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SUBSTOICHIOMETRIC INHIBITION OF MICROTUBULE FORMATION BY ACETALDEHYDE-TUBULIN ADDUCTS

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Abstract—We have shown previously that acetaldehyde forms stable covalent adducts with tubulin, resulting in impaired microtubule formation. The present study explored the mechanism responsible for impaired microtubule formation caused by the substoichiometric stable binding of acetaldehyde to tubulin. The free tubulin dimer was much more reactive with acetaldehyde than microtubules, binding more than twice as much aldehyde. The dimer also formed nearly twice as many stable adducts on its α-chain as on its β-chain, whereas microtubules exhibited an equal distribution of adducts between the two subunits. These data confirm that the α-chain of free tubulin, but not microtubules, has an accessible highly reactive lysine (HRL) residue that is a preferential target of acetaldehyde binding. Adduct formation with the HRL residue also correlated with impaired tubulin polymerization, and only 0.08 moles of acetaldehyde bound per mole of HRL was required for complete inhibition; however, adducts with other lysine residues (bulk adducts) did not affect assembly. Adducts to microtubule-associated proteins (MAPs) also impaired the assembly of tubulin, but were much less effective than HRL adducts. In a copolymerization assay, HRL-adducted tubulin, in addition to being itself assembly incompetent, also interfered with polymerization of normal (unadducted) tubulin. Bulk adducts did not alter assembly and were incorporated normally into the growing polymer. When tubulin was cleaved by the proteolytic enzyme, subtilisin, microtubule formation could readily take place in the absence of MAPs. In this polymerization system, HRL adducts, but not bulk adducts, still markedly inhibited assembly. When low concentrations of acetaldehyde (50 μM) were used to generate HRL adducts, an adduct on only 1 out of 20 tubulin molecules was sufficient to totally block polymerization. These findings indicate that substoichiometric amounts of acetaldehyde bound to HRL of tubulin can markedly inhibit microtubule formation via direct interference of dimer–dimer interactions, and further suggest that low concentrations of acetaldehyde could generate sufficient amounts of HRL adducts in cellular systems to alter microtubule formation and function.

We have proposed that acetaldehyde, the first metabolite of ethanol oxidation, plays a role in alcoholic liver injury by forming stable covalent adducts with hepatocellular proteins [1]. Numerous studies have shown that acetaldehyde reacts with a variety of proteins to form stable and unstable adducts [2–4] and that formation of acetaldehyde-protein adducts occurs during ethanol oxidation in the liver [5, 6]. Unstable protein adducts have been identified to be Schiff bases involving the carbonyl group of acetaldehyde and amino groups of proteins including the ε-amino group of certain lysine residues [7]. Stable adducts have not been fully chemically characterized, but participation of lysine residues in stable binding has been indicated [7, 8]. In addition to establishing the chemical structures of these adducts, identification of target proteins of acetaldehyde binding in the liver as well as the functional consequences of such binding, which could lead to hepatocyte injury, have been the subject of investigation and are important considerations relevant to the verification of our original hypothesis.

To gain information relating adduct formation to functional impairment and eventually to liver injury, we have undertaken studies to examine in detail the acetaldehyde binding properties of model proteins and have related this binding to effects on biologic function. Initially, we showed that enzymes with lysine residues that are essential for catalytic activity are especially susceptible to inhibition by acetaldehyde binding [4]. More recently, we have concentrated our efforts on characterizing the binding of acetaldehyde to tubulin and the subsequent effect on tubulin assembly. Tubulin was chosen as a model protein because many microtubule-dependent processes in the liver are impaired by ethanol administration and these impairments appear to be mediated via acetaldehyde [reviewed in Ref. 9], and because previous work by Sternlicht and coworkers [10–12] has indicated that the α-chain of the dimeric tubulin molecule contains a special lysine residue that is both unusually reactive toward aldehydes and appears to be critically important to tubulin polymerization. In agreement with their studies, we

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† Abbreviations: EGTA, [ethylenebis (oxyethylenenitrilo)] tetraacetic acid; HRL, highly reactive lysine; MAPs, microtubule-associated proteins; MES, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; PC-tubulin, MAP-free tubulin prepared by phosphocellulose chromatography; PC-buffer, for phosphocellulose chromatography consisting of 25 mM MES and 2 mM EGTA at pH 6.7; RAB, reassembly buffer consisting of 20 mM MES, 70 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 2 M glycerol at pH 6.7; and SDS, sodium dodecyl sulfate.

have shown recently that lysine residues in tubulin can be divided into two general classes with regard to their reactivity toward acetaldehyde; those of normal reactivity (bulk lysines) and the highly reactive lysine (HRL†) located on the α-subunit of tubulin [13, 14]. Further studies showed that the HRL adducts were more deleterious to tubulin polymerization than bulk adducts, and that HRL adduct formation on only a small fraction of the total pool of tubulin was sufficient to totally inhibit microtubule formation [15]. The purposes of the current studies were to further establish and characterize the inhibition of microtubule formation caused by the substoichiometric stable binding of acetaldehyde to tubulin and to determine whether this inhibition involves alteration of tubulin-tubulin or tubulin-microtubule-associated protein (MAP) interactions.

