Fluorescence Stopped-Flow Study of the Interaction of Tubulin with the Antimitotic Drug MDL 27048†

Karen Silence, An D'Hoore, and Yves Engelborghs*

Laboratory of Chemical and Biological Dynamics, Katholieke Universiteit Leuven, Celestijnenlaan 200 D, B-3001 Leuven, Belgium

Vincent Peyrot and Claudette Briand

Faculté de Pharmacie, GRIPP, Laboratoire de Physique, Pharmaceutique, 27 Boulevard Jean Moulin, F-13385 Marseille Cedex 5, France

Received April 21, 1992; Revised Manuscript Received July 21, 1992

ABSTRACT: The kinetics of the binding of MDL 27048 to tubulin have been studied by fluorescence stopped flow. The binding is accompanied by a fluorescence increase. The time course can be described by a sum of two exponentials, assumed to be due to the presence of two major tubulin isoforms. The observed rate constants depend in a nonlinear way on the concentration of MDL in pseudo-first-order conditions. This concentration dependence can be described by the presence of a fast equilibrium of low affinity, followed by an isomerization of the initial complex. The dissociation kinetics have been studied by displacement experiments, in which MTC was used as a competitive ligand. The reaction enthalpy change for the first binding equilibrium and the activation energies for the forward and reverse steps of the isomerization were determined from the temperature dependence. This was possible for the two tubulin isotype populations. The kinetics of the binding of MDL to tubulin are slowed down in the presence of 3′,4′,5′-trimethoxyacetophenone, a fast binding analog of the colchicine A-ring, but are not influenced by the binding of tropolone methyl ether, indicating that the binding site of MDL has the A-subsite in common with colchicine, but not the C-subsite.

The binding to tubulin of colchicine and related molecules has been studied in great detail. Equilibrium measurements were performed for those molecules as well as for constituent separated rings (Bhattacharyya & Wolff, 1974; Andreu & Timasheff, 1982a,b; Medrano et al., 1989). Stopped-flow kinetic studies allowed the binding mechanism to be dissected further into two steps: a fast initial binding of relatively low affinity followed by a rather slow conformational change of the initial complex (Garland, 1978; Lambeir & Engelborghs, 1981). The analysis of the kinetics was further complicated by the presence of parallel reactions, which were interpreted as belonging either to two major tubulin isoforms or to two states in slow equilibrium. Later on it was proven that the two parallel phases were due to separable tubulin isoforms (Banerjee & Ludueña, 1987). The temperature dependence of the equilibrium constants and the kinetic parameters obtained from stopped flow experiments allowed the determination of the thermodynamic parameters of the individual steps (Lambeir & Engelborghs, 1981), i.e., the reaction enthalpy of the initial fast equilibrium and the activation parameters for the second step. The strong resemblance between the thermodynamic parameters of the initial step obtained from kinetics and the binding parameters for tropolone methyl ether (Medrano et al., 1989) led Andreu and Timasheff (1982a) to suggest that the first step of colchicine binding must be due to its interaction with the tropolone ring.

MTC[1] is a simple bifunctional analog of colchicine, lacking the middle ring (Fitzgerald, 1976). A thermodynamic study of its binding to tubulin was performed (Menendez et al., 1989). A kinetic study of this compound (Bane et al., 1984; Engelborghs & Fitzgerald, 1986, 1987) revealed interesting differences between MTC and colchicine (See Discussion) and warrants further study of analog compounds.

MDL 27048 is a new antimitotic drug, the structure of which is described in the preceding paper and which has the methoxy benzene (A) ring in common with MTC and colchicine. However, MDL 27048 has only two methoxy groups in para position relative to each other. An equilibrium binding study and a detailed description of its action on microtubules is presented in the preceding paper (Peyrot et al., 1992). Its in vivo effect on PtK2 cells is described by Peyrot et al. (1989).

