The molecular mechanism of interaction of Et_3Pb^+ with tubulin

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Triethyllead ion (Et_3Pb^+) was found to interact with 2 out of 18 thiol groups present in tubulin dimers. Specificity of the interaction was shown by the high affinity of Et_3Pb^+ to tubulin, by the fact that the 16 residual thiol groups in tubulin remained unaffected, and by the observation that other proteins with exposed thiol groups, e.g., actin, did not react with Et_3Pb^+. After complexation of the two thiol groups, tubulin *in vitro* had lost its capability for microtubule assembly. Likewise, polymerized tubulin disassembled on addition of the lead compound.

Triethyllead chloride *Microtubule assembly* *Sulfhydryl group* *Disulfide* *Thiol reagents*

1. INTRODUCTION

The in vitro polymerization of tubulin into microtubules is now a routine technique [1–3] and provides an excellent model system for studying protein self-assembly reactions. A number of drugs, among them alkaloids, antibiotics and other pharmaceutical agents, have been shown to bind to tubulin, thus preventing the assembly into microtubules. The list includes, besides the well known colchicine, colchicine derivatives and vinca alkaloids (review [4]), agents such as podophyllotoxin [5], griseofulvin [6], maytansine [7], rotenone [8], halothane and other anaesthetic drugs [9], methylbenzimidazolylcarbamates [10] and organomercurials such as methylmercury [11].

We have shown that trimethyllead ion and even more so Et_3Pb^+ inhibit microtubule assembly in vitro and cause depolymerization of preassembled microtubules [12,13]. Here we investigated in detail the molecular mechanism of interaction of Et_3Pb^+ with tubulin.

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DNPSSG, 2,4-dinitrophenylglutathionyl disulfide; Et_3Pb^+, triethyllead ion; MAPs, microtubule-associated proteins

2. MATERIALS AND METHODS

2.1. *Tubulin purification*

Microtubule protein was prepared from porcine brain by two cycles of assembly-disassembly as in [3] in a buffer (A) containing 0.1 M Pipes, 1 mM $MgCl_2$, 0.1 mM EDTA and 1 mM EGTA (pH 6.9). The separation of tubulin from MAPs was achieved by phosphocellulose chromatography according to [14]. Purity of the preparations was tested by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels as in [16].

2.2. *Determination of thiol groups in tubulin*

The reaction of tubulin with DNPSSG, prepared as in [15], or with DTNB was started by adding 20 μl of a 0.01 M solution of the reagent in 1% $NaHCO_3$ to 1 ml of 1.5–3.5 μM MAPs-free tubulin in buffer A. The reaction kinetics were followed by measuring the absorbance at 408 or 412 nm, respectively, against a blank without tubulin on an Aminco DW2 spectrophotometer. The molar extinction coefficient of the 2,4-dinitro-

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thiophenolate was $\epsilon_{408} = 12.7\ mM^{-1} \cdot cm^{-1}$, and that of the 3-carboxy-4-nitrothiophenolate $\epsilon_{412} = 13.6\ mM^{-1} \cdot cm^{-1}$. The molar extinction coefficient of tubulin was determined by measuring the protein concentration as in [17] and assuming $M_r = 110000$ (dimer); the value $\epsilon_{280} = 123.0\ mM^{-1} \cdot cm^{-1}$ was used in all cases to determine the tubulin concentration. In experiments with Et_3PbCl (kindly supplied by The Associated Octel Co. Ltd), the lead salt was added in specified concentrations (10–100 μM) 30 min before the reaction with DNPSSG or DTNB was started.

2.3. *Polymerization of tubulin*

Polymerization of MAPs-free tubulin (15 μM in buffer A with 20% dimethylsulfoxide) was started by the addition of GTP to a final concentration of 1 mM and warming the samples from 0 to 37°C. The kinetics of microtubule assembly was monitored by measuring the increase in turbidity at 350 nm [18] against a blank without tubulin. In inhibition experiments Et_3PbCl was added in specified concentrations immediately before GTP. The results of turbidity measurements were confirmed by electron microscopy as in [12].

The inhibition of tubulin assembly was counteracted with glutathione (Boehringer Mannheim) and *t*-butanethiol (Fluka Buchs, Switzerland) added as 0.4 M buffer A solution or 0.1 M methanolic solution, respectively. For gel filtration a Sephadex G-25 column (1 × 40 cm) was used equilibrated with buffer A.