MATERIALS AND METHODS

Materials. Fresh adult beef brains were obtained at the time of slaughter and were rapidly transported to the laboratory on ice. [1,2-^{14}C]Acetaldehyde (3.3 mCi/mmol) and Aquasol were purchased from the New England Nuclear Co. (Boston, MA). Acetaldehyde was received from the manufacturer frozen as an aqueous solution (1 mCi/mL), thawed, and diluted to 200 μCi/mL with distilled water, rapidly refrozen and stored at −80°. GTP type IIS, [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA), 2-(*N*-morpholino) ethanesulfonic acid (MES), non-radioactive acetaldehyde and subtilisin BPN were purchased from the Sigma Chemical Co. (St. Louis, MO). Cellulose phosphate (type P-11) was from Whatman, Inc. (Clifton, NJ). Glycerol was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Electrophoresis equipment and supplies were obtained from Bio-Rad Laboratories (Richmond, CA). Membrane filtration cones (CF-50A) were products of Amicon (Danvers, MA). All other reagents and supplies were of analytical grade.

Tubulin preparation. Microtubule protein was isolated and purified by repetitive cycles of assembly–disassembly from bovine brain following a modification of the method of Shelanski *et al.* [16] as previously described [13]. Free GTP was removed from the cycle-purified tubulin by gel filtration (Sephadex G-25). This preparation was stored under liquid nitrogen in reassembly buffer (RAB) consisting of 20 mM MES, 70 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$ and 2 M glycerol, pH 6.7. The purity of tubulin and microtubules was confirmed using sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and electron microscopy, respectively [13]. Our protein preparation typically contained 80–90% tubulin (α:β dimer) with other bands consisting mainly of high-molecular weight microtubule-associated proteins (MAPs). Tubulin free of MAPs (PC-tubulin) was prepared by phosphocellulose chromatography as described by Szasz *et al.* [10], using precycled cellulose phosphate in PC buffer consisting of 25 mM MES and 2 mM EGTA, pH 6.7. In cases where PC- tubulin was to be stored, Mg^{2+} and glycerol were added to final concentrations of 10 mM and 0.6 M, respectively. MAPs were eluted off the cellulose phosphate column using 0.8 M NaCl in PC-buffer and dialyzed versus PC-buffer. Both PC-tubulin and MAPs were concentrated with membrane filter cones and stored under liquid nitrogen. Protein concentrations were measured by the method of Lowry *et al.* [17], using bovine serum albumin as a standard and applying a correction factor of 1.2 for tubulin. The validity of this correction factor was independently verified by using absorbance measurements and known molar extinction coefficients [13].

Acetaldehyde binding. Standard reaction mixtures consisted of either free tubulin (cycle-purified) or polymerized tubulin (microtubules). Appropriate dilutions of [^{14}C]acetaldehyde were added to the reaction mixtures to give the final desired concentrations. Incubations were carried out in RAB buffer with the final glycerol concentration adjusted to 0.6 M at either 4° or 37° with constant agitation. Reaction vessels were polyethylene and sealed to minimize the loss of volatile radioactivity. At the higher concentrations of acetaldehyde (e.g. 5 mM), incubations were conducted at 37° for up to 120 min, whereas at low concentrations (e.g. 50 μM) incubations were conducted for several hours at 4° in order to achieve sufficient binding of acetaldehyde and to maintain tubulin stability. Postincubation, free and unstable-bound acetaldehyde were separated from stable-bound acetaldehyde by exhaustive dialysis or by gel filtration as previously described [2]. These methods have been shown to adequately quantify stable acetaldehyde-protein adducts [2, 8].

Preparation of bulk, HRL and total (bulk plus HRL) acetaldehyde-tubulin adducts. These acetaldehyde-modified tubulins were prepared as previously described, and this procedure took advantage of the observation that the HRL is reactive when tubulin is in the free dimeric state and unreactive when it is in the microtubule form [15].

