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Kinetics of association and dissociation of colchicine-tubulin complex from brain and renal tubulin

Evidence for the existence of multiple isotypes of tubulin in brain with differential affinity to colchicine

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The kinetics of colchicine binding to bovine brain tubulin have been reported to be biphasic under pseudo first order conditions [(1978) Biochemistry 17, 4466–4472]. Unlike brain tubulin, the kinetics of colchicine binding to bovine renal tubulin are monophasic. The apparent on-rate constant for the binding of colchicine to renal tubulin is found to be very close to that of the faster binding component in brain tubulin. Similarly, the dissociation of colchicine-tubulin complex in the presence of iodide is biphasic for brain tubulin but monophasic for renal tubulin. Since brain and renal tubulin apparently differ in β-tubulin, our results suggest that the biphasic nature of the kinetics for bovine brain tubulin could possibly originate from the existence of multiple isotypes of tubulin differing in drug binding affinity.

Colchicine-tubulin; Biphasic association kinetics; Biphasic dissociation kinetics; Fluorescence; Tubulin isotype; (Brain, Kidney)

1. INTRODUCTION

The antimitotic drug colchicine binds to tubulin in a slow, irreversible and entropy-driven process [1–6]. The binding induces a change in the conformation of both tubulin and colchicine [7–9] and is associated with the promotion of drug fluorescence [10] with a concomitant quenching of the intrinsic protein fluorescence [11].

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Abbreviations: GTP, guanosine-5′-triphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PC-tubulin, tubulin purified through phosphocellulose chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

The native tubulin molecule is a heterodimer of two closely related but distinct polypeptide chains designated α and β [3,4]. Tubulins from various sources have been found to contain microheterogeneity which changes with development and differentiation [12–14]. Tubulin from brain could be resolved into 5–9 isoelectric variants [15–17]; SDS-PAGE analysis and protein sequencing studies revealed the presence of at least four subspecies of α and two subspecies of β (β_1 and β_2) in brain tubulin [18–20]. Recent genetic studies revealed the existence of multiple α- and β-tubulin genes coding for multiple α- and β-polypeptides [21–26], thus indicating the existence of multiple tubulin isotypes. It is not known whether different isotypes of tubulin differ functionally.

The kinetics of colchicine binding to brain tubulin have been found to be biphasic under pseudo first order conditions, where colchicine was

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present in excess over tubulin [7,8]. Similarly the kinetics of dissociation of the colchicine-tubulin complex by iodide have been found to be biphasic [27]. Although several theories have been proposed by earlier investigators in order to explain the biphasic nature of the association and dissociation kinetics (e.g., the presence of ring structures in the tubulin preparation or the existence of multiple isotypes of tubulin), no experimental evidence is available. In this paper we have compared the kinetics of colchicine binding to two different species of tubulin, brain and kidney tubulin, and present evidence that the biphasic nature of the kinetics arises from the existence of multiple isotypes of tubulin with differential affinity to colchicine.

2. MATERIALS AND METHODS

Colchicine, EGTA, GTP, Mes, were obtained from Sigma (St. Louis, MO); EDTA from Aldrich; [^{3}H]colchicine (*ring C, methoxy*-^{3}H) from New England Nuclear (USA); phosphocellulose (P-11) from Whatman (England); and fresh cow brains from Roegelein Co. (San Antonio, TX).

Microtubule protein was purified from bovine brain cortex according to Fellous et al. [28] and from bovine renal medulla according to Barnes and Roberson [29]. Tubulin was purified from microtubule protein by phosphocellulose chromatography [28]. Protein concentration was determined according to Lowry et al. [30].

All studies were carried out in a buffer consisting of 0.1 M Mes-Na (pH 6.4), 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 0.1 mM GTP and 1 mM β-mercaptoethanol. Colchicine was dissolved in water.

All fluorescence measurements were made with a Perkin-Elmer model MPF 44B fluorescence spectrophotometer. Samples were excited at a wavelength of 380 nm to minimize the inner-filter effect and the emission was recorded at 437 nm. Proper corrections were made for quenching due to inner-filter effect. Quench corrections were made according to Lakowicz [31] as follows:

$$F_{corr} = F_{obs} \times \text{antilog}\ [(A_{ex} + A_{em})/2]$$

where F_{obs} and F_{corr} are the observed and corrected values of fluorescence, respectively.

