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Absence of 7-acetyl taxol binding to unassembled brain tubulin

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The effect of taxol on microtubule proteins at 0°C is controversial. In order to determine if taxol is unable to bind to unassembled tubulin, as has been hypothesized, the binding of [^{3}H]acetyl taxol has been studied using equilibrium microdialysis. Ac-taxol bound to microtubules at 37°C and the binding remained stable when the temperature was lowered to 0°C. Ac-taxol bound also at 0°C to microtubules stabilized with rhazinilam. In contrast, there was no binding of Ac-taxol to unassembled tubulin, either free tubulin at 0°C or tubulin, complexed with several microtubule poisons, at 0 and 37°C.

Taxol; Microtubule; Tubulin; Equilibrium dialysis

1. INTRODUCTION

Unlike other microtubule poisons, taxol stabilizes microtubules [1]. Despite its widespread use in biological studies, its mode of action is not well understood [1]. Taxol binds to microtubules in vitro [2] but the sedimentation assay used did not permit study of its interaction with unassembled tubulin. Manfredi et al. [3] showed that drugs causing microtubule disassembly prevented taxol binding in intact cells, suggesting that unassembled tubulin does not bind taxol. However, Thompson et al. [4] reported that microtubules were formed in the presence of taxol at 0°C. Thus, we have carried out a direct analysis of the binding of 7-[^{3}H]-acetyl taxol [5] to assembled and unassembled tubulin, using equilibrium dialysis.

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Abbreviations: Ac-taxol, 7-acetyl taxol; Me_2SO, dimethyl sulfoxide

2. MATERIALS AND METHODS

Microtubule proteins were obtained from sheep brain by three cycles of assembly-disassembly [6] (80% pure by gel electrophoresis) and pure tubulin was obtained according to [7]. Microdialysis chambers (0.2 ml compartments) were prepared as described [8] and filled with dialysis buffer (0.1 M Pipes, pH 6.9/0.5 mM $MgCl_2$/1 mM EGTA/1 mM GTP) containing [^{3}H]Ac-taxol (6–32 μM in 3% (v/v) Me_2SO, 1.3 Ci/mmol) in one compartment and 1.2 mg per ml tubulin in the other compartment. Equilibrium was reached after 6 h with constant shaking at 0 (crushed ice), 5 or 37°C. These conditions affected neither the ability of tubulin kept at 0°C to form microtubules nor the maintenance, at 37°C, of tubulin assembly. The free concentration of Ac-taxol was calculated from radioactivity measured in the compartment without tubulin while the concentration of bound ligand was calculated from the difference of radioactivity between the two compartments. As checked with HPLC (C18 microbondapak column) neither non-radioactive, nor [^{3}H]Ac-taxol were modified during dialysis at 37°C in the absence or presence of tubulin (1–2 mg per ml). Substantial adsorption of [^{3}H]Ac-taxol (1.3 and 32 μM) to the dialysis chambers occurred at 0°C (65 and 71%, respectively) and at 37°C (74 and 70%, respectively) in the absence of tubulin. A similar adsorption (70%) occurred in the presence of tubulin (3 mg per ml) at 0°C but was reduced to 8% at 37°C. The determination of the concentration of [^{3}H]Ac-taxol in each compartment overcame this difficulty. Tubulin adsorption (0.6–1.9 mg per ml of tubulin) to the dialysis chambers was negligible at 0 and 37°C. Determination of the binding constants was per-

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formed using a Scatchard analysis and a curve fitting program. Characterization of tubulin assembly was performed by determination of the amount of sedimentable material (Beckman Airfuge) and observation of assembled tubulin by electron microscopy.

3. RESULTS

The binding of [^{3}H]Ac-taxol to microtubule proteins [6] or pure tubulin [7] was identical. At 37°C, tubulin assembled into microtubules and a strong binding of Ac-taxol occurred (fig.1A). At 5°C, both the Ac-taxol binding and the amount of microtubules assembled were reduced by more than 50% (fig.1A). Other acidic proteins (bovine serum albumin or actin 3 mg per ml) did not bind Ac-taxol at 37°C. Due to the low solubility of Ac-taxol, the Me_2SO concentrations that permitted normal microtubule assembly did not allow us to test whether Ac-taxol could be displaced with an excess of unlabelled taxol. The variations of bound Ac-taxol as a function of free Ac-taxol were used to calculate its binding constants to tubulin at 37°C in 16 independent experiments. The value of the Hill coefficient (0.81, σ: 0.09) was not significantly different from 1, allowing a reliable calculation of the maximum binding capacity (1.03 mol of Ac-taxol per mol of tubulin, σ: 0.22) and of the dissociation constant (0.34 μM, σ: 0.12). But due to the impossibility of performing binding studies in the presence of high concentrations of Ac-taxol, it has not been possible to exclude the existence of an additional binding site characterized by a higher K_d value. Although Ac-taxol did not bind to free tubulin at 0°C, after Ac-taxol binding had occurred to microtubules at 37°C, the amount of microtubules and the extent of Ac-taxol binding decreased by 20 and 30%, respectively when the temperature was lowered to 0°C (fig.1B). From these data the half-life of the microtubule taxol complex was tentatively estimated to be 5 h. Microtubules assembled at 37°C remained fairly stable in the presence of 200 μM rhazinilam [9] even at 0°C: 103 and 67% of the control value for sedimentable protein were found at 37 and 0°C, respectively, after 6 h. In both cases these microtubules were able to bind Ac-taxol: 86 and 47% of the control at 37 and 0°C, respectively, in the presence of 3 μM free Ac-taxol.

