Different Kinetic Pathways of the Binding of Two Biphenyl Analogues of Colchicine to Tubulin†

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ABSTRACT: The kinetics of the interaction of tubulin with two biphenyl analogues of colchicine were measured by fluorescence stopped flow. The ligands were 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB) and 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl (TKB). The binding of both analogues is accompanied by a fluorescence increase with monophasic kinetics, which indicates that these drugs, unlike colchicine, do not discriminate between the isoforms of tubulin. The observed pseudo-first-order rate constant increases in a nonlinear way with the drug concentration, indicating that the binding of the biphenyl analogues to tubulin occurs, like colchicine, in two steps: a fast reversible equilibrium followed by an isomerization of the initial complex. Kinetic analysis shows that TCB and TKB exhibit differences in their K_1 values. At 25 °C, these are 114 000 ± 15 000 M^{-1} for TCB and 8300 ± 900 M^{-1} for TKB. Both molecules show a much higher affinity than colchicine for the initial binding site. Also at 25 °C, the k_2 value is 0.66 ± 0.04 s^{-1} for TCB and 3.0 ± 0.2 s^{-1} for TKB. From the temperature dependence, a reaction enthalpy change for the initial binding ($\Delta H°_1$) of 44 ± 9 kJ·mol^{-1}(TCB) and −40 ± 14 kJ·mol^{-1} (TKB) and an activation energy for the second forward step of 64 ± 2 kJ·mol^{-1} (TCB) and 101 ± 10 kJ·mol^{-1} (TKB) were calculated. The dissociation kinetics were studied by displacement experiments, in which podophyllotoxin was used as a displacing ligand. The rate constant for the second step in the off direction (k_{-2}) is 0.25 ± 0.05 s^{-1} for TCB and 0.093 ± 0.009 s^{-1} for TKB at 25 °C. The activation energies for the backward isomerization of the complexes were found to be 86 ± 20 kJ·mol^{-1} (TCB) and 79 ± 5 kJ·mol^{-1} (TKB). Combination of these results with the kinetic parameters for association gives a full characterization of the enthalpy pathway for the binding of TCB and TKB. The pathway of TCB binding is shown to differ considerably from that of TKB binding. Since their structural difference is located in ring C′, this result points to their use of the ring C′ in the first binding step. The competitiveness of the binding of TCB and TKB with those of podophyllotoxin, MTC, and MDL 27048 indicates that the two biphenyls interact as well with the trimethoxyphenyl-specific subsite.

The alkaloid colchicine, the well-known microtubule inhibitor, binds to its target, the protein tubulin, in an intriguing process. This molecule is a three-ring structure that consists of a trimethoxyphenyl ring (ring A) linked to a tropolone methyl ether ring (ring C) by a seven-membered ring (ring B) (Chart 1).

The binding of colchicine to tubulin results in the promotion of fluorescence, which has been used as a probe to follow the binding kinetics (Bhattacharrya & Wolff, 1974; Arai & Okuyama, 1975). Stopped flow kinetic studies showed that the appearance of fluorescence is biphasic (i.e. has to be described by a sum of two exponentials) (Garland, 1978; Lambeir & Engelborghs, 1981). These two phases were initially interpreted as belonging either to two major tubulin isoforms or to two states in slow equilibrium. Later it was shown that the two parallel phases are caused by the presence of separatable isoforms (Banerjee & Luduena, 1987, 1991, 1992). Recently, studies with isotypically pure tubulin dimers showed that the colchicine binding domain on the isoforms differs (Banerjee *et al*., 1994). Both phases show

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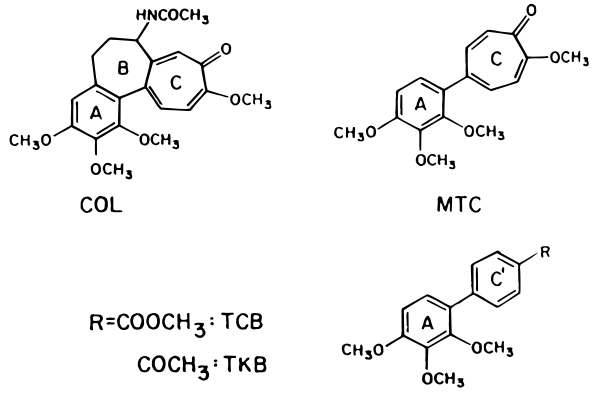
Chart 1



a rate constant that increases in a nonlinear way with the drug concentrations (Lambeir & Engelborhs, 1981). This nonlinear dependence allows the binding mechanism to be dissected into two steps: a fast initial binding of low affinity followed by a rather slow conformational change of the initial complex (Garland, 1978; Lambeir & Engelborghs, 1981).

The temperature dependence of the equilibrium constants and the kinetic parameters allowed the determination of the thermodynamic parameters of the first (Lambeir & Engelborghs, 1981) and second steps (Diaz & Andreu, 1991), whose addition equals global calorimetrically determined values (Menendez *et al*., 1989). The thermodynamics of colchicine binding has been explained on the basis of a bifunctional ligand model (Andreu & Timasheff, 1982a).

MTC[1] is a simple bifunctional analogue of colchicine that lacks the middle B ring (Chart 1) (Fitzgerald, 1976). Its binding to tubulin has been characterized in equilibrium studies (Andreu *et al*., 1984; Bane *et al*., 1984; Menendez *et al*., 1989). A kinetic study of this molecule (Bane *et al*., 1984; Engelborghs & Fitzgerald, 1986, 1987) revealed interesting differences between MTC and colchicine binding. The other two ring analogues that have been studied extensively by equilibrium thermodynamic approaches include TCB and TKB (Medrano *et al*., 1989, 1991). These are related to MTC by replacement of its tropolone methyl ether (ring C) by *p*-carbomethoxybenzene (ring C′) (TCB) (Chart 1) or by *p*-acetylbenzene (ring C′) (TKB) (Chart 1). The binding of these two biphenyls is specific to the colchicine binding site of tubulin, induces a conformational change in the protein and a tubulin GTP-ase activity, similar to colchicine binding (Medrano *et al*., 1989, 1991; Perez-Ramirez *et al*., 1994).

