetylation of ϵ-amino groups of lysine residues.

Tubulin was acetylated for various time intervals to find the duration for acetylation of maximum number of lysine residues. It was observed that acetylation reaction was complete within 20 min, fig.1. To ensure complete acetylation the reaction was continued for a longer time period. It is known that in the reaction of a protein with acetic anhydride in addition to lysines, tyrosine residues are also acetylated. However, it is shown that *O*-acetyl tyrosine derivatives quickly hydrolyze under alkaline condition [11]. In the modified protein, therefore, virtually only the lysine residues are acetylated. It is then reasonable to assume that in our acetylated tubulin preparation only lysine residues were modified by acetic anhydride. Tubulin acetylated for 30 min and 90 min gave similar results. Dinitrophenylation of tubulin acetylated for 90 min showed the value of 8 ± 1 ϵ-DNP lysines, fig.2. This suggests that those lysine residues which were dinitrophenylated were not acetylated and must be buried within the α,β-dimer. About 80% acetylation was achieved in case of tubulin, as also seen in lysozyme [6] but rarely observed in most proteins. Treatment of most native proteins with acetic anhydride seldom leads to the acetylation of more than 60% of the amino groups [11].

In the acetylation of proteins both the α-NH_2 of the N-terminal amino acid and ϵ-NH_2 groups of the lysine residues are modified. However, thin-layer chromatography of the ether soluble components of native and acetylated tubulins showed

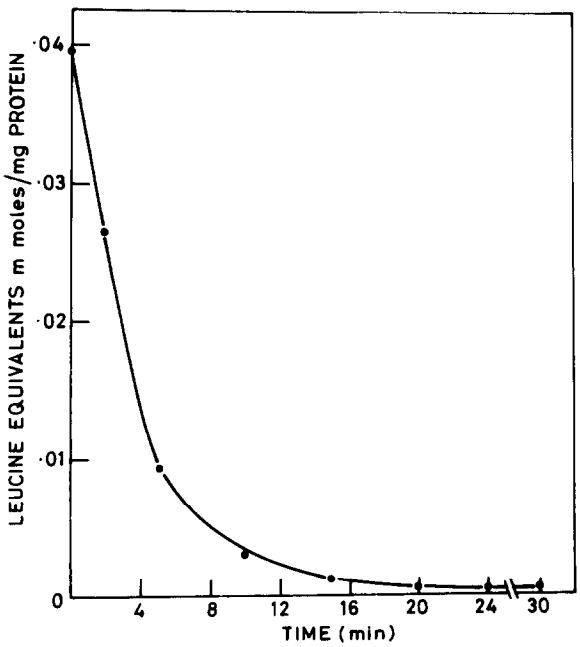

Fig.1. Time course of acetylation of tubulin.

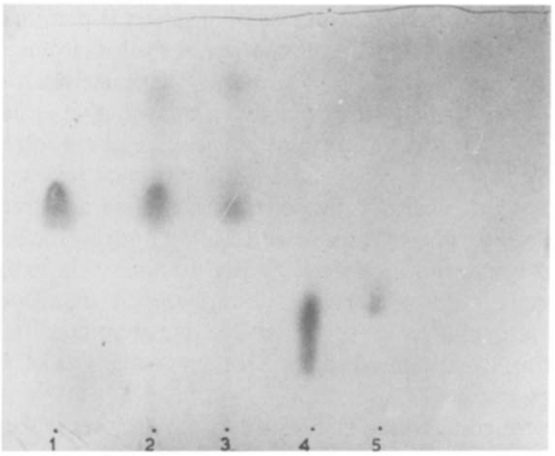

Fig.2. Thin-layer chromatography of water soluble DNP-derivatives. (1) DNP-lysine; (2) DNP-arginine; (3) DNP-derivative of native tubulin; (4) DNP-derivative of acetylated tubulin; (5,6) DNP-tyrosine.

