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INTERACTION OF ETHACRYNIC ACID WITH BOVINE BRAIN TUBULIN

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Abstract—Ethacrynic acid is a diuretic agent that reacts with sulfhydryl groups in proteins, and which shows promise of effectiveness in the treatment of glaucoma. Ethacrynic acid is a known inhibitor of microtubule assembly *in vitro* (Xu *et al.*, *Arch Biochem Biophys* **296:** 462–67, 1992). We have used *N,N'*-ethylenebis (iodoacetamide) (EBI) as a probe to examine the sulfhydryl groups of tubulin; EBI can form two intra-chain cross-links in β-tubulin. One of these, β*, connects Cys^{239} with Cys^{354}; the other, β^{s}, joins Cys^{12} with either Cys^{201} or Cys^{211} (Little and Ludueña, *EMBO J* **4**: 51–56, 1985; *Biochim Biophys Acta* **912**: 28–33, 1987). Formation of β* inhibits microtubule assembly *in vitro*, consistent with the hypothesis that Cys^{239} has an assembly-critical sulfhydryl (Bai *et al.*, *Biochemistry* **28**: 5606–5612, 1989). We have examined the interaction of ethacrynic acid with the sulfhydryl groups of bovine brain tubulin. We found that 130 μM ethacrynic acid gave half-maximal inhibition of assembly, but had no effect on the formation of the β* cross-link by EBI. Ethacrynic acid, however, did inhibit substantially formation of the β^{s} cross-link at this concentration and half-maximally inhibited it at approximately 185 μM. Half-maximal inhibition of the alkylation of tubulin sulfhydryls by iodo [^{14}C]acetamide was obtained at an ethacrynic acid concentration in the range of 190–325 μM. These results indicate that ethacrynic acid can inhibit microtubule assembly by reacting with sulfhydryl groups other than those of Cys^{239} and Cys^{354} and suggest that other sulfhydryl groups in tubulin could be assembly-critical. These results also raise the possibility that these other assembly-critical sulfhydryls may be those of Cys^{12}, Cys^{201} or Cys^{211}.

Key words: tubulin; microtubule assembly; ethacrynic acid; protein cross-linking; *N,N'*-ethylenebis(iodoacetamide); sulfhydryl groups

Microtubules, organelles playing critical roles in a variety of cellular processes, are composed of the 100 kDa protein tubulin, a dimer of 50 kDa α and β subunits [1]. Microtubule assembly both *in vitro* and *in vivo* is highly sensitive to inhibition by sulfhydryl-oxidizing reagents; reaction of a few sulfhydryl groups is sufficient to inhibit microtubule assembly [2]. One such assembly-critical sulfhydryl group has been identified, namely, Cys^{239} on β-tubulin [3]. Reaction of Cys^{239} with DCBT§ is sufficient to inhibit assembly [3]. Similarly, the bifunctional cross-linking reagent, EBI, inhibits assembly by forming a cross-link between Cys^{239} and Cys^{354} [4]. EBI is a useful probe for the sulfhydryl groups of tubulin because, when it reacts with tubulin, it can cause formation of two intra-chain cross-links in β-tubulin [5, 6]. One of these, designated β*, is that between Cys^{239} and Cys^{354} [4]; the other, called β^{s}, is between the sulfhydryls of Cys^{12} and either Cys^{201}or Cys^{211} [7].

Ethacrynic acid (Fig. 1) is a compound that can react with sulfhydryl groups; it is used as a diuretic and as a treatment for glaucoma [8, 9]. Ethacrynic acid has been found previously to inhibit microtubule assembly *in vitro* [10], and it has been reported to cause changes in the shape and in the organization of the microtubule cytoskeleton in cultured cells involved in aqueous humor outflow [11]. Ethacrynic acid appears to react slowly with tubulin; inhibition of microtubule assembly *in vitro* is directly correlated with the reaction of ethacrynic acid with tubulin sulfhydryl groups [10]. Presumably, ethacrynic acid inhibits microtubule assembly by reacting with an assembly-critical sulfhydryl group. Therefore, we

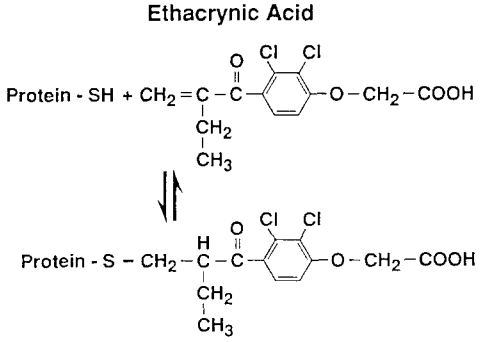

Fig. 1. Structure of ethacrynic acid and its interaction with tubulin.