Complementary to the overall equilibrium binding studies, a detailed examination of the kinetics of TCB and TKB binding should reveal more details of the mechanism of their interaction with tubulin, while competition experiments should give more information about the subsites of colchicine that are involved in the binding site of TCB and TKB.

MATERIALS AND METHODS

Protein. Microtubule protein was purified from pig brain homogenates by two cycles of temperature-dependent assembly/disassembly according to the method of Shelanski *et al*. (1973) and modified as described previously (Engelborghs *et al*., 1977). Glycerol was added only in the first cycle to increase the yield. This preparation contained about 15% of microtubule-associated proteins. Protein concentrations were estimated by the procedure of Bradford (1976).

Pure tubulin was obtained by phosphocellulose chromatography (Whatman P11) according to Weingarten *et al*. (1975) and gel filtration chromatography on Sephadex G-25 in MES buffer. Its purity was checked by sodium dodecyl sulfate electrophoresis. The concentration of pure tubulin·GTP_2 and free nucleotide was determined by two-component analysis using the measured absorption at 278 and 255 nm and the following extinction coefficients: for tubulin, 1.2 $(mg/mL)^{-1}\cdot cm^{-1}$ at 278 nm (Harrisson *et al.*, 1976) and 0.65 $(mg/mL)^{-1}\cdot cm^{-1}$ at 255 nm (our own calibration with the Sephadex G-25-purified tubulin·GTP_2 complex); and for GTP, 12.17 and 7.66 $mM^{-1}\cdot cm^{-1}$ at 255 and 278 nm, respectively.

Ligands. TCB and TKB were prepared by M. J. Gorbunoff, and their purity and structure were checked by chromatography and NMR as described by Medrano *et al*. for TCB (1989) and for TKB (1991). The ligands were dissolved in dimethyl sulfoxide and the resultant solutions stored at −20 °C. The maximum quantity used in the experiments was 2.5% DMSO after dilution in the stopped flow. The concentrations of the biphenyl analogues were determined spectrophotometrically using the following extinction coefficients: TCB, 12 100 $M^{-1}\cdot cm^{-1}$ at 284 nm; and TKB, 14 400 $M^{-1}\cdot cm^{-1}$ at 295 nm. The solubilities of TCB and TKB are limited, independent of the quantity of DMSO (Medrano *et al*., 1989, 1991).

MTC was prepared and purified by T. J. Fitzgerald as described previously (Fitzgerald, 1976). Stock solutions were prepared by dissolving MTC in DMSO. The MTC concentration was determined spectrophotometrically with an extincion coefficient of 18.8 $mM^{-1}\cdot cm^{-1}$ at 350 nm (Bane *et al*., 1984).

POD was purchased from Sigma. It was dissolved in DMSO, and an extinction coefficient of 3700 $M^{-1}\cdot cm^{-1}$ at 290 nm was used (Andreu & Timasheff, 1982b). In the competition experiments, a final DMSO concentration of 5% after dilution in the stopped flow was used.

MDL 27048 was a gift from Merrell Dow Laboratory, and its concentration was measured spectrophotometrically with the extinction coefficient of 21 $mM^{-1}\cdot cm^{-1}$ at 398 nm (Peyrot *et al*., 1989). It was dissolved in DMSO and diluted in MES buffer adjusted to a final DMSO concentration of 10% before dilution in the stopped flow (for the competition experiments).

Ligand Binding Kinetics. All kinetic studies were done with pure tubulin in a buffer consisting of 50 mM MES, 70 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM NaN_3, adjusted to pH 6.4 with NaOH and to 5% DMSO upon mixing in the stopped flow (except some competition experiments; see above). GDP was added to a final concentration of 1 mM to prevent polymerization. The ionic strength of this buffer is 0.1 M.

The kinetics of the binding were measured in a stopped flow apparatus, especially designed for fluorescence measurements and built in the laboratory. A Hamamatsu superquiet mercury−xenon 150 W arc lamp (L2482) was used. For TCB and TKB binding studies, the excitation

[1] Abbreviations: TCB, 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl; TKB, 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl; TXB, either TCB or TKB; MTC: 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; POD, podophyllotoxin; MES, 4-morpholineethanesulfonic acid; DMSO, dimethyl sulfoxide; MDL 27048, *trans*-1-(2,5-dimethoxyphenyl)-3-[(dimethylamino)phenyl]-2-methyl-2-propen-1-one.

monochromator was set at 315 nm. The optical pathway is 2 mm and is perpendicular to the flow direction. The light beam, however, is 8 mm wide along the flow direction. Emission was collected over a wide angle so that a large part of the front surface fluorescence was collected as well. A Kodak Wratten filter 2B (cutoff at 395 nm) was used in the emission pathway. The dead time of the instrument was determined with the reaction of *N*-bromosuccinimide with *N*-acetyltryptophanamide and was found to be about 1.5 ms (Peterman, 1979). All fittings were done with the program Sigmaplot.

Extended Pseudo-First-Order Conditions. In pseudo-first-order conditions, the concentration of the ligand is usually at least 10 times higher than the concentration of the protein. However, to obtain a good signal-to-noise ratio, it is necessary to maintain a minimum protein concentration, 1.5 μM of tubulin in this case . To check how far the pseudo-first-order conditions can be extended, curves were generated by numerical simulation using the program KINSIM and the model for colchicine binding, with ligand/protein ratios between 10/1 and 2/1. The simulated curves were then fitted for a single exponential using the program Sigmaplot. The fits were excellent, and the deviation of the rate constant was not more than 4% at the lowest drug/protein ratio of 2/1. This deviation is smaller than the experimental error ($\pm$10%) due to the noise. Therefore, the pseudo-first-order conditions were pushed to this extreme.