At 0°C neither microtubule nor Ac-taxol binding was observed even after a 24 h dialysis

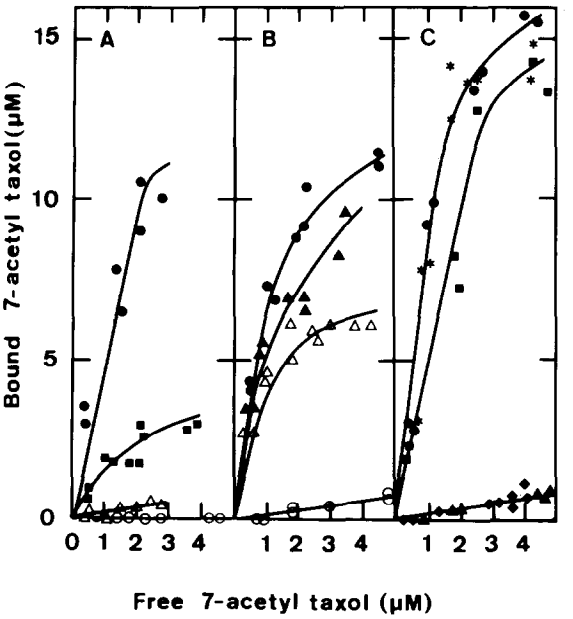

Fig.1. Binding of [^{3}H]Ac-taxol measured by equilibrium dialysis. (A) Effect of temperature. (○ and △) 6 and 24 h dialysis at 0°C, respectively. In both cases 0.10 mg of sedimentable protein per ml were recovered for the highest concentration of Ac-taxol; (■ and ●) 6 h dialysis at 5 and 37°C, respectively (0.39 and 0.86 mg of sedimentable protein per ml respectively). (B) Effect of a shift up to 37°C or a shift down to 0°C, 2.5 h after the beginning of the dialysis. (● and ○) Control at 37 and 0°C, respectively (1.04 and 0.2 mg of sedimentable protein per ml); (▲) 2.5 h dialysis at 0°C and then 2.5 h dialysis at 37°C (0.92 mg of sedimentable protein per ml); (△) 2.5 h dialysis at 37°C and then 2.5 h dialysis at 0°C (0.83 mg of sedimentable protein per ml). (C) Effect of various podophyllotoxin derivatives (20 μM, 37°C, 6 h dialysis). (●) Control; (■) etoposide; (*) teniposide; (▲) podophyllotoxin; and (◆) β-peltatin.

(fig.1A). Likewise Ac-taxol binding was prevented at 0 and 37°C by microtubule inhibitors (fig.1C) acting either at the colchicine binding site (colchicine, podophyllotoxin, β-peltatin, nocodazole) or the vinblastine sites (vinblastine, maytansine) while derivatives unable to prevent microtubule assembly (colchicoside, etoposide, teniposide, methyl benzimidazole carbamate, 16′-epi anhydrovinblastine) did not interfere with Ac-taxol binding.

4. DISCUSSION

In contrast with the sedimentation assay [2],

equilibrium dialysis allows the study of Ac-taxol binding both to assembled and unassembled tubulin. Results obtained using both methods for studying taxol and Ac-taxol binding to microtubules are in close agreement. The K_d of Ac-taxol (0.3 μM) is 3-times lower than the K_d of taxol [2], although Ac-taxol is 2-times less potent than taxol in stabilizing microtubules [10]. Moreover, 1 mol Ac-taxol is bound per mol tubulin, which is 1.7-times the value for taxol [2]. These differences are due most likely to the non-equilibrium assay used for taxol. Despite the highly repetitive structure of microtubules, the Hill coefficient is not different from 1, suggesting the absence of a cooperative mechanism, in agreement with the absence of sigmoidicity in the binding curve of taxol to microtubules [2].

While microtubules formed at 37 and 5°C, none were formed at 0°C. No binding of Ac-taxol was observed at 0°C. Simulation of saturation curves using the measured values for the Hill coefficient and maximum binding capacity, suggests that the dissociation constant of Ac-taxol from free tubulin is higher than 200 μM. Three observations show that Ac-taxol binding does not depend simply on the temperature. (i) When Ac-taxol binding to microtubules has occurred at 37°C, a significant binding persists when microtubules are subsequently incubated at 0°C; under these conditions most microtubules persist at 0°C. (ii) Microtubules stabilized with rhazinilam bind Ac-taxol at both 0 and 37°C. (iii) Whenever tubulin assembly at 37°C is inhibited, no binding of Ac-taxol is observed, as shown by experiments carried out with microtubule poisons acting at two different binding sites [11]. The absence of Ac-taxol binding cannot be due, in all cases, to a direct interference at the level of the taxol binding site. Simulation of saturation curves suggests that, at 37°C, the apparent K_d for Ac-taxol in the presence of colchicine, β-peltatin, maytansine and vinblastine was greater than 30, 50, 50 and 80 μM, respectively, in agreement with an indirect estimation [12] of the K_d of taxol for the tubulin-colchicine complex (13 μM).

Since Ac-taxol does not bind to unassembled tubulin, its binding site is either not accessible on free tubulin molecules or the site is generated during the assembly process. The formation of the Ac-taxol binding site during assembly could involve a conformational change on individual subunits and/or establishment of a site at the interface between adjacent subunits. Thus, it is not possible to compare the binding site for taxol with the binding sites for poisons, that prevent tubulin assembly, as has been done by others [1]. Microtubule assembly at 0°C in the presence of 20 μM taxol [4] or Ac-taxol (Pinet and Wright, unpublished) is puzzling. However, the concentrations of taxol and Ac-taxol which have been used are 7-times higher than the maximum concentration of free Ac-taxol used in equilibrium dialysis experiments. Several hypotheses could account for the effect of high concentrations of taxol and Ac-taxol at 0°C such as the existence of a second binding site with a higher K_d or the stabilization of a transient intermediate playing a role in tubulin assembly.

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