Compared to the overall equilibrium binding studies, a detailed study of the kinetics of MDL 27048 binding will reveal more details of the mechanism of its interaction with tubulin. Hopefully, individual parameters can be obtained for two subpopulations of tubulin isoforms, if the kinetic parameters are sufficiently different. Competition experiments with the constituent rings of colchicine will reveal which subsites of colchicine are involved in the binding site of MDL 27048.

MATERIALS AND METHODS

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)

† This research is supported by the Belgian National Incentive Program on Fundamental Research in Life Sciences initiated by the Belgian State–Prime Minister's Office–Science Policy Program, the program for scientific exchange between the Flemish Community and France (Tournesol T.92.4) and by grants from ARC and La Federation des Centres de Lutte contre le Cancer. A. D'Hoore is supported by the IWONL (Belgium).

* Correspondence should be addressed to this author.

[1] Abbreviations: MDL 27048, *trans*-1-(2,5-dimethoxyphenyl)-3-[4-(dimethylamino)phenyl]-2-methyl-2-propen-1-one; MTC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; TMA, 3′,4′,5′-trimethoxyacetophenone; TME, tropolone methyl ether.

0006-2960/92/0431-11133$03.00/0

and modified as previously described (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 (MAPs). 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 SDS 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 $(mg/mL)^{-1}$ cm^{-1} at 278 nm (Harrisson et al., 1976) and 0.65 $(mg/mL)^{-1}$ cm^{-1} at 255 nm (own calibration with Sephadex G-25 purified tubulin·GTP_2 complex) and for GTP 12.17 and 7.66 mM^{-1} cm^{-1} at 255 and 278 nm, respectively.

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 10% DMSO upon mixing in the stopped flow. The ionic strength of this buffer was 0.1 M. GDP was added to a final concentration of 1 mM to prevent polymerization.

MDL 27048 was a gift of Merrel Dow Laboratory. An extinction coefficient of 21.0 ± 1.1 mM^{-1} cm^{-1} at 398 nm (Peyrot et al., 1989) was used. It was dissolved in DMSO and diluted in MES buffer adjusted to a final DMSO concentration of 20% (v/v) before the 2-fold dilution in the stopped flow.

MTC was a gift of Dr. T. J. Fitzgerald (College of Pharmacy, Florida Agricultural and Mechanical University, Tallahassee, Florida). Its concentration was determined spectrophotometrically with an extinction coefficient of 18.8 mM^{-1} cm^{-1} at 350 nm (Bane et al., 1984). TME was also prepared by Dr. T. J. Fitzgerald, as described by Andreu and Timasheff (1982a), and its purity was checked by NMR. An extinction coefficient of ϵ_{236nm} = 25.9 mM^{-1} cm^{-1} was used (Andreu & Timasheff, 1982a).

Colchicine was purchased from Aldrich Chemical Co. Its concentration was determined spectrophotometrically with ϵ_{350nm} = 16.6 mM^{-1} cm^{-1}. TMA was purchased from Janssen Chimica.

The kinetics of the binding were measured in a stopped flow apparatus described elsewhere (Lambeir & Engelborghs, 1981). For MDL binding studies, excitation was done at 440 nm, and emission was collected through a Kodak Wratten filter nr 4 (cutoff below 480 nm). For colchicine binding studies, excitation was at 380 nm, and a Kodak Wratten filter nr 2B (cutoff below 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 (Peterman, 1979) and was found to be around 1.5 ms.

The kinetic curves were analyzed for multiple exponentials by nonlinear least-squares fitting based on the Marquardt algorithm (Bevington, 1969). The concentration dependence of the observed rate constants was fitted using the program Sigmaplot.