Competition Kinetics. Complex formation between tubulin and a molecule (A) that binds to the colchicine (C) site but does not give an optical signal can be studied by competition kinetics. In this type of experiment, tubulin is mixed with a solution of both ligands together (C + A). Both ligands compete (on the same time scale) for the empty sites of tubulin, and this leads to the following kinetic equations:

$$-\mathrm{d[T]}/\mathrm{d}T = \mathrm{[T]}(k_{+\mathrm{c}}\mathrm{[C]} + k_{+\mathrm{A}}\mathrm{[A]}) \quad (1)$$

$$\mathrm{d[TC]}/\mathrm{d}t = k_{+\mathrm{c}}\mathrm{[C][T]} \quad (2)$$

provided the dissociation rates can be neglected. In pseudo-first-order conditions, integration of this equation leads to the following observed rate constant:

$$k_{\mathrm{obs}} = k_{+\mathrm{c}}\mathrm{[C]} + k_{+\mathrm{A}}\mathrm{[A]} \quad (3)$$

and

$$\text{relative amplitude} = k_{+\mathrm{C}}\mathrm{[C]}/(k_{+\mathrm{C}}\mathrm{[C]} + k_{+\mathrm{A}}\mathrm{[A]}) \quad (4)$$

Therefore, the observed rate constant for colchicine binding will increase, while the relative amplitude will decrease; e.g. MTC and podophyllotoxin behave in this way [see Engelborghs & Fitzgerald (1987)].

In contrast with this prediction, competition experiments can also lead to a reduction of the observed rate constant instead of an increase. This is the case when the competing ligand (B) equilibrates much more rapidily with the protein than the reporter ligand. In that case, a preequilibrium with the second ligand is rapidly established and the rate equation for the binding of the reporter group can be written as follows:

$$-\mathrm{d[T]}/\mathrm{d}t = k_{+\mathrm{C}}\mathrm{[C][T]}/(1 + K_{\mathrm{B}}\mathrm{[B]}) \quad (5)$$

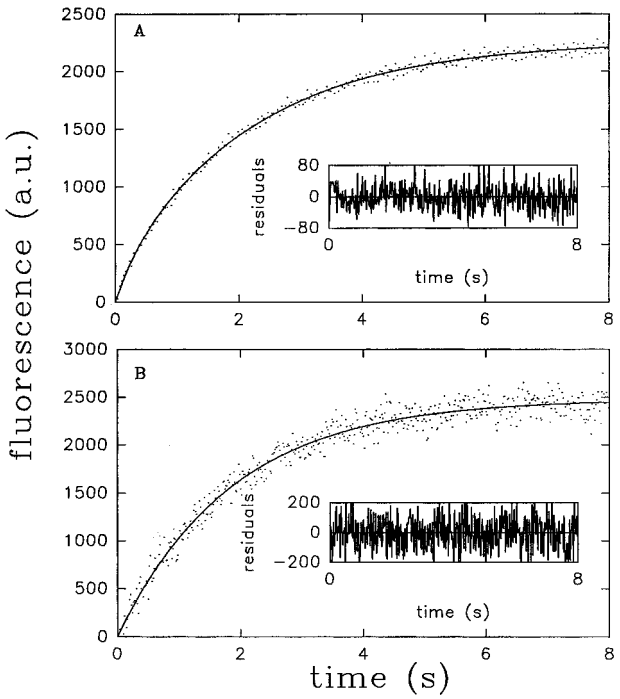


FIGURE 1: Kinetics of association of biphenyl analogues of colchicine to tubulin under pseudo-first-order conditions (excitation at 315 nm; fluorescence above 395 nm). The fluorescence data were analyzed by using a monoexponential model. Experimental and fitted curves are shown. The insets show the residuals between the experimental and theoretical curves. (A) Fluorescence increase upon the binding of TCB (30 μM) to tubulin (5 μM) observed in the stopped flow apparatus at 21.5 °C. (B) Binding of TKB (42 μM) to tubulin (5 μM) at 21.5 °C (all final concentrations). The fluorescence of TKB is weaker than the TCB fluorescence, and the curve was recorded with a gain that was 10 times higher.

with [T]/(1 + K_{B}[B]) being the fraction of tubulin not occupied by B.

$$\mathrm{d[TC]}/\mathrm{d}t = -\mathrm{d[T]}/\mathrm{d}t \quad (6)$$

the observed rate constant will therefore be

$$k_{\mathrm{obs}} = k_{+\mathrm{C}}\mathrm{[C]}/(1 + K_{\mathrm{B}}\mathrm{[B]}) \quad (7)$$

The amplitude does not change (when the affinity of B is negligible compared to the overall affinity of C). From the reduction of the observed rate constant, the binding constant K_{B} can be calculated.

It is clear that the intermediate situation can occur, and in that case, numerical simulations have to be used to obtain the parameters. The simulations were done with the program KINSIM (Dr. Bryce Plapp, Iowa).

RESULTS

(1) Association Kinetics. The binding of the drugs TCB and TKB results in the formation of fluorescent tubulin.drug complexes, which allows the reaction to be followed continuously. Figure 1 shows typical kinetic profiles for the binding of an excess of TCB (30 μM) and TKB (42 μM) to 5 μM tubulin at 21.5 °C. The kinetics were analyzed by using both one- and two-exponential fits. On the basis of the value of the χ^2 and on the spreading of the residuals, the