The kinetics of association of colchicine and tubulin were studied at 25°C under pseudo first order conditions, where colchicine was present in excess over tubulin. The biphasic kinetics were analyzed in terms of two parallel first order reactions according to Lambeir and Engelborghs [8] as:

$$F_{max} - F_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$$

where F_t and F_{max} are the fluorescence values at time t and at equilibrium, respectively; A, B are the amplitudes and α, β are the rate constants for the phases.

The apparent on-rate constants ($k_{on,app}$) were calculated from the pseudo first order rate constant (α) as:

$$k_{on,app} = \alpha/c$$

where c is the concentration of colchicine.

The dissociation of colchicine-tubulin was studied by incubating the [^{3}H]colchicine-tubulin complex with 0.5 M KI at 0°C and subsequent determination of bound colchicine by the DEAE-cellulose filter disc assay procedure of Borisy [32].

3. RESULTS AND DISCUSSION

Earlier studies by Garland [7] and Lambeir and Engelborghs [8] have demonstrated that under pseudo first order conditions, the kinetics of association of colchicine to bovine and pig brain tubulin are biphasic. Although several theories have been proposed in order to explain the biphasic nature of the binding kinetics no evidence is available to show the origin of the two phases. One of the theories which seems to be very reasonable is the existence of multiple species of tubulin dimers. In order to test the validity of this theory one should compare the kinetics of association of colchicine to two different species of tubulin.

It has been demonstrated that bovine renal tubulin differs from bovine brain tubulin at least in the β-subunit [20]; renal tubulin lacks the β_2-subunit which is present in brain tubulin. We thus decided to study the kinetics of colchicine binding to renal tubulin and to compare these with those of brain tubulin. For this, purified tubulins from bovine brain and kidney were incubated with 100 μM colchicine at 25°C and the kinetics of association were followed by monitoring the

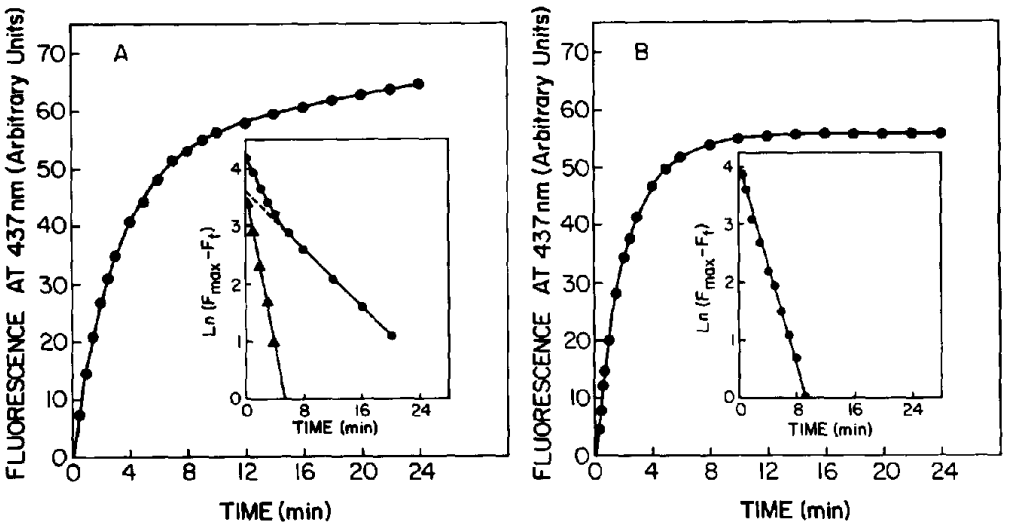

Fig.1. Kinetics of colchicine binding to brain and renal tubulin under pseudo first order conditions. PC-tubulin (2 μM) from either bovine brain (panel A) or bovine kidney (panel B) was incubated with colchicine (100 μM) at 25°C and the kinetics of association were followed by monitoring the fluorescence at 437 nm upon excitation of the samples at 380 nm. The inset shows the semi-logarithmic pattern of the kinetics. The biphasic curve was analyzed as described in section 2 and the fast phase is resolved (▲).

fluorescence at 437 nm. The results (fig.1) show that unlike brain tubulin (panel A) the kinetics for renal tubulin are monophasic (panel B). Analysis of the biphasic curve for brain tubulin yields the pseudo first order rate constants for the fast and the slow phases as 0.58 per min and 0.12 per min, respectively, whereas the rate constant for renal tubulin is 0.45 per min (table 1). The apparent on-rate constants for brain tubulin are 97 $M^{-1} \cdot s^{-1}$ for the fast phase and 20 $M^{-1} \cdot s^{-1}$ for the slow phase, whereas that of renal tubulin is 75 $M^{-1} \cdot s^{-1}$. Our observed k_{on} value for the fast phase is very close to the k_{on} values reported by earlier investigators [7,8,11].