2.4. *Actin*

Actin was prepared from rabbit muscle as in [19]. Polymerization was started by adding to G-actin (0.01 mM, in 0.1 mM ATP, 1 mM Tris–HCl; pH 7.4) $MgCl_2$ to a final concentration of 1 mM, and was followed by viscometry according to [15].

3. RESULTS

The tubulin preparation used for the analysis of thiol groups was >95% pure and free from microtubule associated proteins as shown in fig.1. Based on protein determination according to [17] we measured the molar extinction coefficience as $\epsilon_{280} = 123.0\ mM^{-1} \cdot cm^{-1}$, which is in good agreement with others [20,22]. Related on this value the number of sulfhydryl groups was found to be 17.9 ± 0.2 (mean ± SD, $n = 8$) sulfhydryl groups per tubulin dimer (fig.2a). When tubulin was assayed in the presence of a molar excess of Et_3Pb^+ a value of only 16.0 ± 0.2 ($n = 8$) thiol groups per tubulin dimer was obtained (fig.2b). Obviously, by interaction with Et_3Pb^+, 2 sulfhydryl groups (1.9 ± 0.2) were no longer available for the thiol reagents. The difference in thiol groups was never greater than 2 even in the presence of large excess

Fig.1. SDS-polyacrylamide gels of purified tubulin. (a) Twice-cycled porcine brain tubulin. (b) Phosphocellulose-purified tubulin.

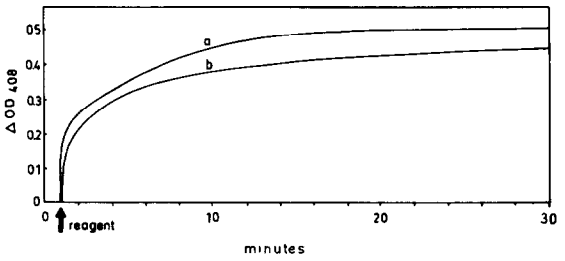

Fig.2. Reaction of excess DNPSSG with 1.9 μM tubulin in buffer A as followed by the increase in A at 408 nm (a) and the corresponding reaction in the presence of 10 μM Et_3Pb^+ (b).

of Et_3Pb^+. This indicates a very specific interaction of the toxin with two distinct thiol groups out of 18. The high specificity was further proved, when actin, another cytoskeletal protein with exposed thiol functions, was assayed under the same conditions. Actin possesses 5 thiol groups, their reactivities depending on the nucleotide and the physical state of the protein [15,21]. On the addition of Et_3Pb^+, no changes in the reactivity of these thiol functions was observed, in both the monomeric and filamentous form, neither was there any change in the rate or extent of actin polymerization (no pictures).

In contrast, addition of Et_3Pb^+ strongly affects the polymerization of tubulin. For example, the formation of microtubules as monitored by light scattering at 350 nm (fig.3a) was almost completely inhibited in the presence of 50 μM Et_3Pb^+ (fig.3b). At higher concentrations of Et_3Pb^+ (200 μM), a steep increase in turbidity was observed (fig.3c), however, not due to the formation of microtubules but of undefined aggregates of tubulin, as shown by electron microscopy (no picture). Undefined tubulin aggregates with a high contribution to the light scattering are also formed when Et_3PbCl is added to the polymerized form of tubulin. Destruction of microtubules is recognized by a decrease of light scattering as shown in fig.3d.

Important for the understanding of the molecular mechanism of interaction was the restoration of polymerizability of tubulin by addition of a large excess of thiols, e.g., *t*-butanethiol or glutathione (fig.4). The thiol-containing compounds do not act as reducing agents as shown by the following gel filtration experiment: tubulin, nonpolymerizable by Et_3Pb^+, was passed through a Sephadex G-25 column, which separated the protein from the lead compound. After ultrafiltration (to achieve the protein concentration necessary for assembly) the tubulin was found to be polymerizable as before the treatment (not shown). This excludes the possibility that nonpolymerizability is caused by the formation of a tubulin disulfide.