Briefly, bulk lysine-adducted tubulin was prepared by first allowing free tubulin (6.00 mg/mL) to polymerize by the addition of 0.5 mM GTP at 37° for 30 min. Half of the formed microtubules served as controls, while the other half was bound with [^{14}C]acetaldehyde at 37° for various time periods. After incubation, both samples were dialyzed against assembly buffer containing 2 mM GTP and 2 M glycerol for 6 hr at 37°. Upon completion of dialysis, the acetaldehyde-modified microtubules (bulk adducts) and control microtubules were placed in separate homogenizing vessels and homogenized at 1000 rpm and placed on ice for 30 min to assure disassembly back to free tubulin. The amount of bulk adducts was quantified and the bulk-adducted tubulin and the identically treated control were subjected to the polymerization assay. HRL adducts on tubulin were prepared by first allowing free tubulin to assemble into microtubules as described above. Microtubules (4.00 mg/mL) were then incubated with 5 mM nonlabeled acetaldehyde at 37° for 90 min. Dialysis was then performed in the presence of GTP at 37° to remove free acetaldehyde. Maintenance of tubulin in the microtubule state throughout the experiment was confirmed at various points by monitoring the absorbance at 350 nm. The microtubules were then disassembled as described

above. The resulting bulk-adducted tubulin (3.00 mg/mL) was then incubated in the presence and absence of [^{14}C]acetaldehyde at 4°. A temperature of 4° was used to assure that the GTP present did not promote reassembly. At various times, the moles of [^{14}C]-acetaldehyde bound to tubulin was determined and the ability of the HRL-adducted tubulin to assemble was compared to the bulk-adducted tubulin. Total adducts (bulk plus HRL) were prepared by incubating free dimeric tubulin (3.00 mg/mL) at 37° with [^{14}C]-acetaldehyde. At various time points, the moles of [^{14}C]acetaldehyde bound per mole of tubulin were measured as was the ability of the modified tubulin to form microtubules compared with control tubulin incubated for an equal amount of time without acetaldehyde.

Tubulin polymerization assay. Tubulin assembly was monitored by measuring the increase in absorbance at 350 nm [18], using a Beckman model DU-70 spectrophotometer. An increase in turbidity at 350 nm has been shown to be directly proportional to the amount of microtubules formed [19]. Polymerization was initiated by warming a solution of depolymerized tubulin to 37° and adding 0.5 mM GTP. Recently, we have shown that unstable adducts do not cause inhibition of polymerization [20]; therefore, some of the acetaldehyde-modified tubulin samples were submitted to polymerization assay directly without attempting to remove unstable adducts. Appropriate controls consisted of samples of tubulin that had been incubated and manipulated exactly as acetaldehyde-modified samples, except that no acetaldehyde was added. The formation of microtubules during the polymerization assays was confirmed by electron microscopy [18].

In some cases, microtubule assembly was also determined by sedimentation [21]. Following incubation at 37°, reaction mixtures were ultracentrifuged (28,000 *g*) to separate the microtubules from the soluble, free tubulin. The protein content and radioactivity were then determined in the pellet and supernatant.

Proteolytic digestion. Limited proteolysis was performed with subtilisin BPN at a concentration of 1% (w/w) tubulin [22]. Both free PC-tubulin and acetaldehyde-adducted PC-tubulin were used as substrates for the protease. Digestion was performed in 50 mM MES buffer (pH 7.0) containing 2 mM GTP, 0.5 mM $MgCl_2$, 1 mM mercaptoethanol and 4 M glycerol at 30° for 30 min. The reaction was terminated by adding phenylmethyl sulfonyl fluoride [23]. The specificity of the cleavage of tubulin by subtilisin was verified by SDS–polyacrylamide gel electrophoresis.

Gel electrophoresis. Various reaction mixtures were subjected to SDS–polyacrylamide gel electrophoresis using the modified Laemmli system [13] and stained with Coomassie blue. The α and β bands of tubulin were well resolved using this system. The bands corresponding to the α- and β-subunits of tubulin were cut out and solubilized using 0.2 mL of 30% H_2O_2 at 75°. After cooling to room temperature, 0.5 mL of thiourea (2 g/100 mL) was added to each sample directly into a scintillation vial. Samples were counted with 15 mL of Aquasol after neutralization with acetic acid. The relative distribution of radioactivity between the α- and β-polypeptides was expressed as a ratio.