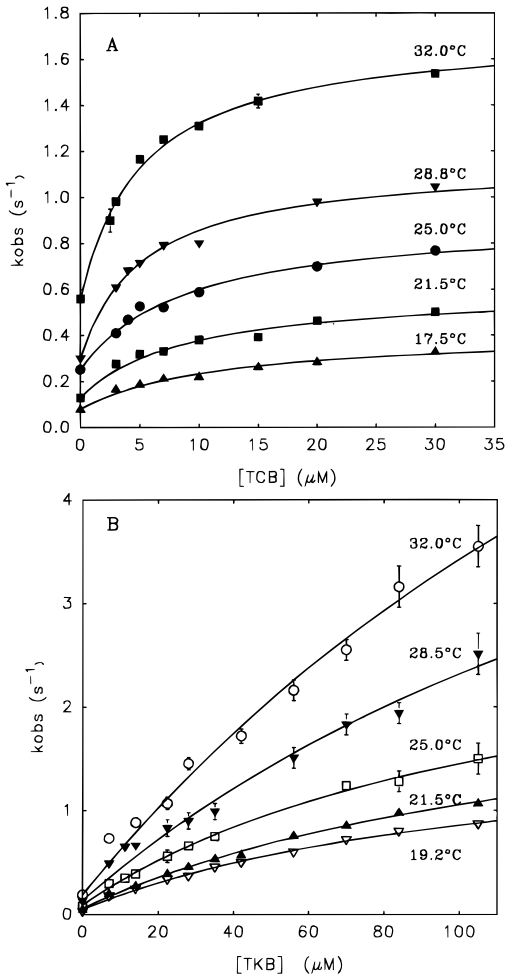


FIGURE 2: Nonlinear concentration dependence of the observed rate constants for the binding of TCB (A) and TKB (B) to tubulin at different temperatures. The tubulin concentration was 1.5 μM for the lowest ligand concentrations (<10 μM) and 5 μM for the higher concentrations (Materials and Methods; extended pseudo-first-order conditions). Each point represents the mean of ten experiments. The data were fitted to the hyperbolic model. The rate constants for isomerization in the off direction (k_{-2}) (intercept) were obtained separately from the dissociation studies.

best fit was single exponential (monophasic kinetics):

$$F = F_\infty - \Delta F \exp(-k_{obs}t) \qquad (8)$$

where F_∞ is the fluorescence at time infinite and ΔF and k_{obs} are the amplitude and the observed rate constant, respectively.

The mechanism of the binding reaction was tested with respect to a one- or two-step process as had been done for colchicine (Garland, 1978; Lambeir & Engelborghs, 1981). The pseudo-first-order rate constants were measured at different drug concentrations and temperatures and the data of TCB and TKB binding were plotted as shown in panels A and B of Figure 2, respectively. At concentrations lower than 10 μM drug, the tubulin concentration was lowered to 1.5 μM. The lowest ratio of drug/protein is therefore 2 (see Materials and Methods; extended pseudo-first-order kinetics). It is evident from the figures that the plots of k_{obs} vs concentration are not linear. This indicates that these biphenyl analogues follow the same two-step mechanism as colchicine, MTC, and MDL 27048 (Garland, 1978; Engelborghs & Fitzgerald, 1987; Silence *et al.*, 1992):

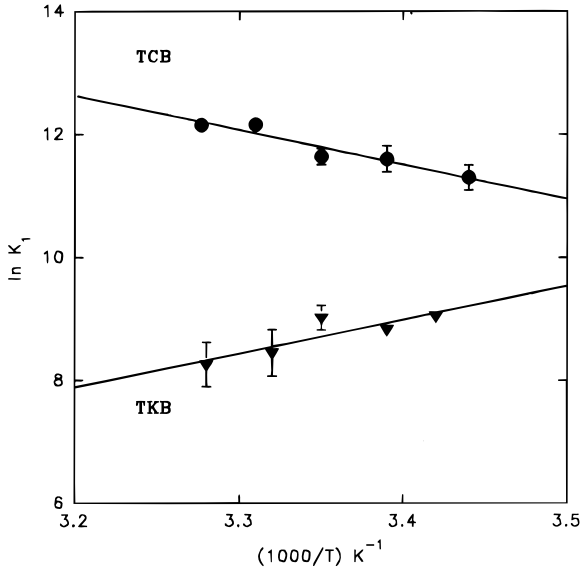


FIGURE 3: Van't Hoff plot for the initial equilibrium binding of TCB and TKB to tubulin.

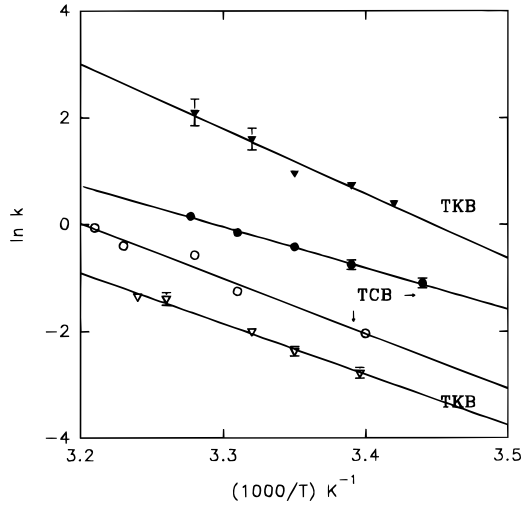


FIGURE 4: Arrhenius plot for the rate constants for the formation (filled symbols) and the disappearance (open symbols) of the final TKB or TCB complexes.

$$T + TXB \overset{K_1}{\rightleftharpoons} T{\cdot}TXB \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} T{\cdot}TXB^* \qquad (9)$$

where K_1 is the association constant for initial binding and k_2 and k_{-2} are the rate constants for the formation and disappearance of the final complex, respectively. The observed rate constant for the two-step scheme under pseudo-first-order conditions is given by the following hyperbolic expression:

$$k_{obsd} = k_{-2} + \frac{k_2 K_1[TXB]}{1 + K_1[TXB]} \qquad (10)$$

Measurement of the binding kinetics at different temperatures allows the calculation of $\Delta H°_1$ of the fast initial equilibrium (Figure 3) and the activation energy (E_{a2}) of the second step in the association direction (Figure 4). The thermodynamic and kinetic parameters are listed in Table 1.