RESULTS

Figure 1 shows the time course of the increase of MDL 27048 fluorescence when binding to tubulin. The fluorescence increase has to be fitted with a sum of two exponentials, based on the value of the χ^2 and on the spreading of the residuals:

$$F = F_0 + \Delta F_f \exp(-k_{obs,f}t) + \Delta F_s \exp(-k_{obs,s}t) \quad (1)$$

where F_0 is the fluorescence at time zero and ΔF_f, $k_{obs,f}$, ΔF_s,

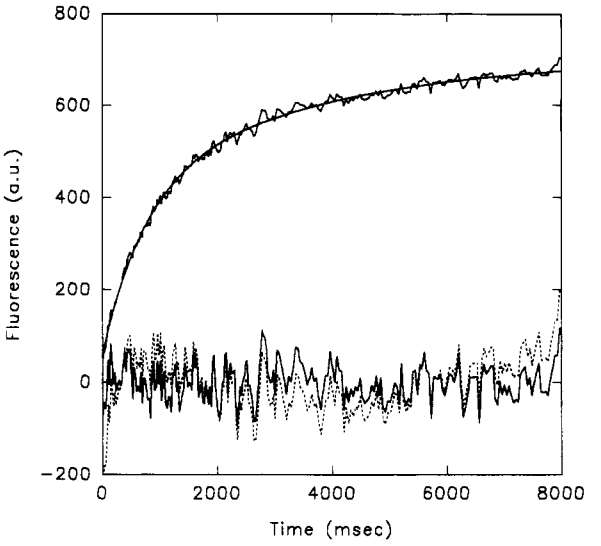

FIGURE 1: Fluorescence increase upon the binding of MDL 27048 (25 μM) to tubulin (5 μM), observed in the stopped flow experiment at 25 °C. The experimental curve is fitted to a sum of two exponentials. The calculated rate constants are $k_{obs,fast}$ = 1.32 ± 0.10 s^{-1} and $k_{obs,slow}$ = 0.27 ± 0.05 s^{-1}. The residuals (experimental curve − calculated curve) are 4-fold amplified and plotted near the origin of the *Y* axis. The full line represents the residuals for a two-exponential fit, the dotted line for a single-exponential fit.

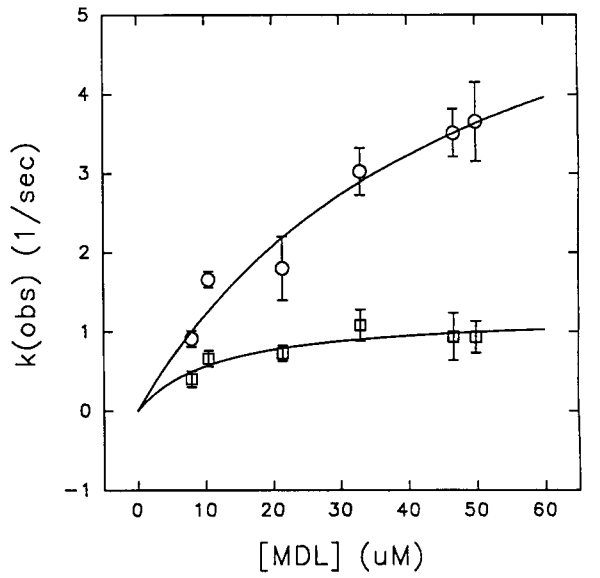

FIGURE 2: Nonlinear concentration dependence of the observed rate constants (k_{obs}) for the binding of MDL to the fast- and the slow-reacting isoforms of tubulin. Measurements were done at 313 K. (○) Fast phase; (□) slow phase.

and $k_{obs,s}$ are the amplitudes and the observed rate constants of the fast and slow phases, respectively. Figure 2 shows the concentration dependence of the observed rate constants for the two phases. Both show a very pronounced deviation from the linear relation expected for a simple pseudo-first-order concentration dependence. Therefore, for each phase, the following two-step mechanism is suggested, analogously to colchicine binding (Garland, 1978):

$$T + MDL \underset{}{\overset{K_1}{\rightleftharpoons}} T{\cdot}MDL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} T{\cdot}MDL^* \quad (2)$$

where K_1 is the association constant for the first fast equilibration step and k_2 and k_{-2} are the forward and backward rate constants for the isomerization of the initial complex. For such a scheme, the following rate constant can be derived