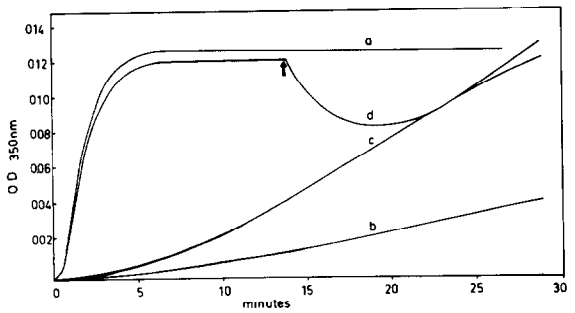

Fig.3. Inhibition of microtubule assembly and disassembly of preformed microtubules by Et_3Pb^+ as measured by the turbidity assay at 350 nm and 37°C. Concentration of tubulin: 15 μM. (a) Control, (b) 50 μM Et_3Pb^+, (c) 200 μM Et_3Pb^+, (d) addition of 200 μM Et_3Pb^+ (arrow) to preassembled microtubules.

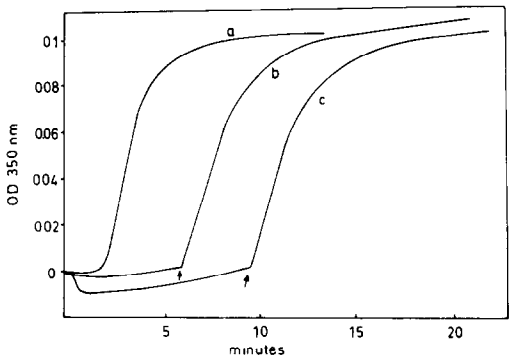

Fig.4. Restoration of microtubule assembly capacity on addition of excess thiols. Control (a), inhibition by 50 μM Et_3Pb^+ (b,c) restored by addition of 1 mM glutathione (b) or 1 mM *t*-butanethiol (c), at times indicated by arrows.

4. DISCUSSION

The sulfhydryl groups of tubulin have been established as essential for microtubule assembly. For the analysis of thiol groups of the present tubulin preparation a new thiol reagent, DNPSSG, was used, in addition to the well known Ellman's reagent, DTNB. In 8 experiments (tubulin of 3 different preparations) we determined, with either reagent and high reproducibility, 18 sulfhydryl groups per dimer. In the presence of Et_3Pb^+ the number of sulfhydryl groups was decreased by 2. This value was reproduced in all experiments, also in the presence of excess lead compound. We conclude that tubulin and Et_3Pb^+ form a complex of the tentative structure $(\alpha+\beta)$ tubulin $(S^- Et_3Pb^+)_2$, in which two thiolate groups of tubulin form ion pairs with the monovalent lead ions. In addition to the electrostatic interaction the 3 ethyl residues of Et_3Pb^+ probably interact with lipophilic domains

of the protein. This is strongly suggested by the fact that only large excess of various thiols added to the tubulin solution restored polymerizability. From the 100-fold excess required we can estimate that the additional lipophilic interaction increases the affinity of the two thiol groups of tubulins for Et_3Pb^+ by a factor of 10^2.

The possibility arises that the complex ($\alpha+\beta$) tubulin (S^{-2} $Et_3Pb^+)_2$ undergoes a redox reaction under formation of tubulin disulfide, ($\alpha+\beta$) tubulin (S-S), and hexaethyldilead, Et_3Pb-$PbEt_3$. This possibility was excluded by the gel filtration experiment described above, showing that after separation of the lead compound the tubulin is still in a polymerizable state. Further evidence for the exclusion of a disulfide was drawn from the fact that after the restoration of polymerizability with glutathione the equivalent of glutathione disulfide was not detectable in an NADPH/glutathione reductase assay (not shown).

By the interaction of Et_3Pb^+, the tubulin dimers lose their capability for microtubule assembly (fig.3). Tubulin complexed to Et_3Pb^+ forms aggregates which make their own contribution to light scattering. Electron microscopy shows a dense population of small granules but no microtubules.

In conclusion, among the 18 thiol groups present in the tubulin ($\alpha+\beta$) dimer only two are suited to react with Et_3Pb^+ at μM concentrations. The high affinity of Et_3Pb^+ for these two thiol groups may be understood by additional lipophilic interactions between the ethyl groups of lead salt and the protein. With the two thiol groups blocked tubulin is no longer capable of assembling into microtubules. The high specificity of interaction, which is further supported by the fact that Et_3Pb^+ does not react with the thiol groups of actin, places the toxic activity of Et_3Pb^+ in the vicinity of microtubule poisons of the alkaloid or antibiotic type rather than in relation to the typical reactivity of heavy metal ions.

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