INHIBITION OF THE SELF-ASSEMBLY OF TUBULIN BY DIETHYLPYROCARBONATE AND PHOTOOXIDATION

Young C. Lee, L. L. Houston, and Richard H. Himes
Department of Biochemistry
University of Kansas
Lawrence, Kansas 66045

Received February 24, 1976

SUMMARY

Chemical modification of tubulin by photooxidation and by reaction with diethylpyrocarbonate inhibits the *in vitro* formation of microtubules. This inhibition apparently results from the modification of histidine residues, since the inhibition by diethylpyrocarbonate is reversed by hydroxylamine and the pH dependence of the rate of photooxidation shows the involvement of a group with a pKa value of about 6.5. The inhibition of self-assembly results from the modification of not more than three histidine residues. Sulfhydryl residues are not modified under the experimental conditions used. Colchicine and GTP binding by tubulin were not greatly affected under conditions which completely inhibited the polymerization.

INTRODUCTION

Various parameters and requirements of the *in vitro* polymerization and depolymerization of microtubules have been studied in several laboratories. The self-assembly of tubulin from bovine brain is highly sensitive to the ionic strength of the medium and has optimum of 0.08-0.1 μ (1). The reaction also has a sharp pH optimum of 6.4 (1). These results suggest the importance of ionic interactions in the formation of microtubules and that a group which undergoes deprotonation and protonation in the pH region of 6.5 plays a role in these interactions. Since the imidazole ring of hisitidine is the most likely to undergo ionization at this pH we undertook a study of the effect of modifying these residues on the self-assembly process.

Diethylpyrocarbonate (DEP) is a reagent that is commonly used in active site modification studies. Although it reacts with a number of nucleophiles at pH values of 7.0 and above, it is fairly specific for the imidazole ring of histidine around pH 6.0, resulting in the formation of N-ethoxyformyl-

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histidine (2, 3). The N-ethoxyforml- group is removed and the histidine residue regenerated by hydroxylamine (2). Photochemical oxidation of imidazole groups is another reaction commonly used to modify histidine residues. This reaction can be performed under mild conditions in the presence of a sensitizing dye and is frequently quite specific for the modification of histidine residues. The present study describes the effects of photochemical oxidation and modification by DEP on the polymerization of tubulin and provides some evidence for the involvement of histidine residues in the formation of microtubules in vitro.

MATERIAL AND METHODS

DEP and methylene blue were purchased from Sigma Chemical Co. and Matheson, Coleman and Bell Co., respectively. [^{32}P] GTP, [^{3}H] GTP and [^{3}H] colchicine were obtained from New England Nuclear Co. Tubulin was prepared from fresh beef brain according to the method of Shelanski et al. (4). Homogenates were made in reassembly buffer which contained 20 mM 2[N-morpholino] ethane sulfonic acid (MES), 70 mM NaCl, 1 mM ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra-acetic acid (EGTA) and 0.5 mM $MgCl_2$, pH 6.4. After two cycles of purification tubulin was stored at -80° in reassembly buffer containing 2 M glycerol. The DEP reaction with tubulin was performed in reassembly buffer and was stopped by the addition of imidazolium chloride. Photooxidation was carried out in the presence of methylene blue. A fluorescent lamp, 15 cm from the reaction vessel, was used as a light source. At the completion of the reaction excess methylene blue was removed by the addition of charcoal to a final concentration of 1 mg/ml. For the GTP binding studies, 50 to 70 μM tubulin was incubated in the presence of 0.9 mM [^{3}H]- or [^{32}P] GTP at room temperature for 20 minutes. Protein bound nucleotide was separated from free nucleotide by passing the mixture through a Sephadex G75 (40-120 μ) column (0.7-30 cm) equilibrated with 50 mM $TrisH^{+}$ chloride (pH 7.0) containing 5 mM $MgCl_2$ and 100 mM KCl. Aliquots of fractions were counted in Brays solution and used for protein determination by the Lowry method. The free sulfhydryl content was determined using the Ellman reagent (5) and colchicine binding was measured by the DEAE filter method (6). Amino acid analyses of tubulin were carried out on samples which were hydrolyzed in 6N HCl for 6 or 12 hours at 110°.