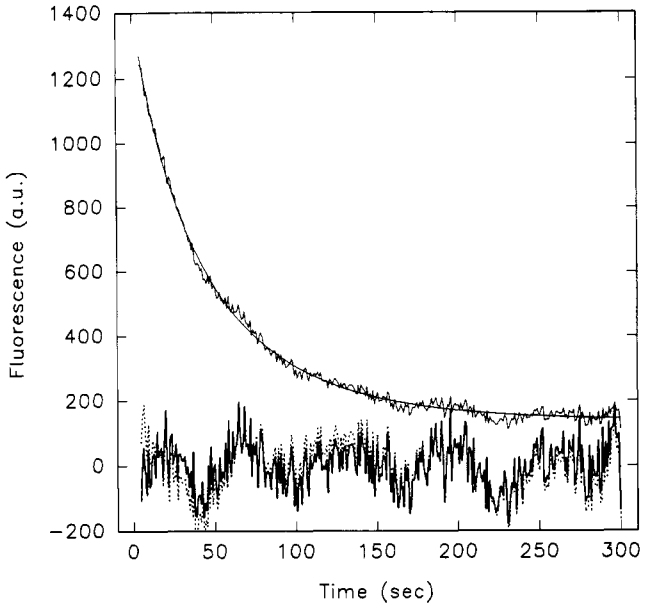

FIGURE 3: Displacement of bound MDL by excess MTC at 30 °C. The fluorescence decrease is shown, with the fit to the sum of two exponentials. The observed rate constants are 0.049 ± 0.009 s^{-1} and 0.017 ± 0.001 s^{-1}. The remaining least-squares sum was 367 for a single- and 323 for a double-exponential fit. The residuals (experimental curve – calculated curve) are 4-fold amplified and plotted near the origin of the Y axis. The full line represents the residuals for a two-exponential fit, the dotted line for a single-exponential fit. The single-exponential fit is practically as good and gives rate constants 12% higher than those of the slow phase.

(Garland, 1978):

$$k_{obs} = k_{-2} + k_2K_1[MDL]/(1 + K_1[MDL]) \quad (3)$$

As in the case of colchicine and its bicyclic analog (MTC), the two phases are attributed to the presence of two major isoforms of tubulin. So, for each isoform, the same equation for k_{obs} can be used. The intercept characterizing the dissociation rate constant (k_{-2}) is too small to allow for an unambiguous determination in this way. Therefore, the dissociation rate constant was determined by displacement experiments, where an excess of MTC is added to a preformed complex of tubulin and MDL 27048. Bound MDL will then be replaced by MTC (Peyrot et al., 1992). When the overall association rate of MTC [$(K_1k_2)_{MTC}$[MTC]] is much larger than the overall association rate of MDL [$(K_1k_2)_{MDL}$[MDL]], every dissociating molecule of MDL will be replaced by a molecule of MTC, and the dissociation of MDL will be the rate-limiting step. In our conditions of 12.8 μM MDL 27048 and 3.85 mM MTC, at 25 °C,

$$(K_1k_2)_{MTC}[MTC] = 273\ M^{-1} \times 58.5\ s^{-1} \times 3.85 \times 10^{-3}\ M = 61.5\ s^{-1}$$

and

$$(K_1k_2)_{MDL}[MDL] = 2.5 \times 10^4\ M^{-1} \times 2.14\ s^{-1} \times 12.8 \times 10^{-6}\ M = 0.684\ s^{-1}$$

Consequently, the observed rate constant k_{obs} equals k_{-2}. A typical fluorescence curve is shown in Figure 3. Fitting the data with a sum of two exponentials gives a much smaller residual least-squares sum and a somewhat better spreading of the residuals than with one exponential, despite the fact that the two rate constants do not differ more than a factor of 2–4. (Since the rate constants are so close, this splitting in two exponentials is not essential.)