(2) Displacement Experiments with Podophyllotoxin. The dissociation process can be studied more directly by displacement experiments in the presence of a large excess of podophyllotoxin. In these experiments, a solution of tubulin

Table 1: Kinetic and Thermodynamic Parameters of the Individual Steps of TCB and TKB Binding (25 °C)

	TCB	equilibrium ligand fluorescence[a]	TKB	equilibrium binding studies[b]
K_1 (M^{-1})	$(11.4 \pm 1.5) \times 10^4$		$(0.83 \pm 0.09) \times 10^4$	
k_1 ($M^{-1}\cdot s^{-1}$)[c]	$57\,000 \pm 10\,000$		$>10^6$	
k_{-1} (s^{-1})[c]	0.5 ± 0.1		>120	
$\Delta H_1°$ ($kJ\cdot mol^{-1}$)	44 ± 9		-40 ± 14	
$\Delta S_1°$ ($J\cdot mol^{-1}\cdot K^{-1}$)	240 ± 30		-60 ± 45	
k_2 (s^{-1})	0.66 ± 0.04		3.0 ± 0.2	
E_{a2} ($kJ\cdot mol^{-1}$)	64 ± 2		101 ± 10	
k_{-2} (s^{-1})	0.25 ± 0.05		0.093 ± 0.009	
E_{a-2} ($kJ\cdot mol^{-1}$)	86 ± 20		79 ± 5	
$K_{overall}$ (M^{-1})	$(3.0 \pm 0.4) \times 10^5$	$(1.15 \pm 0.27) \times 10^5$	$(2.7 \pm 0.4) \times 10^5$	$(2.38 \pm 0.65) \times 10^5$
$\Delta H°_{overall}$ ($kJ\cdot mol^{-1}$)	22 ± 20	3 ± 2	-18 ± 18	8 ± 3

[a] Data from Medrano *et al.* (1989). [b] Data from Medrano *et al.* (1991). [c] From numerical simulations of competition experiments.

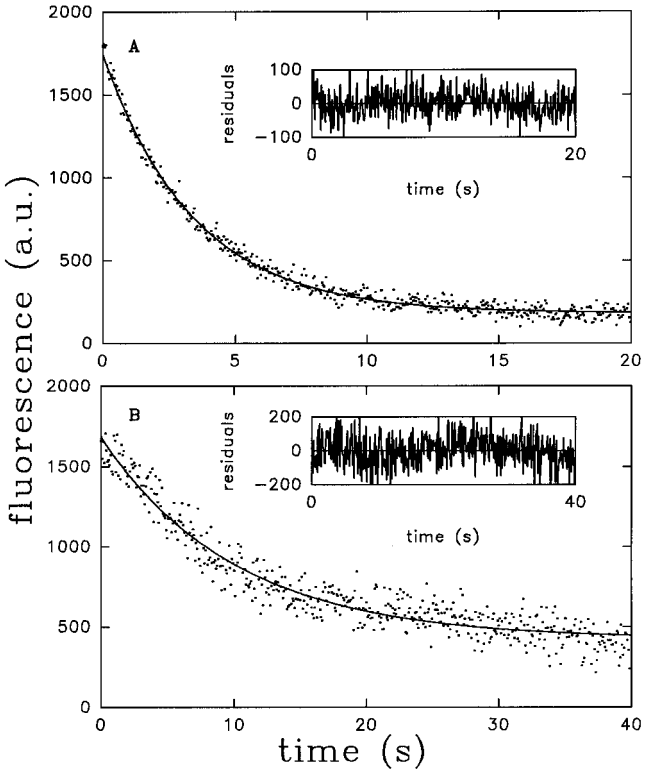


FIGURE 5: Displacement kinetics of bound TCB (A) or TKB (B) by podophyllotoxin at 28 and 25 °C, respectively. In the stopped flow, tubulin was incubated with TCB (5 μM) or TKB (5 μM) to form the complexes. The dissociation was initiated by the addition of POD to a final concentration of 1 mM. The experimental and theoretical curves are drawn. The insets show the residuals between the experimental and theoretical curves.

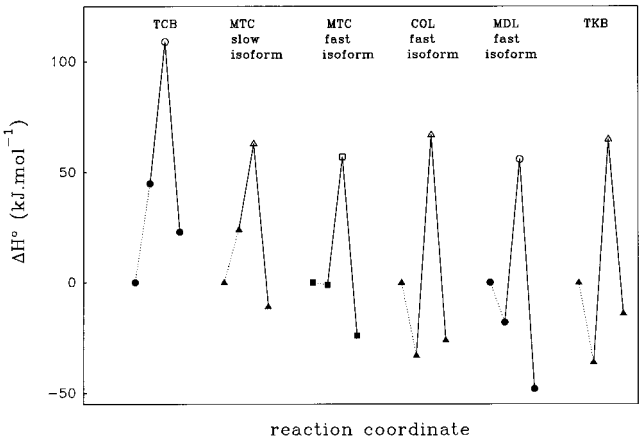


FIGURE 6: Reaction path for the binding of TCB (to tubulin), MTC (to the fast and slow tubulin isoform), colchicine (to the fast tubulin isoform), MDL 27048 (to the fast tubulin isoform), and TKB (to tubulin). The open symbol represents the transition state for the second step.

(5 μM) containing a low concentration of the biphenyl analogue (5 μM) was mixed in the stopped flow apparatus with the largest excess of POD possible (1 mM in MES buffer at 5% DMSO) (final concentrations). It is known that POD binds in one step to tubulin (Engelborghs & Fitzgerald, 1987). In such experiments, every binding site that becomes free upon the dissociation of the TCB or TKB molecule from the complex should immediately be occupied by POD. As shown in Figure 5, the dissociation reaction followed single exponential (monophasic) kinetics. The rate constants at 25 °C were found to be 0.25 ± 0.05 s^{-1} for TCB and 0.093 ± 0.009 s^{-1} for TKB. Since K_1 is a very fast equilibrium, it is k_{-2} which is the rate-limiting step for these displacement experiments. These experiments were repeated at several temperatures. When the logarithms of the rate constants were plotted as a function of $1/T$, a linear Arrhenius plot (Figure 4) was obtained. From this plot, activation energies of 86 ± 20 and 79 ± 5 $kJ\cdot mol^{-1}$ were calculated for the TCB and TKB backward isomerization, respectively.

The determination of the activation energies for the forward and the reverse reactions of the second step resulted in the knowledge of all the thermodynamic parameters for the two steps (see Table 1). The individual thermodynamic parameters permitted the construction of the pathway (Figure 6) and their comparison with those of colchicine (fast tubulin isoform), MTC (slow and fast tubulin isoform), and MDL 27048 (fast tubulin isoform).

(3) Effect of DMSO. In order to obtain the rate constants under identical final conditions, all the experiments were done in 2.5% DMSO. Doubling the DMSO concentration had no effect on the dissociation rate constant, while the association rate constant was decreased by about 5%. The effect is thus rather limited.