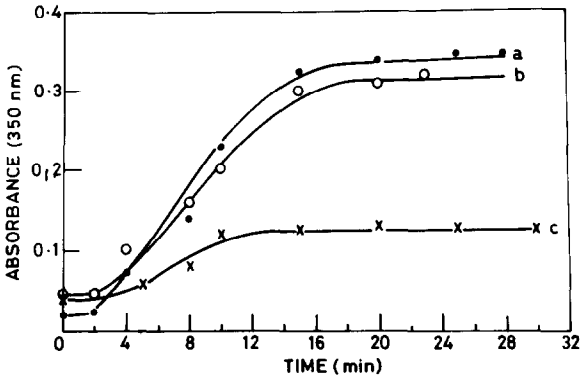

Fig.3. Thin-layer chromatography of ether soluble DNP derivatives. (1) DNP-methionine, R_f 59; (2) DNP-derivative of native tubulin, R_f 59; (3) DNP-derivative of acetylated tubulin, R_f 59; (4) DNP-serine, R_f 33; (5) DNP-glycine, R_f 39.

single spot having R_f value identical to that of DNP-methionine, fig.3. It, therefore, appears that the α-NH_2 group of the terminal methionine was not available for acetylation. Thus at 4°C (pH 7.5–8), tubulin assumes a conformation in which the N-terminal amino acid of the α- and β-subunits are buried within the molecule.

Acetylation of tubulin led to a significant reduction in the efficiency of colchicine binding. Specific activity of colchicine binding to native and acetylated tubulin is shown in fig.4. Acetylated tubulin binds colchicine with only 10% efficiency as compared to native protein. Colchicine bound to

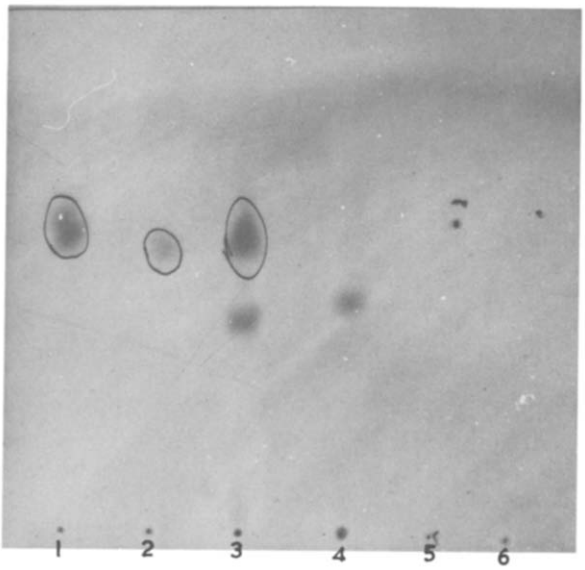

Fig.4. Colchicine binding activity of native and acetylated tubulins (90 min).

native tubulin was calculated to be 0.8 mol per dimer and that bound to acetylated tubulin was 0.08 mol per dimer.

The course of polymerisation is depicted in fig.5. Acetylation of tubulin reduced the assembly competence by about 65%. The rate of assembly also declined and reached a maximum by 12 min, whereas the polymerisation of native tubulin progressed up to 20 min. The lag period for assembly process of acetylated protein was also longer as compared to the native protein.

A sample of native tubulin was treated in a manner identical to that of acetylation, except that acetic acid was added in place of acetic anhydride to serve as a reagent control. Polymerisation of this control sample was seen to be nearly the same as that of the native untreated protein. This eliminated the possibility of drastic alterations in tubulin caused due to its exposure to alkaline pH and acetic acid.