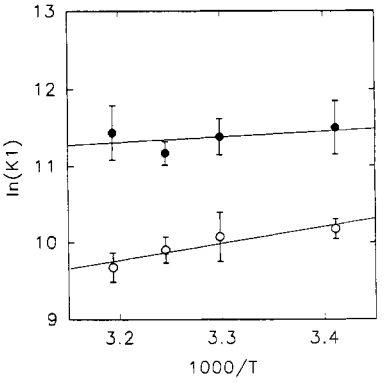

FIGURE 4: van't Hoff plot for the initial low-affinity equilibrium binding of MDL to tubulin, for the fast (○) and slow (●) reacting isoforms of tubulin.

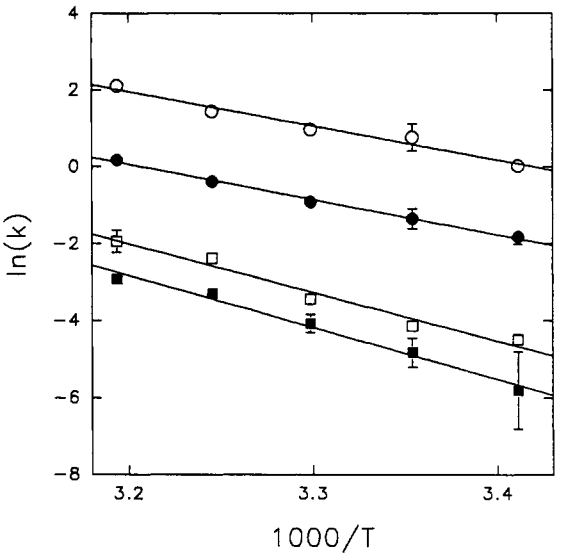

FIGURE 5: Arrhenius plot for the rate constants of the conformational change of the initial complex in forward (○ and ● = k_2) and backward (□ and ■ = k_{-2}) direction for the fast (open symbols) and slow (filled symbols) reacting isoforms of tubulin.

The study of the binding kinetics at different temperatures allows for the calculation of the $\Delta H°_1$ of the fast initial equilibration and the activation energy $\Delta E_{a,2}$ of the conformational change in the association direction. The activation energy ($\Delta E_{a,-2}$) of the conformational change in the dissociation direction can be obtained from the temperature dependence of the displacements experiments (for the fast and the slow tubulin isoforms separately). The equilibrium constant K_1 and the rate constants obtained at different temperatures are plotted in Figures 4 and 5. The thermodynamic and activation parameters derived from these plots are shown in Table I. From these data, the reaction (enthalpy) pathway for the binding of MDL 27048 can be constructed. This is shown in Figure 6 and 7 together with the pathway for MTC and colchicine.

In order to obtain a more detailed localization of the binding site, kinetic experiments were performed in the presence of the constituent rings of colchicine. TME binds to the C-subsite of colchicine. The kinetics of colchicine binding were studied at 0.4 mM colchicine and 5.4 μM tubulin preincubated for 30 min with 10 mM TME. The amplitude of the reaction is found to be decreased to 36% of the reference reaction in the absence of TME (64% of the amplitude was recovered in an extremely slow displacement reaction of bound TME). The same preincubation with TME, however, does not influence the amplitude of the MDL binding kinetics, studied at 40 μM

Table I: Thermodynamic and Kinetic Parameters Describing the Two-Step Binding of MDL 27048 to the Two Populations of Tubulin Isoforms (Fast and Slow Isoforms)[a]

	fast phase	slow phase
K_1 (M^{-1})	2.5 (± 1) × 10^4	9.1 (± 3) × 10^4
$\Delta H°_1$ (kJ mol^{-1})	−18 ± 4	−6 ± 8
$\Delta S°_1$ (J mol^{-1} K^{-1})	23 ± 16	76 ± 26
k_2 (s^{-1})	2.14 ± 0.77	0.26 ± 0.07
$E_{a,2}$ (kJ mol^{-1})	74 ± 7	75 ± 3
k_{-2} (s^{-1})	0.016 ● 0.001	0.008 ± 0.003
$E_{a,-2}$ (kJ mol^{-1})	104 ± 10	111 ± 10
K_1K_2 (M^{-1})	3.34 × 10^6	2.95 × 10^6

[a] The equilibrium constant K_1 and the rate constants k_2 and k_{-2} are given at 25 °C.