(4) Competition Experiments. In order to obtain more detailed information about the localization of the binding site of TCB and TKB, kinetic experiments were performed in the presence of some analogues that contain ring A (POD, MTC, and MDL 27048), although MTC also contains ring C.

Competition experiments with POD were done as follows. In the stopped flow apparatus, tubulin (5 μM) was mixed with a solution containing both TCB (10 μM) and POD. Only TCB binding contributes to the amplitude of the fluorescent signal. Figure 7 shows that the observed rate constant

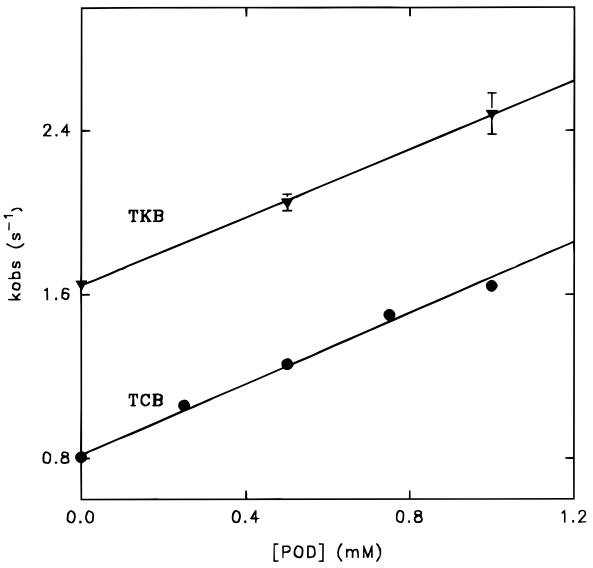


FIGURE 7: Competition kinetics of TCB (●) and TKB (▼) with podophyllotoxin. In the stopped flow, tubulin (5 μM) was mixed with a solution of TCB (10 μM) or TKB (56 μM) containing increasing concentrations of POD (final concentrations) (28 °C). The observed rate constant increases linearly with the concentration of POD up to 1 mM (limited by solubility).

Table 2: Competition Experiments with MTC and MDL 27048

T (°C)	ligand	[TCB] (μM)	[TKB] (μM)	$\gamma_{\text{amplitude}}$ [a]	$\gamma_{k_{\text{obs}}}$ [b]
25	MTC (15 μM)	−	−	1	1
		20	−	0.88	0.72
		30	−	0.85	0.68
25	MTC (15 μM)	−	−	1	1
		−	30	0.56	0.70
		−	60	0.38	0.58
25	MDL (11 μM)	−	−	−	1
		10	−	0.90	1.75
		30	−	0.87	2
		−	30	0.62	2.41

[a] $\gamma_{\text{amplitude}}$ is the ratio of the observed amplitude of MTC or MDL 27048 in the presence of TCB or TKB relative to the blank. [b] $\gamma_{k_{\text{obs}}}$ is the ratio of the observed rate constant relative to the blank.

follows a linear increase up to the highest concentration which is limited to about 1 mM by the solubilty of POD in 5% DMSO. In this case, POD is in competition with TXB for the free tubulin binding sites. From the slope, the bimolecular binding rate constant for POD is found to be 866 ± 38 $M^{-1}\cdot s^{-1}$ at 28 °C. This is in good agreement with the rate constant found by Engelborghs & Fitzgerald (1987). The ratio of the amplitudes (absence vs presence of POD) is in fair agreement with the calculated ratio (eq 4). The same experiments were done with TKB but at 56 μM. A bimolecular binding rate constant for the association of POD of 830 ± 22 $M^{-1}\cdot s^{-1}$ at 28 °C was calculated (Figure 7).

Competition experiments were also performed with MTC. Due to the strong absorption of MTC at 315 nm, these experiments were performed with excitation at 365 nm and MTC was used as the fluorescent probe. Tubulin was mixed in the stopped flow apparatus with a solution of MTC (15 μM) together with varying concentrations of TCB or TKB. Under these conditions of low MTC and low tubulin concentrations, the two exponential phases of MTC binding could not be resolved. The results are listed in Table 2. Surprisingly, the competition leads here to a decrease of the amplitude and the rate constant. This result suggests that

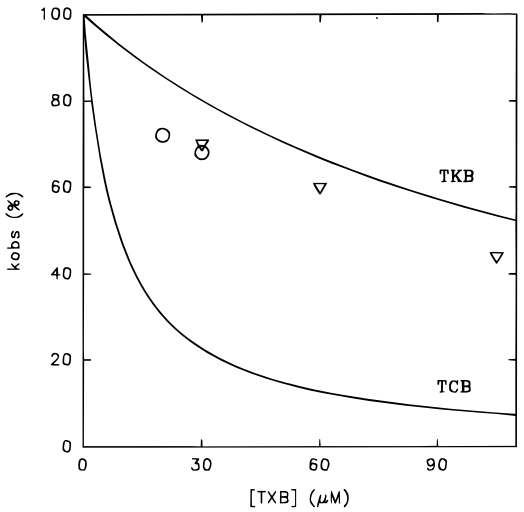


FIGURE 8: Competition kinetics of MTC with TXB. The theoretical reduction of the observed rate constant of the MTC binding in the presence of TXB, calculated with the saturation function at 25 °C, with K_1 the association constant of the initial binding of TXB, is shown by the lines. The open symbols are the ratio of the experimental observed rate constant of MTC binding in the presence of TCB (○) or TKB (▽) to that of the blank.

TCB and TKB bind much more rapidly to tubulin than MTC. If the observed association rate constant of this situation is described by eq 7 and a value of 3×10^5 M^{-1} is used for the global association constant (K_B) of TXB, the observed rate constant has to be decreased at least 10 times compared with the blank at a TXB concentration of 30 μM. The experiments (see Table 2) show, however, that the observed rate constant in the presence of 30 μM TXB decreases only 1.5 (TCB) or 1.4 (TKB) times compared with that of the blank. This indicates that the competition is not with the full affinity of TXB binding. The next possibility is a competition for the initial binding of TXB only. Figure 8 shows the ratio of the observed rate constant of MTC binding in the presence of TXB to that of the blank. The lines show the theoretical reduction of k_{obs} calculated with the saturation function $[K_1[\text{TXB}]/(1 + K_1[\text{TXB}]))$, where K_1 is the association constant of the initial binding of TXB. The ratio of the experimental data is given by the points. The TKB data fit fairly well the theoretical curve, while the experimental results of the TCB competition are situated far off the theoretical curve. These results suggest that there is a very fast preequilibrium (K_1) for the binding of TKB to tubulin, while the initial binding of TCB is slower. Numerical simulations should give more information (see below).