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§ Abbreviations: DCBT, 2,4-dichlorobenzyl thiocyanate; EBI, *N,N'*-ethylenebis(iodoacetamide); FSBG, 5′-*p*-fluorosulfonylbenzoylguanosine; MAP, microtubule-associated protein; and MPMAP, 2-(4-methyl-1-piperazinylmethyl) acrylophenone.

used ethacrynic acid to investigate further the role of assembly-critical sulfhydryl groups in microtubule assembly. We found that ethacrynic acid inhibited overall alkylation of tubulin sulfhydryls by iodo[^{14}C]-acetamide at concentrations comparable to those at which it inhibits microtubule assembly. However, at these concentrations, ethacrynic acid had no effect on EBI-induced formation of the β^* cross-link but could inhibit formation of the β^s cross-link. Hence, it seems likely that ethacrynic acid reacts with an assembly-critical sulfhydryl group other than that of Cys^{239}.

These results have been presented in preliminary form elsewhere [12].

MATERIALS AND METHODS

Materials. Ethacrynic acid was purchased from the Sigma Chemical Co., St. Louis, MO, and was dissolved in dimethyl sulfoxide immediately prior to use. All other materials were obtained as previously described [13]. Microtubules were purified from bovine brain cerebra by cycling according to the procedure of Fellous *et al.* [14], and were stored as pellets at −80°. Tubulin was purified from these pellets by chromatography on phosphocellulose [14]. These same pellets were also used as the source of unfractionated boiled MAPs (a mixture of tau and MAP 2), which were prepared as previously described [14]. Unless otherwise indicated, all experiments were performed in buffer A [0.1 M 2-(*N*-morpholino)-ethanesulfonic acid, pH 6.4, 1 mM EGTA, 0.1 mM EDTA, 1 mM GTP and 0.5 mM $MgCl_2$]. Some of the cross-linking experiments were performed in the above buffer minus GTP and $MgCl_2$.

Microtubule assembly. Aliquots of tubulin (1.16 mg/mL) were incubated in the absence or presence of ethacrynic acid for 1 hr at 0°. They were then diluted to 1.0 mg/mL and made 0.35 mg/mL in unfractionated boiled MAPs. The samples were incubated for 1 hr at 37° in centrifuge tubes. The tubes were then centrifuged at 39,000 *g* for 40 min at 30°, and the protein concentration of the supernatants was determined [15]. By subtracting this concentration from that of the uncentrifuged material, the concentration of polymerized protein was calculated.

Cross-linking. Aliquots (0.25 mL) of tubulin (0.66 mg/mL) purified in the absence of GTP and $MgCl_2$ were incubated for 1 hr at 30° in the presence of 0.91 mM EBI. All samples contained reduced and carboxymethylated conalbumin (0.20 mg/mL) as an internal standard. In experiments where the goal was the measurement of the formation of the β^* cross-link, the samples contained 1 mM GTP to prevent β^s cross-link formation. In experiments measuring the formation of the β^s cross-link, samples contained 50 μM podophyllotoxin to prevent formation of the β^* cross-link. Samples were then dialyzed, reduced and carboxymethylated, and subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. Samples in which the yield of the β^* cross-link was to be measured were analyzed on gels by the system of Laemmli [16], while those in which the yield of the β^s cross-link was to be determined were analyzed by the modified system of Banerjee *et al.* [17]. Gels were stained with fast green. The yields of β^* and β^s were calculated as previously described [6].