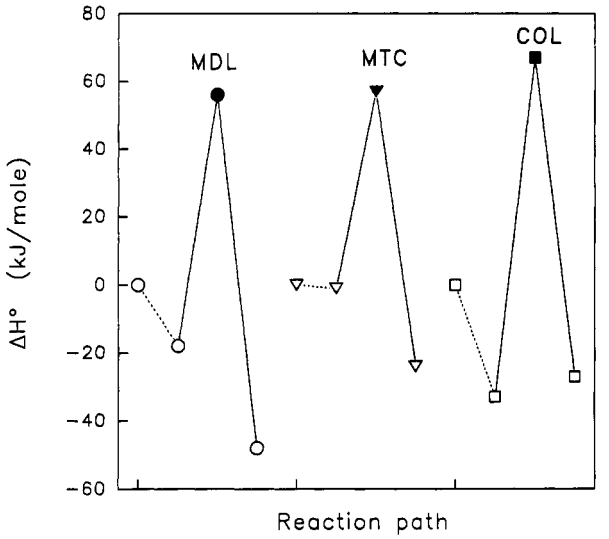

FIGURE 6: Reaction path for the binding of colchicine, MTC, and MDL 27048 to the fast isoforms of tubulin. (○) MDL 27048; (▽) MTC; (□) colchicine. Dotted lines are used to represent initial binding because the activation barriers, although small, are unknown. The filled symbol represents the transition state for the second step as well as for overall binding.

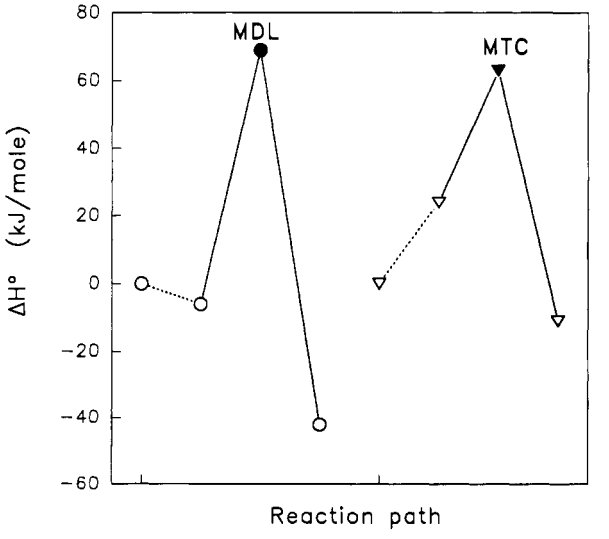

FIGURE 7: Reaction path for the binding of MDL 27048 and MTC to the slow isoforms of tubulin. (○) MDL 27048; (▽) MTC. The filled symbol represents the transition state for the second step, as well as for overall binding.

MDL. This clearly shows that MDL does not use the C-subsite of colchicine. In contrast, addition to tubulin of TMA at 4.16 mM slows down the binding of MDL, giving rate constants that are reduced to 71% of the reference values (for the two phases), without influencing the amplitudes. This clearly indicates that TMA competes very fast for the same subsite as MDL.

DISCUSSION

Equilibrium binding to tubulin and the effect on tubulin behavior of many analogs of colchicine have been studied. These properties are described in an excellent review (Bane-Hastie, 1991). However, detailed kinetic studies have only been performed for colchicine (Garland, 1978; Lambeir & Engelborghs, 1981), MTC (Engelborghs & Fitzgerald, 1986, 1987), and in this paper for MDL 27048.