RESULTS

Previous studies have shown that in the depolymerized state, tubulin forms both bulk and HRL adducts with acetaldehyde, but in the microtubular state, it forms only bulk adducts with the HRL being protected [15]. This phenomenon was again confirmed in this study. For example, when free tubulin was incubated with 5 mM acetaldehyde for 90 min, 0.189 ± 0.05 moles of acetaldehyde was bound per mole of tubulin, whereas under identical reaction conditions tubulin, in the polymerized state, formed less than one-half the stable adducts (0.088 ± 0.05 mole/mole tubulin). Furthermore, consistent with the HRL location on the α chain [15], the free tubulin dimer bound 1.85 ± 0.05 times more acetaldehyde on its α chain than on its β chain, whereas microtubules exhibited an equal distribution of stable adducts between the two subunits (α/β ratio of adducts 1.0 ± 0.02). These results allowed us to specifically prepare and quantify these different tubulin adducts (bulk or HRL) and to study their effects on tubulin assembly. In general agreement with our previous studies [15], 0.15 and 0.08 moles of acetaldehyde bound per mole of tubulin for total (HRL plus bulk) and HRL adducts, respectively, completely inhibited tubulin assembly into microtubules; however, bulk adducts up to 0.5 moles of acetaldehyde bound per mole of tubulin did not affect polymerization.

Since only a small mole ratio of acetaldehyde binding to tubulin was sufficient to completely inhibit the polymerization process, it appeared that HRL-adducted tubulin was not only polymerization incompetent, but also by its presence, prevented the assembly of unmodified tubulin as well. Therefore, a copolymerization assay was developed to study in depth this substoichiometric inhibition of assembly. Using this copolymerization assay, we were able to study the effects of modified tubulin on the assembly of normal (unmodified) tubulin. The components of the reconstituted assay system were control (unmodified) PC tubulin (2.5 mg), MAPs (0.8 mg) and the test (modified) PC tubulin (0.5 mg). The reconstitution of this system (using control PC tubulin as the test protein) resulted in tubulin polymerization that was similar to that observed for the same amount of cycle-purified tubulin assayed immediately following its isolation from brain (Fig. 1). Two additional control samples included a deletion control (the addition of the 0.5 mg of test PC tubulin was omitted) and a denatured control (0.5 mg of test PC tubulin was thermally denatured at 37° for 24 hr and was unable to bind colchicine). The deletion control polymerized normally, but a smaller total change in absorbance was observed (Fig. 1). This observation can be explained by the decreased amount of PC tubulin in this system, but indicates that we are still above the critical tubulin concentration for polymerization in this system. Assembly of the denatured control also appeared normal, and the observed total change in absorbance fell between the reconstituted and deletion controls

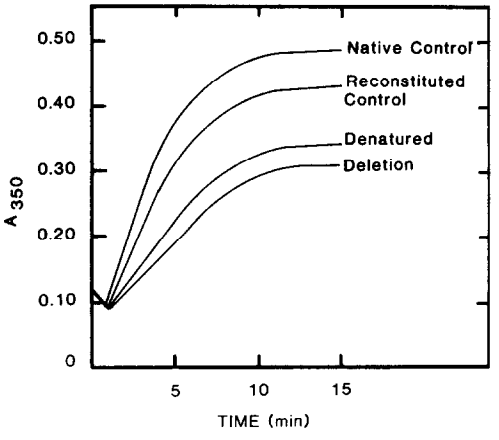

Fig. 1. Standardization and verification of the copolymerization assay. Cycle-purified tubulin (native control) was used for establishing the standard polymerization curve, and then was separated into its constituents, PC tubulin and MAPs, by phosphocellulose chromatography as described in Materials and Methods. All samples were subjected to the standard polymerization assay at 37° by the addition of 0.5 mM GTP. The native control was 3.8 mg/mL of cycle-purified tubulin and yielded the standard polymerization curve. The reconstituted control was the recombination of 3.0 mg/mL control PC tubulin and 0.8 mg/mL MAPs. The denatured control was the mixture of 2.5 mg/mL control PC tubulin, 0.5 mg/mL thermally denatured PC tubulin (incubated at 37° for 24 hrs) and 0.8 mg/mL MAPs. The deletion control was the recombination of 2.5 mg/mL control PC tubulin and 0.8 mg/mL MAPs. These samples were observed for their ability to form microtubules by measuring the change in absorbance at 350 nm. The polymerization curves shown are a typical representation of 6 determinations.