RESULTS

Effect of DEP. The inhibition of the in vitro self-assembly of tubulin by DEP is shown in Fig. 1A. Short term (10 minutes) preincubation of 15 μM tubulin with 0.8 mM DEP before initiating polymerization with GTP results in almost complete inhibition. To determine if the inhibition by DEP is due to the acylation of histidine residues, tubulin was treated with NH_2OH after the reaction with DEP. Upon treatment with NH_2OH, a 50% recovery of self-

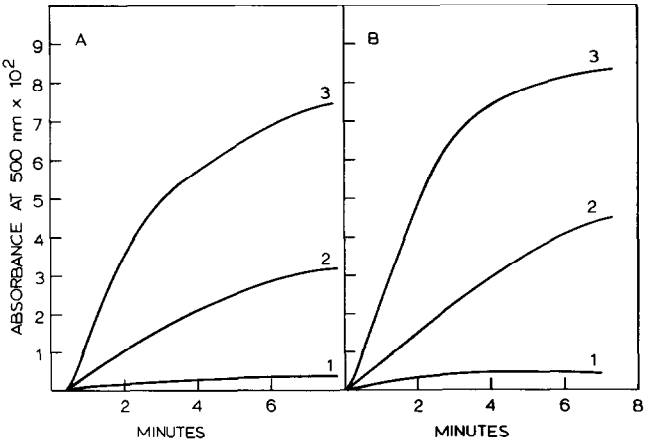

Fig. 1 A. Inhibition of polymerization of tubulin by DEP and reversal by NH_2OH. Curve 1, DEP (final concentration, 0.8 mM) was reacted with 1.6 mg of tubulin in 1 ml of reassembly buffer, pH 6.4, for 10 minutes at room temperature. The reaction was stopped by the addition of imidazolium chloride to a final concentration of 10 mM. The sample was then dialyzed at 4° for 4 hours against reassembly buffer containing 1 M glycerol. Polymerization was performed at 37° using 0.5 mM GTP. Curve 2, tubulin was reacted with DEP and imidazolium chloride as in 1. NH_2OH (final concentration , 0.1 M) was then added and the mixture was dialyzed against 0.1 M NH_2OH in 0.02 M sodium phosphate buffer containing 1 M glycerol, pH 7.0 for 3 hours at 4°. This was followed by dialysis against reassembly buffer as described in 1 above. Curve 3, tubulin was treated as in 2 except that DEP was omitted.

B. Effect of DEP on polymerized tubulin. Curve 1, tubulin (final concentration, 1.3 mg/ml) was reacted with 0.8 mM DEP for 10 minutes at 37° and the excess DEP was removed by the addition of imidazolium chloride to a final concentration of 10 mM. The sample was dialyzed against reassembly buffer containing 1 M glycerol at 4° overnight and then repolymerized at 37° using 0.5 mM GTP. Curve 2, tubulin (1.3 mg/ml) was polymerized at 37° and was then treated as in 1. Curve 3, tubulin was treated as in 1 except that DEP was omitted.

assembly can be obtained when the self-assembly had been completely inhibited by DEP (Fig. 1A). NH_2OH treatment of partially inhibited tubulin brought about a complete recovery. In one experiment for example, tubulin which had lost 36% of its polymerizing activity was fully active after the reaction with NH_2OH. The reversal of DEP inhibition by NH_2OH was confirmed by electron microscopic examination of negatively stained samples (Fig. 2).