For reasons of limited solubility of MDL 27048, the kinetics studies were all done with the same final concentration of 10% DMSO. However, DMSO only has a very limited effect on the binding of these drugs. The overall binding constant of 3.3 × 10^6 M^{-1} determined from these kinetic studies (Table I) perfectly agrees with the value of 2.8 × 10^6 M^{-1} determined by equilibrium titrations at 1% DMSO in practically the same buffer (Peyrot et al., 1989). Previous studies (Engelborghs & Fitzgerald, 1987) also showed that DMSO has a very limited effect on the kinetics of MTC binding.

The overall enthalpy change for MDL binding deduced from the kinetic experiments is −48 (±20) kJ/mol for the fast tubulin isoform and −42 (±20) kJ/mol for the slow isoform (errors were calculated by addition of the individual errors of the parameters ΔH_1, $E_{a,2}$, and $E_{a,-2}$, themselves obtained from the fitting program Sigmaplot). On average for the two isoforms, a value of −26 (±7) kJ/mole was obtained form calorimetric experiments (Peyrot et al., 1992). In view of the highly different techniques used, the agreement can be considered to be fair.

The kinetic behavior of MDL is formally very analogous to that of colchicine and MTC. In pseudo-first-order conditions, two phases are observed which are attributed to the binding to different isoforms (or classes of isoforms) of tubulin. For colchicine, this has been confirmed by Banerjee and Luduena (1987).

The nonlinear concentration dependence of the two 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 second step is accompanied by a change in the protein far-UV circular dichroism and by the appearance of GTPase activity, clearly proving the involvement of a conformational change of the protein, which propagates from the colchicine site to the GTP-binding site. The binding of MDL does not lead to CD changes or to induced GTPase activity (Peyrot et al., 1992). This fact indicates that the second step is not to be attributed to the same conformational change of tubulin. The second step of MDL binding must therefore represent a smaller conformational change or a deformation of the drug only.

The experiments with the constituent rings of colchicine, TMA and TME, clearly demonstrate that MDL only uses the A-subsite of colchicine: TMA rapidly competes for binding and slows down MDL binding, while TME has no influence at all on the binding kinetics. This observation with MDL also confirms that the two molecules (TMA and TME) act at different sites. It is tempting to assume that the dimethoxybenzene ring of MDL occupies the A-subsite, even if there is no strict evidence for this assumption, and it should be noted that the two methoxy groups are at different positions compared to colchicine.

As discussed in the preceding paper (Peyrot et al., 1992), MDL 27048 binding to tubulin has much in common with podophyllotoxin. This is also a molecule that shares the

A-subsite of colchicine (Andreu & Timasheff, 1982a). In contrast to MDL, its binding kinetics show a linear concentration dependence up to 1 mM (Engelborghs & Fitzgerald, 1987), leading to a bimolecular association rate constant of 195 M^{-1} s^{-1} at 15 °C. This molecule, therefore, fits into its binding site in a single step. The small value of the rate constant is to be understood on the basis of a simple model where a fast equilibrium is assumed between an open and a closed site, with the equilibrium position strongly poised toward the closed conformation. MDL has an apparent bimolecular rate constant of 5.3×10^4 M^{-1} s^{-1} at 25 °C, but its binding rate reaches a plateau below 0.1 mM. This molecule has easier access to an initial site, but the initial complex needs to be deformed or repositioned to enter the final state.

For colchicine and MTC, the affinities of the initial step are rather low, of the order of 2×10^2 M^{-1}, and a big gain in affinity occurs upon the second step. (Only MTC binding to the slow isoform of tubulin gives a higher initial binding constant of 1.19×10^3 M^{-1}). For MDL the situation is different: the affinity in the first step is already 10^4 M^{-1}, and the gain in the second step is 100 or smaller.

The differences in the kinetic behavior of MDL 27048, MTC, and colchicine are shown in Figures 6 and 7, where the molar (activation) enthalpy changes along the reaction pathway are shown. Sofar only three compounds have been studied in detail (MDL, colchicine, MTC). The different behavior of colchicine and MTC has been discussed in terms of the two-site model by Andreu et al. (1991), who suggested it is due to the theoretical possibility of MTC to enter its binding site in two ways: with either the A- or the C-ring first.

