Biphasic Kinetics of the Colchicine−Tubulin Interaction: Role of Amino Acids Surrounding the A ring of Bound Colchicine Molecule†

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Received April 1, 2005; Revised Manuscript Received June 13, 2005

ABSTRACT: Isotypes of vertebrate tubulin have variable amino acid sequences, which are clustered at their C-terminal ends. Isotypes bind colchicine at different on-rates and affinity constants. The kinetics of colchicine binding to purified (unfractionated) brain tubulin have been reported to be biphasic under pseudo-first-order conditions. Experiments with individual isotypes established that the presence of β_{III} in the purified tubulin is responsible for the biphasic kinetics. Because the isotypes mainly differ at the C termini, the colchicine-binding kinetics of unfractionated tubulin and the β_{III} isotype, cleaved at the C termini, have been tested under pseudo-first-order conditions. Removal of the C termini made no difference to the nature of the kinetics. Sequence alignment of different β isotypes of tubulin showed that besides the C-terminal region, there are differences in the main body as well. To establish whether these differences lie at the colchicine-binding site or not, homology modeling of all β-tubulin isotypes was done. We found that the isotypes differed from each other in the amino acids located near the A ring of colchicine at the colchicine-binding site on β tubulin. While the β_{III} isotype has two hydrophilic residues (serine^{242} and threonine^{317}), both β_{II} and β_{IV} have two hydrophobic residues (leucine^{242} and alanine^{317}). β_{II} has isoleucine at position 318, while β_{III} and β_{IV} have valine at that position. Thus, these alterations in the nature of the amino acids surrounding the colchicine site could be responsible for the different colchicine-binding kinetics of the different isotypes of tubulin.

The heterodimeric protein tubulin consists of two similar but distinct polypeptide chains called α and β (*1*, *2*). In many organisms, both α and β tubulin exist as numerous isotypes, distinguished by their unique C-terminal sequences (*3*−*9*). Thus, tubulin prepared from mammalian brain contains four β-tubulin classes designated as β isotype I (β_I),[1] β_{II}, β_{III}, and β_{IV} accounting respectively for 3, 58, 25, and 13% of brain tubulin (*10*). The kinetics of colchicine binding to purified (unfractionated) brain tubulin have been reported to be biphasic under pseudo-first-order conditions (*11*). The origin of the two phases in the binding kinetics was not clear until it was shown that the bovine kidney tubulin, which lacks the β_{III}-tubulin isotype (which accounts for 25% of the total brain tubulin) binds colchicine in a monophasic manner (*12*). To know the functional significance of the isotypes, the individual dimer $\alpha\beta_{II}$, $\alpha\beta_{III}$, and $\alpha\beta_{IV}$ have been separated and purified using immunoaffinity columns (*10*, *13*). Detailed studies on the colchicine-binding properties of these purified isotypes have shed light on the observed biphasic kinetics of the colchicine−tubulin interaction (*14*). The results of colchicine binding to purified β_{III} and β_{III}-depleted isotype fraction clearly demonstrated that the on-rates and the affinity constants for the binding of colchicine to the different tubulin isotypes differ considerably and hence account for the origin of the biphasic kinetics observed in unfractionated tubulin (*14*−*16*).

The β-tubulin isotypes differ from each other apparently at the C termini ($\beta^{430-444}$). This C-terminal part of the β subunit, containing a large number of glutamic acid residues, has been found to be flexible, solvent-exposed, and sensitive to proteolysis (*17*, *18*). The carboxy termini of tubulin attract attention for their role in tubulin polymerization. The binding of microtubule-associated proteins as well as Ca^{2+} takes place at the C termini and regulates the self-assembly of tubulin (*19*, *20*). Colchicine does not bind to the C termini of tubulin; however, several properties of colchicine−tubulin interac-

† The financial support from CSIR, Government of India, is thankfully acknowledged. Additional support was provided by grants to A.B. (National Institutes of Health CA59711 and U.S. Army Breast Cancer Research Program DAMD17-98-1-8244) and to R.F.L. (Welch Foundation AQ-0726 and U.S. Army Breast Cancer Research Program DAMD17-01-1-0411).

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[1] Abbreviations: C termini, carboxy termini; β_I, β isotype I; β_{II}, β isotype II; β_{III}, β isotype III; β_{IV}, β isotype IV.