MDL 27048 is a drug which has the methoxybenzene (A) ring in common with MTC, TCB, and TKB. However, MDL 27048 has only two methoxy groups in para position relative to each other. In previous studies (Peyrot *et al*., 1992; Silence *et al*., 1992), it was shown that MTC, colchicine, and podophyllotoxin inhibit the binding of MDL 27048 to tubulin. As shown in Table 2, when a mixture of TCB or TKB was added to tubulin and the MDL 27048 binding was followed, a decrease in amplitude and an increase in the observed rate constant were observed.

These experiments clearly indicate that TCB and TKB compete for the same subsite as MTC, POD, and MDL 27048, which all have the A subsite in common.

(5) Determination of k_1 and k_{-1} from Numerical Simulations. Since only the competition experiments between MTC

and TCB behaved unexpectedly, these experiments were analyzed further by numerical simulations. For TKB, numerical simulations were used to estimate a lower limit for k_1. The binding mechanism and the rate equations are described in the Apppendix. In the simulations, the unknown rate constants were varied over a wide range. For the kinetic parameters of MTC binding, the values as described by Engelborghs and Fitzgerald (1987) were used. When for the association rate constant of TCB a value of 57 000 $M^{-1}\cdot s^{-1}$ was used, and for the dissociation rate constant a value of 0.5 s^{-1}, the simulations showed a decrease of the concentration of the complex at equilibrium (decrease of the amplitude of the signal) and the expected decrease of the observed rate constant of MTC binding. For a TCB concentration of 30 μM, the ratio of k_{obs} (absence vs presence of TCB) (0.70) is in good agreement with the ratio of the observed rate constants from the experiments (0.68) (the observed rate constant of the MTC binding is determined by fitting the numerical KINSIM curve with Sigmaplot). The same numerical experiment was done with lower (20 μM) and higher (60 μM) TCB concentrations. From these experiments it can be concluded that the acceptable range for the association rate constant of the initial binding of TCB can be estimated at 57 000 $\pm$ 10 000 M^{-1} s^{-1}.

The competition experiments between MTC and TKB were simulated by using a fast preequilibrium for TKB binding (see the Appendix). When a two-step mechanism is used in the simulation, the association rate constant for the first binding has to be at least about 10^6 $M^{-1}\cdot s^{-1}$.

With these parameters, the competition experiments with MDL 27048 and POD were simulated. For MDL 27048, the initial binding equilibrium K_1 is known, but not the rate constants. These, therefore, had to be estimated. The binding mechanism is expressed in the Appendix. When for the association rate constant of the initial binding of MDL 27048 the same value was taken as for the association rate constant of the initial binding of TCB, the theoretical results seemed to fit the data. However, when the first step of MDL 27048 was taken as a fast equilibrium, the observed rate constant showed a decrease instead of an increase (data not shown).

These results clearly demonstrate that the kinetics of the initial binding of TCB and TKB are highly different.

DISCUSSION

The binding of colchicine to tubulin has been interpreted in terms of a bifunctional ligand binding to two subsites on the protein (Andreu & Timasheff, 1982a). Previous studies have shown that the binding of TCB and TKB reaches binding equilibrium within seconds (Medrano *et al*., 1989, 1991). The current study gives a detailed kinetic analysis of the process.

The overall kinetically estimated binding constants ($K_1 \times K_2$) at 25 °C for TCB and TKB are compatible with the equilibrium values measured by Medrano *et al*. (1989, 1991) (see Table 1). In view of the different solution conditions, techniques and protein preparations used, the agreement can be considered fair. The overall enthalpy change for TCB binding deduced from the kinetic experiments is 22 ($\pm$20) and $-$18 ($\pm$18) kJ$\cdot$mol^{-1} for TKB binding. The overall $\Delta H°$ values agree less. Of course, the calculation of overall parameters accumulates numerous errors. The systematic difference may be due to the presence of DMSO, which might influence the temperature dependence of the binding (2.5% here, compared to 1% in the equilibrium studies).

An intriguing result is the difference between the kinetics of the development of fluorescence in the presence of colchicine or MTC and TXB. With colchicine, two phases are observed, which are attributed to the binding to different isoforms of tubulin. For colchicine, this has been confirmed by Banerjee and Luduena (1987, 1991, 1992). In the case of TCB and TKB, only one phase was observed, which suggests that these drugs are insensitive to the differences between the isoforms.

The nonlinear concentration dependence of the observed association rate constants can again be explained by a two-step binding mechanism: an initial fast equilibration followed by a slow second step. In the case of colchicine and MTC, the binding is accompanied by the appearance of GTPase activity (David-Pfeuty *et al*., 1977) and by a change in the protein far-UV circular dichroism (CD) (Andreu & Timasheff, 1982b; Andreu *et al*., 1984). The former indicates that a conformational change occurs in the protein (Perez-Ramirez *et al*., 1994). The interaction of TCB and TKB with tubulin also leads to weak CD changes and to induced GTPase activity (Medrano *et al*., 1989, 1991). The protein CD pertubation induced by TCB is weaker than that induced by TKB. This result suggests a difference in the mutual alignments of the transition vectors of the two biphenyls with those of a protein chromophore.