DEP, at concentrations which completely inhibit self-assembly, had little effect on preformed microtubules. There was no decrease in turbidity

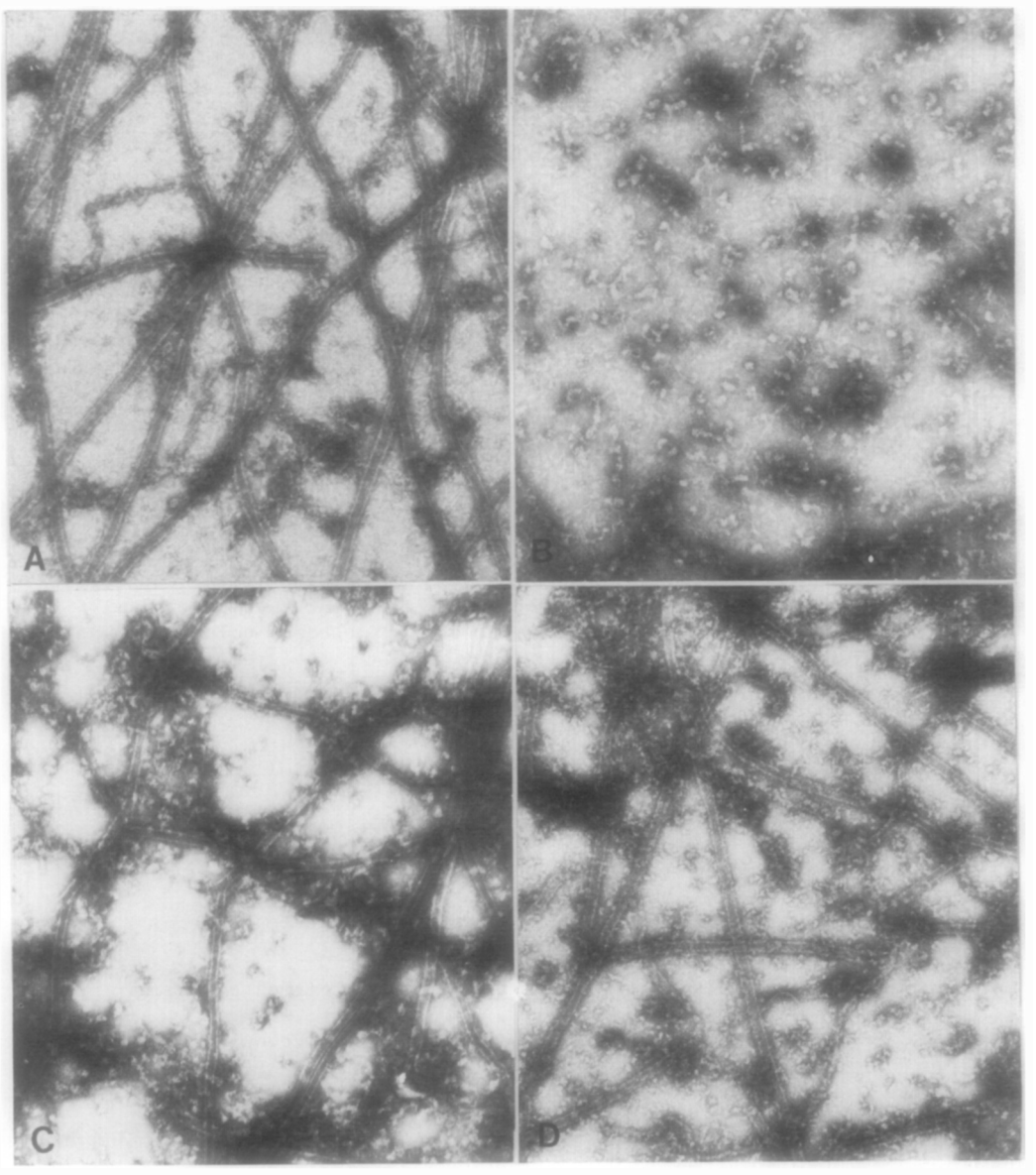

Fig. 2. Electron micrographs of microtubules formed in vitro. A. Tubulin polymerized in the absence of DEP. B. Tubulin polymerized in the presence of DEP. C. Reversal of DEP inhibition by NH_2OH. Experimental conditions of A, B, and C correspond to Curves 3, 1, and 2 in Fig. 1A, respectively. D. Effect of DEP on polymerized tubulin. After the completion of polymerization the sample was incubated with 1.2 mM DEP for 10 minutes at 37°. Samples were negatively stained with 2% uranyl acetate. Magnification of A, B, and C is 72,439 and of D is 60,365.