10.1021/bi050599l CCC: $30.25
Published on Web 07/08/2005

tions such as pH sensitivity, off-rate, on-rate, and the stability of the colchicine-binding site are regulated by the C-terminal region of α tubulin (*21*). Recently, Pal et al. suggested that the negatively charged C terminus of α tubulin is involved in ion-pair formation with the positively charged residues of the main body of tubulin (*22*). They termed this interaction as the "tail−body interaction", which was shown to control many properties of tubulin. Because the β isotypes also differ mainly at their C termini, we wanted to find out whether the C terminus of β tubulin is involved in a similar type of interaction as is the C terminus of α tubulin and thereby affect the colchicine-binding kinetics. Although the C termini are not a part of the colchicine-binding site, it is possible that through this "tail−body interaction" they can influence the kinetics of the colchicine−tubulin interaction. Thus, it would be interesting to see whether the kinetics of colchicine binding become monophasic with C-termini-depleted tubulin.

In the present study, we therefore measured the kinetics of colchicine binding to unfractionated tubulin lacking the C-terminal region of either only β ($\alpha\beta_s$) or both α and β tubulin ($\alpha_s\beta_s$). In this paper, we will refer to these two forms of tubulin as hybrid tubulin and tubulin S, respectively. Surprisingly, we found that like $\alpha\beta$ tubulin, the colchicine binding to $\alpha\beta_s$ and $\alpha_s\beta_s$ tubulin remained biphasic under pseudo-first-order conditions. The C termini play no role in controlling the colchicine−tubulin-binding kinetics. Therefore, our next focus was to compare amino acid sequences at the colchicine-binding site on the tubulin main body among different isotypes. Amino acid residues defining the binding site of colchicine on tubulin have been recently identified from the crystal structure of tubulin−colchicine−stathmin-domain complex, solved at 3.5 Å resolution (*23*). From the crystal structure, it has been observed that the colchicine-binding site is mostly buried in the intermediate domain of the β subunit, while the B-ring side chain of colchicine interacts with the α subunit. Analysis of all of the β-tubulin isotype sequences along with the renal β-tubulin sequence was performed using the β subunit of the crystal structure as a template. It has been reported that the colchicide (Figure 1B) where the C-10 methoxy group of ring C has been replaced by a hydrogen atom and MD [2-methoxy-5-(2′,4′-dimethoxyphenyl)-2,4,6-cycloheptatriene-1-one] (Figure 1E), an A-ring analogue of AC (Figure 1D), which has a hydrogen in place of the 3′-methoxy group of ring A, do not recognize different isotypes (*24*). Therefore, it was also important to analyze the amino acids lying at the binding site of different colchicine analogues having different substituents in the A and C ring of colchicine. To unravel and decipher this mystery, the structure of the colchicine molecule and also the amino acids at the colchicine-binding site of tubulin have been scrutinized in detail.

MATERIAL AND METHODS

PIPES, GTP, EGTA, PMSF, colchicine, and subtilisin were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Tubulin Preparation and Estimation. Tubulin was isolated from goat brain by two cycles of temperature-dependent assembly and disassembly in PEM buffer (50 mM PIPES, 1 mM EGTA, and 0.5 mM $MgCl_2$ at pH 6.9), in the presence of 1 mM GTP, followed by two more cycles in 1 M glutamate buffer (*25*). The purified tubulin, free of MAPs, was stored in aliquots at −70 °C. The protein concentration was estimated by the method of Lowry et al. (*26*) using bovine serum albumin as the standard.

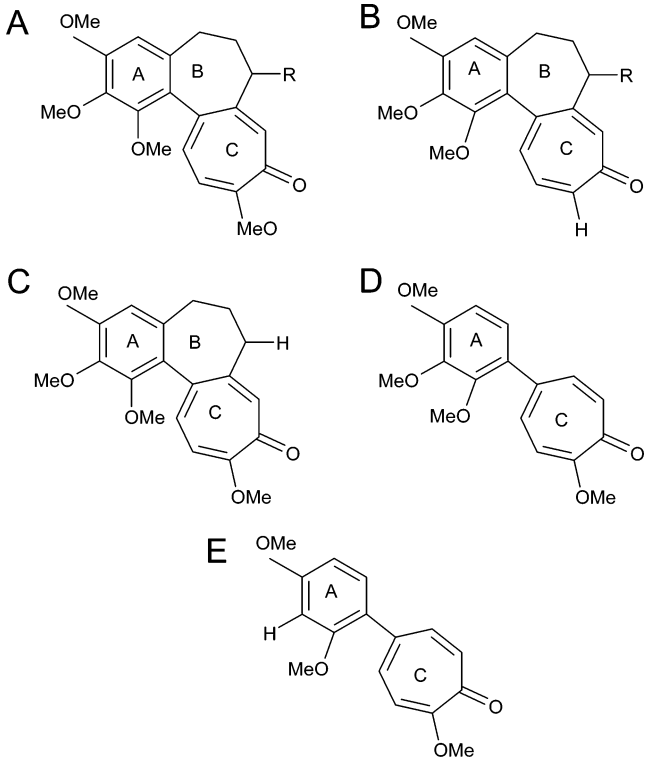

FIGURE 1: Structure of drugs. (A) colchicine, (B) colchicide, (C) DAAC (desacetamido colchicine), (D) 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (AC), and (E) 2-methoxy-5-(2′,4′-dimethoxyphenyl)-2,4,6-cycloheptatriene-1-one (MD).