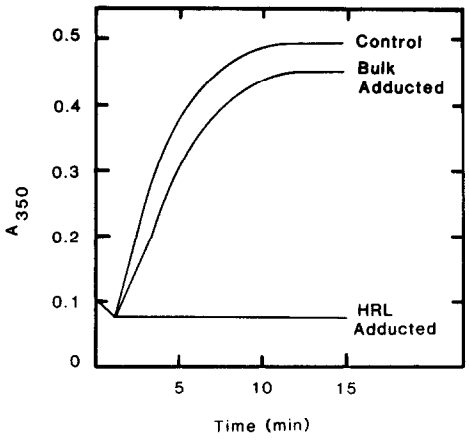

Fig. 2. Copolymerization of bulk-adducted and HRL-adducted tubulin with unmodified tubulin. Tubulin (6.00 mg/mL) and microtubules (6.00 mg/mL) were incubated with 5 mM acetaldehyde at 37° for a length of time to produce 1 mole acetaldehyde adduct per mole of tubulin. Free acetaldehyde and unstable acetaldehyde adducts were removed by gel filtration. HRL-adducted tubulin and bulk-adducted tubulin were then obtained as described in Materials and Methods, and the MAPs removed by phosphocellulose chromatography to yield HRL-adducted and bulk-adducted PC tubulin. The control sample was the recombination of 3.0 mg/mL PC tubulin (unmodified) and 0.8 mg/mL MAPs. The bulk-adducted sample was a mixture of 2.5 mg/mL control PC tubulin, 0.5 mg/mL bulk-adducted PC tubulin and 0.8 mg/mL MAPs. The HRL-adducted sample was a mixture of 2.5 mg/mL control PC tubulin, 0.5 mg/mL HRL-adducted PC tubulin and 0.8 mg/mL MAPs. All polymerization assays were conducted as stated in Materials and Methods. The polymerization curves presented are a typical representation of 6–8 determinations.

(Fig. 1). These data indicate that some of the denatured PC tubulin was excluded and some incorporated into the growing microtubule polymer; however, denatured PC tubulin showed no inhibitory influence on the ability of the control PC tubulin to form microtubules.

Using this copolymerization assay system, we investigated the effects of bulk and HRL adducts on the assembly of unmodified tubulin. With the addition of bulk-adducted tubulin to the system, no inhibitory effect on assembly was observed (Fig. 2), indicating that bulk adducts of tubulin had no detrimental effect on the ability of control tubulin to polymerize and apparently also retained its own functional capacity to assemble. On the other hand, when HRL-adducted PC tubulin was added to the system, compete inhibition of assembly was observed (Fig. 2). These results show that not only was HRL-adducted tubulin unable to polymerize, but that it also interfered with the assembly of normal (unadducted) tubulin. Additional experiments, in which labeled bulk or HRL adducts were added to the copolymerization assay and the microtubules separated from the free soluble tubulin by ultracentrifugation after polymerization had taken place, allowed us to determine the distribution of protein content and radioactivity between the free tubulin (supernatant) and the microtubules (pellet). In the case of bulk adduct addition, 85% of the protein and 80% of the radioactivity were located in the pellet, whereas when HRL adducts were added, only 17% of the protein and 7% of the radioactivity were located in the pellet (Fig. 3). These data further show that bulk-adducted tubulin copolymerizes normally, but that HRL-adducted tubulin inhibits the assembly process.

Most of the data presented so far suggests that HRL adducts impair assembly via alteration in tubulin dimer–dimer interactions; however, when acetaldehyde adducts with MAPs were added to unmodified tubulin, impaired assembly was also observed (Table 1). But in this case, tubulin polymerization was much less sensitive to adducted MAPs than to adducted tubulin since ten times the level of adduct formation to MAPs compared to tubulin had less than half the inhibitory effect (Table 1). Therefore, to distinguish between alterations in dimer–dimer interactions and tubulin–MAPs interaction as the major mechanism for inhibited assembly, we adapted a system to study tubulin polymerization that did not require the presence of MAPs. This assay system included the treatment of