upon the addition of DEP and as shown in Fig. 2D there was no apparent change upon electron microscopic examination. These results suggest that

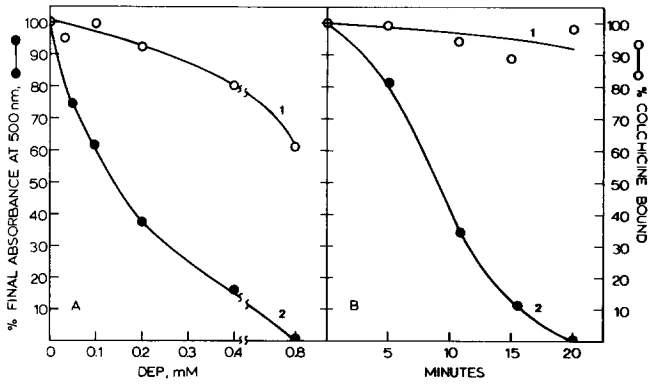

Fig. 3. Effect of chemical modification on colchicine binding and polymerization. A. Effect of DEP. DEP was reacted with 1.8 mg of tubulin in 1 ml of reassembly buffer at 22° for 6 minutes. The reaction was then stopped by the addition of imidazolium chloride pH 7.0 to a final concentration of 100 mM. All samples were dialyzed against reassembly buffer containing 1M glycerol for 4 hours at 4° before colchicine binding and polymerization experiments. Curve 1. Colchicine binding. [^{3}H] Colchicine (final concentration, 2 x 10^{-5} M, 0.5 μC/ml) was incubated with 0.18 mg of tubulin in 1 ml of reassembly buffer for 2 hours at 37°. The amount of bound colchicine was determined by the filter assay technique (6). Curve 2. Polymerization. Polymerization was performed at 37° with 0.4 ml of each sample using 0.5 mM GTP. B. Effect of photooxidation. Tubulin (final concentration, 1.8 mg/ml) was reacted with 50 μM methylene blue in 50 mM potassium phosphate buffer, pH 7.0, at 4° and the excess dye was removed by the addition of charcoal. Samples were dialyzed as in A. Curve 1, colchicine binding. Curve 2, polymerization. Conditions for these assays were the same as in A.

the residues which are modified by DEP are much less accessible to the reagent when tubulin is in the polymerized state. This was confirmed by another experiment. After reacting unpolymerized and polymerized tubulin with DEP and removing excess reagent with imidazolium chloride the solutions were dialyzed at 4°. The resulting tubulin solutions were compared in their ability to reassemble (Fig. 1B). Clearly tubulin derived from DEP-treated microtubules assembles to a much greater extent and at a greater rate than DEP-treated tubulin.

To determine whether the inhibition by DEP could be due to structural changes in the protein, the ability of tubulin to bind colchicine after treatment with the reagent was examined. Incubation of tubulin with 0.8 mM DEP for 6 minutes completely inhibited self-assembly whereas it produced only a 40% inhibition of colchicine binding (Fig. 3A).

Tubulin prepared by the Shelanski method consists of the 6S dimer and aggregates. In preparations from porcine brain the aggregate has sedimentation coefficient of 36S (7). We have found that in our beef brain preparations the aggregate is an 18-21 S component. Upon treatment with DEP to cause complete inhibition there was a shift in the relative amounts of the two components. In one experiment, for example, upon DEP treatment the amount of the aggregate dropped from 72% to 46% of the total and was accompanied by a corresponding increase in the 6S dimer. 5,5'-Dithio-bis (2-nitrobenzoic acid) (DTNB) was used to determine whether DEP caused modification of free sulfhydryl groups. Within experimental error, the number of sulfhydryl residues was unchanged by DEP modification.

Photooxidation of Tubulin. Photooxidation using a ratio of 3 moles of methylene blue per mole of tubulin resulted in a complete inhibition of self-assembly of tubulin within 20 minutes at 4° at pH 7.0, whereas colchicine binding was almost unaffected under these conditions (Fig. 3B). The rate of photoinactivation of tubulin increases with increasing pH, as shown in Fig. 4A. The pH rate profile for the photoinactivation process is shown in Fig. 4B. The shape of this curve with an inflection point at pH 6.5 indicates that the destruction of histidine residues (8) is the event responsible for loss of the ability of tubulin to self-assemble.