The kinetics of dissociation were followed by incubating the [^{3}H]colchicine-tubulin complex in the presence of 0.5 M KI at 0°C. Here the temperature was kept at 0°C in order to minimize the association reaction. A semi-logarithmic plot of the dissociation data yields a biphasic curve for brain tubulin but a monophasic one for renal tubulin (fig.2). Analysis of the biphasic kinetics shows that the rate constants for the fast and the slow phases are 0.26 and 0.09 per min, respectively, whereas the rate constant for renal tubulin is 0.09 per min.

Since the brain and the renal tubulin differ apparently in the β-subunit, it is possible that the existence of the two phases in brain may be correlated with the presence of β_2-tubulin. The fact that the rate constant for renal tubulin corresponds to that of one of the phases in brain tubulin is also in favor of this hypothesis.

In this context it should be mentioned that although we do not have any evidence we cannot completely rule out the possibility that the biphasic kinetics may also originate from the existence of different α-chains as reported by Ponstingl et al. [18].

Thus our results are consistent with the hypothesis that the biphasic nature of the colchicine-binding kinetics for brain tubulin

Table 1

Comparison of the on-rate constants for the binding of colchicine to brain and renal tubulin

Binding parameters	Brain	Renal
Pseudo 1st order rate constant (min^{-1})	0.58, 0.12	0.45
$k_{on,app}$ ($M^{-1} \cdot s^{-1}$)	97, 20	75

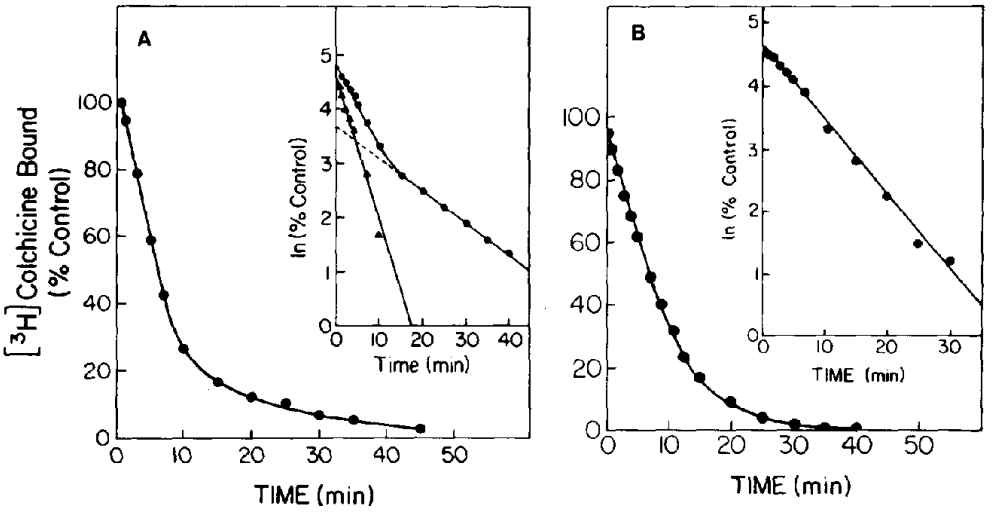

Fig.2. Kinetics of dissociation of the colchicine-tubulin complex from brain and renal tubulin. PC-tubulin (1.4 mg/ml) from either bovine brain (panel A) or bovine kidney (panel B) was incubated with [^{3}H]colchicine (100 μM) at 37°C for 30 min. The reaction mixtures were subsequently incubated with 0.5 M KI at 0°C. Aliquots were withdrawn at different times, diluted 50-fold with 10 mM sodium phosphate buffer (pH 7.0) and filtered immediately through DEAE-81 filter discs, and processed as described in section 2. Data are presented as percent control as compared to an identical sample in the absence of iodide. The inset in each panel shows the semi-logarithmic pattern of the dissociation data. The biphasic kinetics were analyzed as described in the text. The resolved fast phase is shown in the inset (▲).

originates from the existence of multiple tubulin isotypes and it is conceivable that the difference in rate constants may arise from the existence of multiple β-tubulins.

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