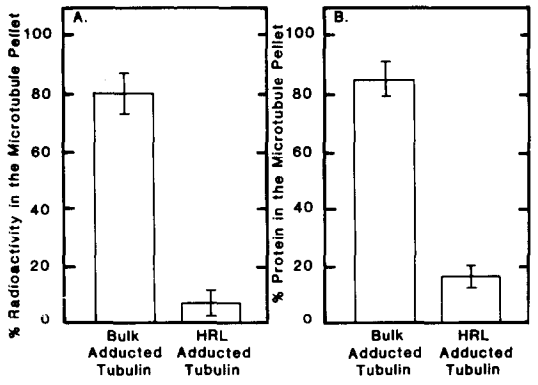

Fig. 3. Influences of HRL-adducted PC tubulin and bulk-adducted PC tubulin on tubulin polymerization as determined by the sedimentation assay. Tubulin and microtubules were incubated at 37° with 5 mM [^{14}C]-acetaldehyde for a length of time to produce 1 mole acetaldehyde per mole tubulin of stable adduct. Labeled HRL adducts and bulk adducts of PC tubulin were isolated as described in Materials and Methods. The experimental assay was done using two conditions: 2.5 mg/mL control PC tubulin, 0.5 mg/mL of labeled bulk-adducted PC tubulin and 0.8 mg/mL MAPs; and 2.5 mg/mL control PC tubulin, 0.5 mg/mL of labeled HRL-adducted PC tubulin and 0.8 mg/mL MAPs. Both samples were allowed to polymerize as described in Materials and Methods. Upon completion, each sample was centrifuged (28,000 *g*) to separate soluble nonpolymerized tubulin from insoluble microtubules. The microtubules (in the pellet) were then solubilized in 0.5 mL of 0.5 N NaOH at 75°. Panel A shows the percentage of radioactivity located in the solubilized pellets corresponding to the amount of bulk-adducted PC tubulin or HRL-adducted PC tubulin incorporated into the microtubules. Panel B shows the percentage of total protein found within the solubilized microtubule pellet. Results are shown as means ± SEM for 6–8 determinations.

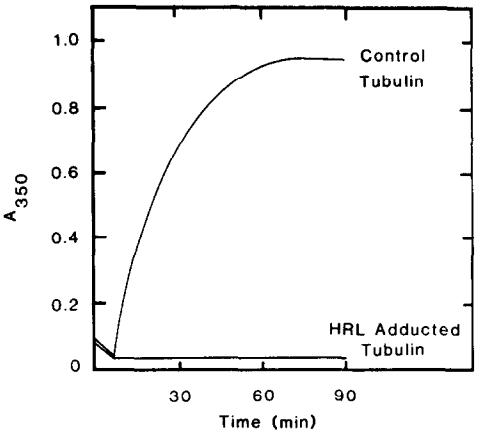

Fig. 4. The effects of HRL adducts on the subtilisin-induced polymerization of tubulin in a MAP-free system. HRL-adducted PC tubulin was prepared as described in Materials and Methods. Control PC tubulin was prepared identically, but was not exposed to labeled acetaldehyde. The HRL-adducted PC tubulin (2.00 mg/mL) and the control PC tubulin (2.00 mg/mL) were then exposed to 2 mM GTP and subtilisin proteolysis in the absence of MAPs, and the changes in absorbance at 350 nm were recorded at 30°. The polymerization curves presented are a typical representation of 4–6 determinations.

PC tubulin with the proteolytic enzyme, subtilisin, which has been shown to remove a small fragment from the carboxyl terminus of the α- and β-chains of tubulin [22]. This cleavage removes the natural inhibition of polymerization and allows microtubule formation to take place in the absence of MAPs or chemical agents, such as Me_2SO [22, 23]. Using this system, we observed that HRL-adducted PC tubulin still completely inhibited the polymerization process (Fig. 4). However, bulk-adducted PC tubulin polymerized normally in this system (data not shown). SDS–polyacrylamide gel electrophoresis of the subtilisin-treated tubulins showed that cleavage of HRL, bulk and control PC tubulin by this enzyme was identical in all three cases (data not shown). These data demonstrate that HRL adducts of tubulin impair assembly via alterations in dimer–dimer interaction; however, it does not completely exclude alterations in MAP–tubulin interactions as a minor contributing factor in this impairment.

Since acetaldehyde concentrations in the liver

Table 1. The effects of acetaldehyde-tubulin adducts and acetaldehyde-MAP adducts on microtubule formation

Protein combinations*	Binding†		% Inhibition‡
	nmol acetaldehyde/mg tubulin	nmol acetaldehyde/mg MAPs	
Adducted PC tubulin plus adducted MAPs	1.6 ± 0.39	15.6 ± 2.90	100
Control PC tubulin plus adducted MAPs	0	15.6 ± 2.90	40
Adducted PC tubulin plus control MAPs	1.6 ± 0.39	0	100

Acetaldehyde (5 mM) binding experiments were performed with cycle-purified tubulin (tubulin with MAPs) at 37° for 90 min. Post incubation, the tubulin was passed through a G-25 gel filtration column to remove free acetaldehyde. The tubulin and MAPs were then isolated by phosphocellulose chromatography.

* PC tubulin and MAPs (80%:20% w/w) were recombined at a final protein concentration of 3.8 mg/mL.