Detailed kinetic studies have shown that the affinities of the initial step for colchicine and MTC are rather low, on the order of 200 M^{-1}, and a big gain in affinity occurs within the second step for MTC and colchicine (only MTC binding of the slow isoform of tubulin gives a higher initial binding constant of 1.19×10^3 M^{-1}) (Lambeir & Engelborghs, 1981; Engelborghs & Fitzgerald, 1986, 1987). For TCB, the situation is different. At 25 °C, the affinity of the first step is 1.1 ($\pm$0.1) $\times$ 10^5 M^{-1} and the second step is only about 3. This is comparable to MDL 27048 binding [$K_1 = 2.5(\pm1) \times 10^4$ (fast phase) and 9.1 ($\pm$3) $\times$ 10^4 M^{-1} (slow phase) and K_2 is 100 or smaller (Silence *et al*., 1992)]. The affinity of the initial binding of TKB [$K_1 = 8.3$ ($\pm$0.9) $\times$ 10^3 M^{-1}] is also high, and K_2 has a value of about 30. Comparison of the clearly different kinetics of binding of TCB and TKB, which only differ by an oxygen in the 4′ substituent, indicates that ring C′ contributes to the first kinetic step of binding of these ligands. The strong affinity found in the first step of binding, however, points to a lot of interactions and therefore to the likelihood of some participation of ring A as well. This notion is further supported by the comparison of the thermodynamic parameters of the two steps to those of the single ring analogues of rings A and C of colchicine (Andreu & Timasheff, 1982a).

The initial binding of TCB is endothermic, while that of TKB is exothermic. The binding of TKB is driven by enthalpy, while the binding of TCB is driven by entropy. Since the trimethoxyphenyl ring (ring A) is common to the two compounds, all the differences in the properties of the initial binding must stem from the small difference in ring C′. One way of interpreting these data is to assume that the larger molecule (TCB) cannot fit, for steric reasons, the binding site available to the smaller one (TKB), which is consistent with the structural analysis of Rossi *et al*. (1995). The smaller one seems to get in very easily (high k_1) and

interacts directly with specific groups ($\Delta H < 0$). TCB gets in more slowly and does not find immediately the specific groups to interact with ($\Delta H > 0, \Delta S > 0$). This behavior is practically reversed in the second part of the binding process. Indeed, the second step of TCB binding is driven by enthalpy, for TKB, it is driven entropy.

While the above analysis is consistent with the use by TCB and TKB of the colchicine C subsite, the competition experiments with MTC, POD, and MDL 27048 demonstrate that TCB and TKB also interact with the A subsite of colchicine. Numerical simulations of these competition experiments show that the association and the dissociation rate constant of the initial binding of TXB, k_1 and k_{-1}, respectively, are different. The k_1 of TKB is more than 20 times the k_1 of TCB, and k_{-1} is more than 100 times the k_{-1} of TCB (see Table 1).

The differences in the kinetic behavior of TCB, MTC, colchicine, MDL 27048, and TKB are shown in Figure 6, where the activation enthalpy changes along the reaction pathway are shown. TKB mimics colchicine better than does TCB.

These studies illustrate that the energetic properties of a binding pathway can be highly sensitive to small alterations in the ligand molecule. The question remains whether an energetic pathway can be strictly correlated with a topological pathway.

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APPENDIX

Kinetic Equations for Competition

(1) Competition of TXB and POD. TXB binding was shown to occur in two steps, and it is known that POD binds slowly in one step to tubulin. This leads to the following scheme:

Scheme 1

$$\mathrm{T} + \mathrm{TXB} \underset{}{\overset{K_1}{\rightleftharpoons}} \mathrm{T{\cdot}TXB} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{T{\cdot}TXB^*}$$

$$+ \mathrm{POD} \underset{k_{-p}}{\overset{k_p}{\rightleftharpoons}} \mathrm{T{\cdot}POD}$$

In this case, the observed rate constant can be defined as:

$$k_{obs} = k_{obs(TXB)} + k_{obs(POD)}$$

$$k_{obs} = \frac{k_2 K_1 [\mathrm{TXB}]}{1 + K_1[\mathrm{TXB}]} + k_{-2} + k_p[\mathrm{POD}] \qquad (11)$$

The dissociation rate constant of POD is about 6×10^{-4} s^{-1} and therefore, it can be neglected (Engelborghs & Fitzgerald, 1987).

(2) Competition Kinetics of MTC and TCB. At the low MTC concentrations used, the association kinetics for MTC are described by the product K_1k_2. The binding of TXB occurs in two steps as represented by Scheme 2.

Scheme 2

$$\mathrm{T} + \mathrm{C} \underset{k_{-2}}{\overset{K_1k_2}{\rightleftharpoons}} \mathrm{TC^*}$$

$$\mathrm{T} + \mathrm{TCB} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{T{\cdot}TCB} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{T{\cdot}TCB^*}$$

Numerical simulations of this scheme give a good agreement with the experiments. The following parameters were used. The rate constants of the MTC binding were taken as described by Engelborghs and Fitzgerald (1987). k_3 and k_{-3} are the unknown rate constants of the initial binding. k_4 and k_{-4} are 0.66 and 0.25 s^{-1} at 25 °C, respectively (see Table 1). The time factors used are as follows: δ time, 0.1, one iteration for one point; and flux tolerance, 0.1; and integration tolerance, 0.01.

(3) Competition Kinetics between MTC and TKB. The binding of TKB occurs in two steps, with a fast initial binding.

Scheme 3

$$\mathrm{T} + \mathrm{C} \underset{k_{-2}}{\overset{K_1k_2}{\rightleftharpoons}} \mathrm{TC^*}$$

$$\mathrm{T} + \mathrm{TKB} \underset{}{\overset{K_3}{\rightleftharpoons}} \mathrm{T{\cdot}TKB} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{T{\cdot}TKB^*}$$

with K_3 being the association constant of the initial binding. k_4 and k_{-4} are 3 and 0.093 s^{-1} at 25 °C, respectively (see Table 1). The time factors used are as follows: δ time, 0.1; one iteration for one point; and flux tolerance, 0.1.

(4) Competition Kinetics of MDL 27048 and TXB. As stated before, the binding of MDL 27048 occurs in two steps. This leads to the following scheme:

Scheme 4

$$\mathrm{T} + \mathrm{MDL} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{T{\cdot}MDL} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{T{\cdot}MDL^*}$$

$$\mathrm{T} + \mathrm{TXB} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{T{\cdot}TXB} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{T{\cdot}TXB^*}$$

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