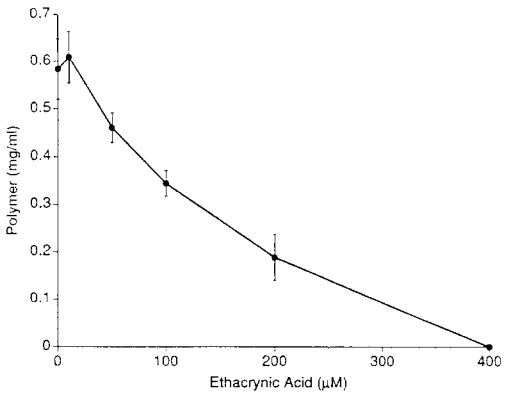

Fig. 2. Effect of ethacrynic acid on microtubule assembly. Aliquots of tubulin (1.16 mg/mL) were incubated with the indicated concentrations of ethacrynic acid for 1 hr at 0°. They were then diluted to 1.0 mg/mL and made 0.35 mg/mL in unfractionated boiled MAPs (a mixture of tau and MAP 2) and incubated at 37° for 1 hr. Next they were centrifuged at 39,000 *g* for 40 min at 30°, and the concentration of polymerized protein was determined. All incubations were done in duplicate; the range of values is shown.

Alkylation. Aliquots of tubulin were reacted with iodo[^{14}C]acetamide as previously described [18]. They were then precipitated and filtered, and the radioactivity of the filters was determined [18].

Other methods. The protein concentrations of the samples were determined using a modified version [19] of the procedure of Lowry *et al.* [15].

RESULTS

We first examined the effect of ethacrynic acid on microtubule assembly (Fig. 2). We found that half-maximal inhibition was obtained at an ethacrynic acid concentration of approximately 130 μM. Similar results were obtained by Xu *et al.* [10], who report half-maximal inhibition at 70 μM ethacrynic acid. To obtain the inhibition shown in Fig. 2, it was necessary to preincubate the tubulin with ethacrynic acid for 1 hr at 0°. Without preincubation, very little inhibition was seen.

To determine if ethacrynic acid reacted with the assembly-critical sulfhydryl group of Cys^{239}, we examined the effect of a series of ethacrynic acid concentrations on the EBI-induced formation of the β^* cross-link. If the tubulin was reacted with EBI without preincubation with ethacrynic acid, there was very little effect; even at a concentration of 1 mM, ethacrynic acid caused only 15% inhibition of β^* formation. The effect of ethacrynic acid on the formation of the β^s cross-link was significantly greater. Close to half-maximal inhibition of formation of the cross-link was obtained at about 1 mM ethacrynic acid. However, in order to parallel the

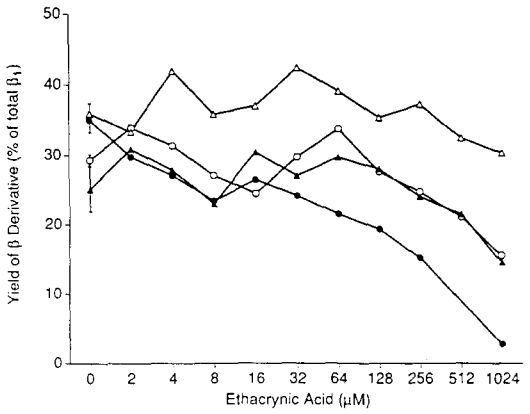

Fig. 3. Effect of ethacrynic acid concentration on the formation of the β^s and β^* cross-links. Aliquots (250 μL) of tubulin (0.67 mg/mL) containing reduced and carboxymethylated conalbumin (0.2 mg/mL) were incubated for 1 hr at 0° with the indicated concentrations of ethacrynic acid in the presence of either 1 mM GTP (▲) or 50 μM podophyllotoxin (●). The samples were then made 0.91 mM in EBI and incubated for 1 hr at 30°. The final tubulin concentration was 0.66 mg/mL. The samples were then processed as described in Materials and Methods. Another experiment was performed, identical to the one above, except that the preincubation at 0° was omitted. The figure shows the yields of the β^* (▲, △) and β^s (●, ○) cross-links in the experiments performed with (●, ▲) and without (○, △) preincubation. Yields obtained in the absence of ethacrynic acid represent incubations done in duplicate; the range of values is shown for these samples.

conditions in which the effect of ethacrynic acid on microtubule assembly was measured, tubulin was preincubated with ethacrynic acid for 1 hr at 0° prior to reaction with EBI. When this was done, it was clear that ethacrynic acid was able to partially inhibit formation of both cross-links (Fig. 3). The effect on β^* formation was the smallest; even at a concentration of 1 mM, ethacrynic acid was only able to inhibit β^* formation by 42%. In contrast, ethacrynic acid inhibited β^s formation half-maximally at a concentration of approximately 185 μM. In short, at the ethacrynic acid concentrations that caused half-maximal inhibition of microtubule assembly, there was little or no effect on the formation of the β^* cross-links, but there was significant inhibition of the formation of the β^s cross-link. This result means that at concentrations at which ethacrynic acid inhibited polymerization half-maximally, very little reaction with either Cys^{239} or Cys^{354} was occurring.