† [^{14}C]Acetaldehyde binding was determined following phosphocellulose separation of PC tubulin and MAPs as described in Materials and Methods, and results are expressed as means ± SEM of 6–8 determinations.

‡ The inhibition of tubulin polymerization was determined by comparing the change in absorbance at 350 nm of the different protein combinations as compared to identically treated controls with the exception of acetaldehyde exposure.

during *in vivo* ethanol oxidation are in the micromolar range [24], we focused this last set of experiments to examine the effects of acetaldehyde binding on tubulin assembly using low ("relevant") concentrations of the aldehyde. Because of the temperature sensitivity of tubulin *in vitro* and because of the extended time periods required to obtain adequate binding, these binding experiments were conducted at 4°. When tubulin was treated with 200 μM acetaldehyde under conditions that yielded total (bulk + HRL) adducts, inhibition of tubulin assembly correlated with adduct formation (Fig. 5). Complete inhibition was observed, under these conditions, when 0. 11 moles of acetaldehyde bound per mole of tubulin. This mole ratio of binding (0.11) required for complete inhibition of polymerization compared favorably to that of 0.15 which was observed when high concentrations (e.g. 5 mM) were used. When HRL adducts were generated using a concentration of 50 μM acetaldehyde, HRL binding also correlated to impaired assembly (Fig. 5). In this case, the 0.05 moles of acetaldehyde bound per mole HRL required for complete inhibition also compared favorably to the value (0.08) obtained using higher concentrations of the aldehyde. These results confirm that adduct formation and not the reaction conditions or acetaldehyde concentrations used is the most important factor in determining the degree of impaired tubulin polymerization and further emphasize the substoichiometric effect of acetaldehyde binding on microtubule formation.

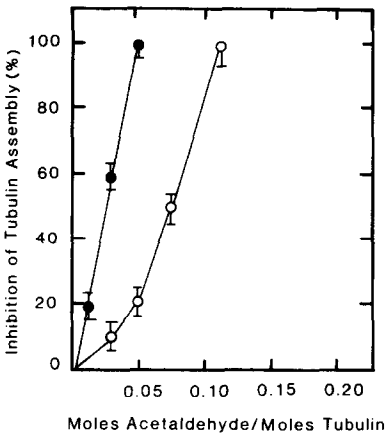

Fig. 5. The inhibition of tubulin polymerization by stable adducts formed by low, "physiological" concentrations of acetaldehyde. The effects of total adducts (bulk plus HRL) on tubulin assembly were determined by incubating cycle-purified tubulin (3.00 mg/mL) at 4° with 200 μM [^{14}C]-acetaldehyde. At various time points, the moles of [^{14}C]-acetaldehyde bound per mole of tubulin were measured as was the ability of the modified tubulin to form microtubules compared with control tubulin incubated for an equal amount of time without acetaldehyde. The effects of HRL adducts on assembly were determined by first allowing free tubulin to assemble into microtubules. Microtubules (4.00 mg/mL) were then incubated at 37° for 2 hr with 5 mM unlabeled acetaldehyde. Following dialysis and depolymerization procedures as described in Materials and Methods, the resulting unlabeled bulk-adducted tubulin (with HRL reactive) was then exposed to a [^{14}C]-acetaldehyde concentration of 50 μM. At various times during the 4° incubation, [^{14}C]acetaldehyde adduct formation and the ability of the adducted tubulin to polymerize were determined. The ability of the HRL-adducted tubulin to polymerize was compared to an identically treated control with the exception of exposure to labeled acetaldehyde. The results represent the means ± SEM of 6–8 determinations for each acetaldehyde concentration. Key: (○) total adducts; and (●) HRL adducts.

DISCUSSION

The results of the present study demonstrate the special acetaldehyde-binding properties of the cytoskeletal protein, tubulin, and further indicate that microtubule formation is especially susceptible to inhibition by low level acetaldehyde-tubulin adduct formation.

Previous studies have shown that acetaldehyde can bind to proteins, including tubulin, and form both unstable and stable adducts [2–4, 13, 14]. The epsilon-amino group of internal lysines and the amino group of N-terminal amino acids appear to be the major residues that participate in aldehyde binding [7, 13, 25]. Unstable adducts are mainly Schiff bases [2, 7], but the chemical identity of stable adducts has not been established. It does appear, however, that stable adducts formed in the absence of a reducing agent, such as $NaCNBH_3$, are distinctly different from the ethylated amino groups formed under reductive conditions [7]. Therefore, all the acetaldehyde-binding experiments in this study were conducted in the absence of a reducing agent to more closely simulate the physiological situation.