The most remarkable feature of the comparison of the reaction pathways of all three compounds is that the overall activation enthalpy change is between 60 and 70 kJ/mol for all of them. This overall activation barrier corresponds to the process $T + X \rightarrow TX^{*,\ddagger}$, where X refers to each of the three ligands, and $TX^{*,\ddagger}$ refers to the transition state toward the final complex. This means that the negative enthalpy changes in the initial binding step are largely compensated by a large positive activation enthalpy for the second step. So it is suggested that some of the bonds formed in the initial complex have to be broken or distorted again in going to the transition state and possibly also to the final complex. This is an aspect that has not been considered in previous two-site binding models, where initial binding is always assumed to be going straight to the final complex.

ACKNOWLEDGMENT

We are grateful to Dr. J. M. Andreu for helpful discussions, to Dr. H. W. Bohme for provision of MDL 27048, and to Dr. Fitzgerald for MTC and TME.

REFERENCES

Andreu, J. M., & Timasheff, S. N. (1982a) *Biochemistry 21*, 534–543.

Andreu, J. M., & Timasheff, S. N. (1982b) *Biochemistry 21*, 6465–6476.

Andreu, J. M., Gorbunoff, M. J., Medrano, F. J., Rossi, M., & Timasheff, S. N. (1991) *Biochemistry 30*, 3777–3786.

Bane, S., Puett, D., Macdonald, T. L., & Williams, R. C. (1984) *J. Biol. Chem. 259*, 7391–7398.

Bane-Hastie, S. (1991) *Pharmacol. Ther. 51*, 377–401.

Banerjee, A., & Ludueña, R. F. (1987) *FEBS Lett. 219*, 103–107.

Bevington, P. R. (1969) *Data Reduction and Error Analyses for the Physical Sciences*, pp 235–240, McGraw-Hill, New York.

Bhattacharyya, B., & Wolff, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2627–2631.

Bradford, H. H. (1976) *Anal. Biochem. 72*, 248–254.

Diaz, F. J., & Andreu, J. M. (1991) *J. Biol. Chem. 266*, 2890–2896.

Engelborghs, Y., & Fitzgerald, T. J. (1986) *Ann. N.Y. Acad. Sci. 466*, 709–717.

Engelborghs, Y., & Fitzgerald, T. J. (1987) *J. Biol. Chem. 262*, 5204–5209.

Engelborghs, Y., De Maeyer, L. C. M., & Overbergh, N. (1977) *FEBS Lett. 80*, 81–85.

Fitzgerald, T. J. (1976) *Biochem. Pharmacol. 25*, 1383–1387.

Garland, D. L. (1978) *Biochemistry 17*, 4266–4272.

Harrisson, C. M. H., Page, B. M., & Keir, H. M. (1976) *Nature 260*, 470–476.

Lambeir, A., & Engelborghs, Y. (1981) *J. Biol. Chem. 256*, 3279–3282.

Medrano, F. J., Andreu, J. M., Gorbunoff, M. J., & Timasheff, S. N. (1989) *Biochemistry 28*, 5598–5599.

Menendez, M., Laynez, J., Medrano, F. J., & Andreu, J. M. (1989) *J. Biol. Chem. 264*, 16367–16371.

Peterman, B. F. (1979) *Anal. Biochem. 93*, 442–444.

Peyrot, V., Leynadier, D., Sarrazin, M., Briand, C., Rodriguez, A., Nieto, J. M., & Andreu, J. M. (1989) *J. Biol. Chem. 264*, 21296–21301.

Peyrot, V., Leynadier, D., Sarrazin, M., Briand, C., Menendez, M., Laynez, J., & Andreu, J. M. (1992) *Biochemistry* (preceding paper in this issue).

Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.

Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.

Registry No. MDL 27048, 124711-23-5.