When the effect of ethacrynic acid on the overall alkylation of tubulin with iodo[^{14}C]acetamide was assayed (Fig. 4), it was found that half-maximal inhibition of alkylation occurred at about 325 μM ethacrynic acid. In this experiment, tubulin was incubated for 1 hr at 0° with the indicated concentrations of ethacrynic acid prior to the addition of the iodo[^{14}C]acetamide; by performing the preincubation at 0°, the conditions paralleled those in which the effects of ethacrynic acid on microtubule assembly and cross-link formation were assayed. In an analogous experiment, in which the preincubation step was omitted and the ethacrynic acid was added at the same time as the iodo[^{14}C]acetamide, half-maximal inhibition of alkylation was obtained at an ethacrynic acid concentration of 190 μM. In this type

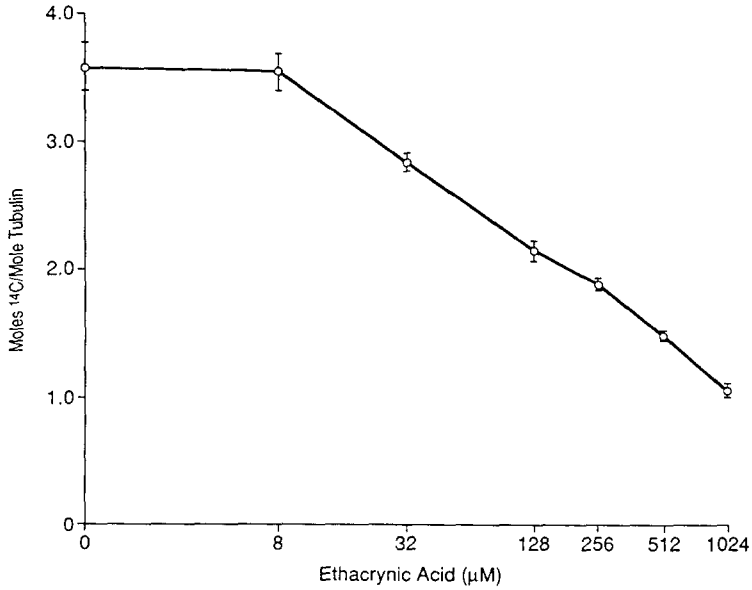

Fig. 4. Effect of ethacrynic acid on the alkylation of tubulin. Aliquots (250 μL) of tubulin (0.67 mg/mL) were incubated for 1 hr at 0° in the presence of the indicated concentrations of ethacrynic acid. After 1 hr, iodo[^{14}C]acetamide (0.66 Ci/mol) was added to each sample to a final concentration of 1.36 mM. The final tubulin concentration was 0.66 mg/mL. The samples were incubated for 1 hr at 37° and then precipitated and filtered [18]. The radioactivity of the filters was determined. Values are means ±SD of quadruplicate samples.

of assay, iodo[^{14}C]acetamide reacts with a substantial number of sulfhydryl groups, on both α and β [5].

DISCUSSION

Our results and those of Xu *et al.* [10] indicate that ethacrynic acid inhibits microtubule assembly, presumably by reacting with a sulfhydryl group on the tubulin molecule. The fact that the level which produced half-maximal inhibition of assembly was close to that which gave half-maximal inhibition of alkylation suggests that the alkylation and assembly effects are correlated. However, ethacrynic acid inhibited assembly at concentrations where it had little or no effect on β^* cross-link formation. This suggests that ethacrynic acid does not inhibit microtubule assembly by reacting with the sulfhydryls of Cys^{239} or Cys^{354}. Hence, ethacrynic acid must be reacting with some other assembly-critical sulfhydryl. The results in Fig. 3, however, suggest that ethacrynic acid inhibits β^s formation almost as strongly as it inhibits assembly. Is it possible that one of the β^s sulfhydryls is assembly-critical?