The course of depolymerization is presented in fig.6. Depolymerization of native tubulin and the control sample was nearly 60% whereas that of acetylated protein was almost negligible, 10–5%. This apparently indicated that unlike the native protein, acetylated tubulin was perhaps insensitive to cold. Such alterations in disassembly process suggest that blocking of the lysine residues leads to conformational changes of tubulin. This was fur-

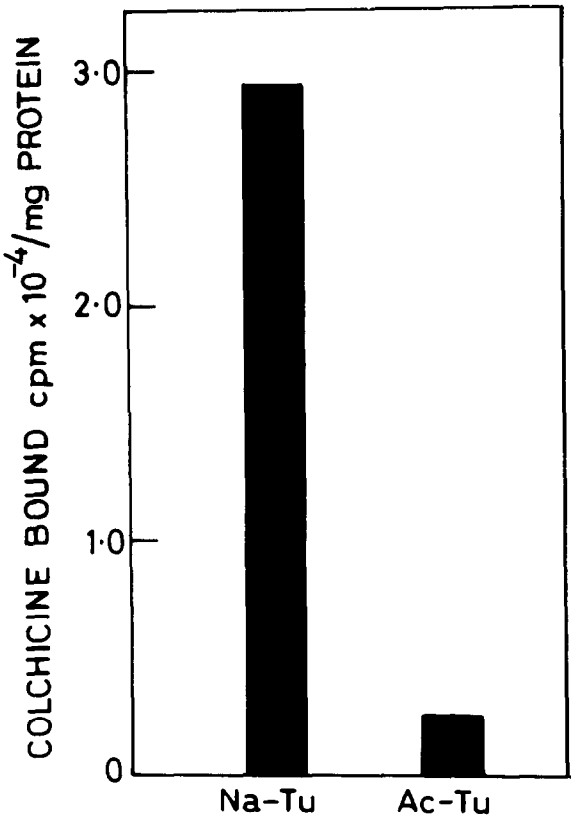

Fig.5. Polymerisation of native and acetylated tubulin. (a) Native untreated tubulin; (b) native tubulin as a reagent control; (c) acetylated tubulin; 2 mg/ml in MES buffer at pH 6.4 (0.1 MES, 1 mM EGTA, 1 mM GTP and 0.05 mM Mg^{2+}) in 4 M glycerol.

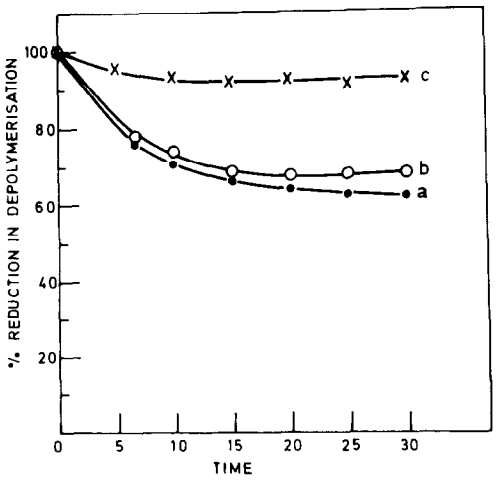

Fig.6. Percent reduction in depolymerisation. (a) Native untreated tubulin; (b) native tubulin as reagent control; (c) acetylated tubulin.

ther supported by the fact that acetylated tubulin aggregated much faster than native tubulin on storage at −40°C.

Thus it is clear that blocking of the amino groups leads to the conformational alteration in the protein molecule. It is, therefore, difficult to conclude whether the colchicine binding and polymerization processes were affected due to blocking of lysine residues alone or due to alteration in the conformation or both. It is apparent that tubulin conformation is very sensitive to protein modification, as compared to some other proteins, e.g., lysozyme [12].

Since blocking the amino groups of lysine residues causes specific alterations in the tubulin molecule preventing both formation of a stable drug protein complex, and inhibiting the assembly process and also stalling the disassembly process, it implies that lysine residues and/or conformational alterations are in some way involved in both these processes. It, therefore, follows that colchicine may inhibit polymerization by blocking or masking these specific amino acid residues critical for assembly interaction.

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