Photoinactivation of tubulin to 50% of its original polymerizing activity followed by amino acid analysis shows, in fact, that only 1-2 hisitidines per tubulin dimer are destroyed. The number of histidine residues destroyed by photooxidation was also determined with DEP. After photooxidation and removal of dye as described in Fig. 4 the protein was reacted with DEP in 0.02 M MES-0.1% SDS, pH 6.2 and the histidine concentration was determined by measuring the total increase in absorbance at 240 nm (3). These results showed that tubulin which had been 90% inactivated had lost a maximum of 3 histidine residues. The number of cysteine residues determined with DTNB were not changed after the photooxidation. In a series of experiments in which

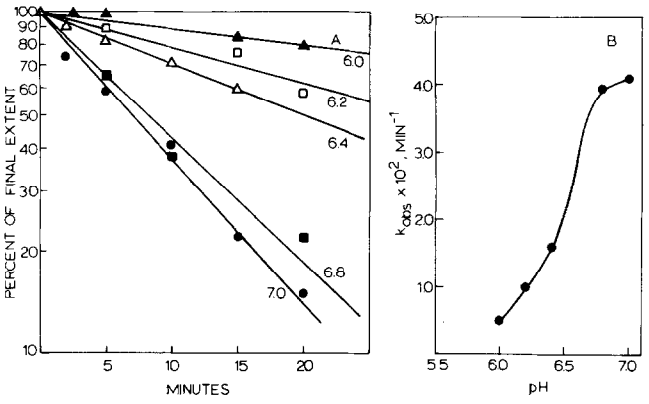

Fig. 4. Rate of photoinactivation as function of pH. Photooxidation of tubulin (final concentration, 12 to 17 μM) was carried out in the presence of 50 μM methylene blue in a buffer containing 25 mM MES and 25 mM potassium phosphate. After removing excess dye with charcoal, samples were dialyzed against reassembly buffer containing 1 M glycerol for 4 hours at 4°. Polymerization was then performed at 37° C using 0.5 mM GTP.

the tubulin was inactivated from 20 to 100%, it was found that GTP binding activity was unchanged. In a number of experiments the amount of [^{32}P]- or [^{3}H] GTP bound per tubulin dimer ranged between 0.5 to 1.

DISCUSSION

Chemical modification of tubulin either by DEP or by photooxidation with methylene blue inhibits the self-assembly reaction. Modification of histidine residues seems to be responsible for this inhibition. The partial recovery of activity by NH_2OH after complete inhibition by DEP strongly suggests that histidine residues are involved in the self-assembly of tubulin. The involvement of histidine residues in polymerization is further substantiated by the distinctive pH-rate profile of photoinactivation. However, the destruction of only a few histidine residues causes the inhibition. Sulfhydryl groups which are suggested to be functional in polymerization and in colchicine binding (9) were not modified either by photooxidation or by the reaction with DEP under the experimental conditions we used. The inhibition of polymerization by modification is unlikely to be due to the denaturation of the protein. This is indicated by the following

observations: (a) the colchicine-binding activity was not greatly affected when the polymerization was almost completely inhibited, (b) GTP binding was not changed by photooxidation, and (c) the sedimentation rates of molecular species present were not changed although there was a shift of relative amounts in 6S tubulin and aggregates. The results reported here could be explained by assuming that the modifications of a few histidine residues prevent the necessary interactions between tubulin dimers in the formation of microtubules. However, in view of the fact that the self-assembly of tubulin requires other proteins which copurify with tubulin in very small amounts (10, 11) this assumption may not be valid. Histidine modification could also result in a decreased interaction between tubulin and these proteins.

ACKNOWLEDGEMENT

This research was supported by NIH grant NS 11360 and by a grant from the University of Kansas General Research Fund. L. L. H. is a recipient of Career Development Award, CA 00114.

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