It is worth comparing the effects of ethacrynic acid with those of cystamine and MPMAP, both of which inhibit microtubule assembly by reacting with sulfhydryl groups on tubulin [17, 20]. Cystamine inhibits microtubule assembly at concentrations at which it has little effect on β^* formation, but its effect on β^s formation is comparable to its effect on assembly. As with ethacrynic acid, preincubation at 0° (in the case of cystamine for only 30 min) is required to see these effects. In contrast, MPMAP gives half-maximal inhibition of both microtubule assembly and tubulin alkylation by iodo[^{14}C]-acetamide at a concentration of 15 μM [21]. At these concentrations, MPMAP has no effect on the formation of either the β^s or β^* cross-links [22]. MPMAP reacts sufficiently rapidly with tubulin so that no preincubation is needed to inhibit assembly. In short, MPMAP appears to inhibit microtubule assembly by reacting with sulfhydryl groups other than those in the β^s or β^* cross-links.

How likely is it that one of the β^s sulfhydryls is assembly-critical? Contrasting arguments can be advanced. On the one hand, in order to obtain significant inhibition of β^s formation, it was necessary to preincubate tubulin with ethacrynic acid in the absence of GTP (to permit β^s formation) before reaction with EBI. When the preincubation step was omitted, very little inhibition of β^s formation was observed. GTP is likely to stabilize tubulin [22, 23]. Hence, incubating tubulin in the absence of GTP could accelerate the decay which would include exposure of many sulfhydryl groups, including perhaps one or more of those involved in the β^s cross-link. Ethacrynic acid could then react non-specifically with these sulfhydryl groups during the preincubation. In the experiment shown in Fig. 3, the tubulin contained 50 μM podophyllotoxin to inhibit β^* formation; podophyllotoxin has a small stabilizing effect, but still permits a good deal of decay [24]. In short, the fact that ethacrynic acid differs from MPMAP in inhibiting β^s formation and assembly almost concomitantly could simply reflect that MPMAP reacts very quickly with tubulin sulfhydryls before the tubulin molecule has time to decay.

If one of the β^s sulfhydryls is assembly-critical, a good argument could be made that it is either Cys^{201} or Cys^{211} but not Cys^{12}; recent work by Shivanna *et al.* [25], using direct photoaffinity labeling, indicates that Cys^{12} is immediately adjacent to the guanine moiety of GTP when the latter binds to tubulin. This makes it unlikely that a relatively bulky reagent such as ethacrynic acid could react with this sulfhydryl to inhibit assembly in the presence of GTP. There is no reason to imagine, however, that a portion of the GTP molecule comes close to either Cys^{201} or Cys^{211}. Hence, these could be candidates for reaction with ethacrynic acid. If this is the case, we must note that they are not distant in the sequence from Cys^{239}, which also appears to be critical to assembly; conceivably there could be some kind of mechanism that involves all of these sulfhydryl groups, or else they could be at a single tubulin–tubulin interaction site.

On the other hand, it is conceivable that there are several assembly-critical sulfhydryl groups on the tubulin molecule, including Cys^{239}, with which EBI and DGBT react, a β^s sulfhydryl, with which ethacrynic acid reacts, and another sulfhydryl, neither β^* nor β^s, with which MPMAP and ethacrynic acid react. It is important to note that although GTP inhibits formation of the β^s cross-link, this does not mean that GTP inhibits access to the sulfhydryls involved in the cross-link. It is certainly possible that these sulfhydryls are available in the presence of GTP, but that GTP sterically hinders EBI from making a covalent cross-link between them. The role of GTP in assembly, together with the probable proximity of the β^s sulfhydryls to GTP, makes the possibility that one of them could be regulatory a highly attractive one. 5′-*p*-Fluorosulfonylbenzoyl-guanosine (FSBG), an affinity analogue of GTP which reacts with sulfhydryl groups, can react with tubulin [26]. In FSBG, the phosphate moiety of GTP is replaced with a fluorosulfonylbenzoyl group. These results suggest that the phosphate moiety of GTP may bind to tubulin near a sulfhydryl group. If this sulfhydryl group is one of the β^s sulfhydryls, then it is possible that reaction of ethacrynic acid with this sulfhydryl could interfere with GTP hydrolysis and inhibit assembly.

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