In agreement with a previous study [15], tubulin in its free dimeric state exhibited markedly different binding properties than in its polymerized form. The tubulin dimer bound more than twice the acetaldehyde than did microtubules when the concentrations of acetaldehyde and time of incubation were adjusted to result in the minimum amount of binding sufficient to completely inhibit tubulin assembly. Under these same conditions, the α-chain bound nearly twice the amount of acetaldehyde than the β-chain when the dimer reacted with acetaldehyde, despite the essentially equal number of amino groups in the two chains [26]. An equal distribution of stable adducts between the two chains was observed when binding occurred to microtubules. Overall, these results further confirm that the α-chain of free tubulin, but not microtubules, has an accessible HRL residue as previously indicated by Szasz *et al.* [10, 12] and Jennett *et al.* [13–15]. Therefore, the HRL on the α-chain appears to be a preferential site of acetaldehyde binding to tubulin and likely accounts for the unique binding properties of this protein. Furthermore, it is likely that acetaldehyde adducts to HRL are necessary for impaired tubulin assembly and that only substoichiometric binding to HRL is sufficient to totally inhibit polymerization.

A copolymerization assay was used in this study to further characterize the apparent substoichiometric

inhibition of assembly by acetaldehyde-HRL adducts of tubulin. The results obtained with this assay showed that HRL-adducted tubulin, in addition to being itself assembly incompetent, also interfered with polymerization of normal (unadducted) tubulin. On the other hand, bulk-adducted tubulin copolymerized normally with unadducted tubulin. These conclusions were supported by measurements of copolymerization by both turbidity and sedimentation assays. The latter assay clearly showed that HRL-adducted tubulin was not itself incorporated into the growing polymer and impaired the polymerization process and, conversely, that bulk-adducted tubulin was incorporated into microtubules and did not affect assembly. Proper controls verified that the reconstituted system used in the copolymerization assay behaved appropriately and especially assured us that we were above the critical concentration of tubulin for polymerization in all cases. A previous report by Zeeberg *et al.* [27] did not indicate an effect of acetaldehyde-treated tubulin on its ability to copolymerize with normal tubulin. However, their treatment consisted of reaction conditions of low temperature and short incubation times followed by reduction with $NaBH_4$. Their conditions would likely favor bulk adduct formation and would be in agreement with our findings which showed no effect of bulk adducts on coassembly.

The prominent role of HRL adducts in impaired tubulin assembly strongly suggests that dimer–dimer interactions are altered in such a way that inhibits the polymerization process. However, the data indicating that adducts to MAPs also can decrease the assembly of unadducted tubulin, although to a much lesser extent than HRL adducts to α-tubulin (Table 1), does not rule out the possibility that altered dimer–MAP interactions could also be a factor in impaired assembly. To distinguish between these two possibilities, an assay system was used that did not require the presence of MAPs for polymerization to take place. This system consisted of treatment of tubulin with the proteolytic enzyme, subtilisin, which cleaves a small fragment at the C-terminal region of both the α- and β-subunits. This treatment results in the increased ability for tubulin to assemble and, most importantly, does not require the presence of MAPs to facilitate polymerization [22, 23, 28]. When this assay system was used, HRL-adducted tubulin markedly impaired assembly, whereas bulk adducts had no effect. Therefore, in this MAP-free system, HRL adducts retained their ability to markedly inhibit polymerization, indicating that altered dimer–dimer interactions are mainly responsible for decreased microtubule formation. This, however, does not rule out a possible minor contribution by adducted MAPs to impaired assembly. The mechanism by which HRL adducts inhibit tubulin assembly via alterations in dimer–dimer interactions is unknown, but could be related to the mechanism that describes the substoichiometric inhibition of polymerization induced by the classical antimicrotuble agent, colchicine [29].

When low concentrations of acetaldehyde were used to generate tubulin adducts, impaired assembly was also evident (Fig. 5), strongly supporting the conclusion that the extent of adduct formation, especially HRL adducts, is the most important factor governing the degree of impaired microtubule formation. When a concentration of 50 μM acetaldehyde (levels in the range of those observed in the liver during ethanol oxidation [24]) were used to form HRL adducts, an adduct on only 1 out of 20 tubulin molecules was sufficient to totally block polymerization. This further emphasizes the substoichiometric nature of this inhibition and indicates that the HRL residue is a very selective target for binding at low aldehyde concentrations. These findings suggest that in cellular systems, such as the hepatocyte, low concentrations of acetaldehyde could generate sufficient amounts of HRL adducts to alter microtubule formation and function.

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