Purification of $\alpha\beta_{III}$. $\alpha\beta_{III}$ isotype was purified from bovine brain tubulin according to Banerjee et al. (*13*). Briefly, PC-tubulin was passed successively through two immunoaffinity columns, first through an anti-β_{II} column that removes $\alpha\beta_I$ and $\alpha\beta_{II}$ and followed by a second anti-β_{IV} column that removes $\alpha\beta_{IV}$. Purified $\alpha\beta_{III}$ was concentrated using an Amicon XM 50 ultrafiltration membrane, dialyzed in MES buffer containing 8 M glycerol, and frozen in aliquots at −80 °C. Before use, the purified isotype was passed through a Sephadex G-25 column equilibrated in PEM buffer to remove the glycerol.

Proteolysis and the Preparation of Hybrid Tubulin ($\alpha\beta_s$) and Tubulin S ($\alpha_s\beta_s$). Tubulin was digested with subtilisin in PEM buffer containing 1 mM GTP (*27*). Digestion of tubulin with subtilisin was done using a tubulin−enzyme ratio of 100:1 (w/w). The reaction was terminated by the addition of 1 vol % of 1% (w/v) PMSF in DMSO. Digestion at 4 °C resulted in the cleavage of the C terminus of the β subunit only, and the product $\alpha\beta_s$ is termed hybrid tubulin. Digestion at 30 °C cleaved the C terminus of both the subunits resulting in tubulin S ($\alpha_s\beta_s$). The products were subjected to SDS−polyacryamide slab gel containing 9% acrylamide. The picture of the gel is shown in Figure 2.

Colchicine-Binding Kinetics. The kinetics of colchicine binding to tubulin samples were studied at 37 °C under pseudo-first-order conditions where the drug was present in large excess over tubulin. The samples were excited at 380 nm to reduce the inner-filter effect because of the high drug

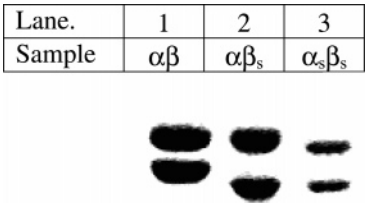

FIGURE 2: Subtilisin digestion of tubulin. Lane 1, undigested tubulin ($\alpha\beta$); lane 2, hybrid tubulin obtained by enzymatic digestion of tubulin at 4 °C ($\alpha\beta_s$); lane 3, tubulin S obtained by the digestion of tubulin at 30 °C ($\alpha_s\beta_s$).

concentration, and the emission was monitored at 437 nm. All fluorescence values were corrected for the inner-filter effect using the equation (*28*)

$$F_{corr} = F_{obs}\ \text{antilog}(A_{380} + A_{437})/2$$

where F_{obs} and F_{corr} were the observed and corrected fluorescence values and A_{380} and A_{437} were the absorbances at the excitation and emission wavelengths, respectively. The biphasic plot was analyzed (*29*) in terms of two parallel reactions as

$$F_{max} - F_t = Ae^{-\alpha t} + Be^{-\beta t}$$

where A and B were the amplitudes and α and β were the rate constants for the fast and slow phases, respectively. The analysis was performed using nonlinear curve-fitting softwares Origin 5.0 and MINSQ (from MicroMath Scientific Software, Salt Lake City, Utah). The apparent on-rate constants were calculated as $k_{on,app} = \alpha/c$, where c was the concentration of the drug.

Multiple Sequence Alignment. Sequences corresponding to the β chain of the crystal structure (PDB 1SA0) and kidney β tubulin (GenBank accession number P05217) were extracted from www.rcsb.org/pdb and www.ncbi.nlm.nih.gov, respectively. Sequences corresponding to all β-tubulin isotypes were collected from the literature (*7*). All sequences were aligned using the multiple sequence alignment program CLUSTALW (*30*).

Three-Dimensional Model Building. Homology modeling of different isotypes was performed using the β subunit of the crystal structure as the template using MODELLER (*31*). The stereochemical quality of the models were analyzed by PROCHECK (*32*) and the corresponding Ramachandran plots (*33*). The homology-modeled isotypes were then subjected to energy minimization using the conjugate-gradient method until the final energy derivative reached 0.001 kcal/mol. Each of the modeled β isotypes was then superimposed separately on the β subunit of the crystal structure. The rms deviation of superimposition was in the range of 0.2−0.3 Å for the isotypes. The colchicine-binding sites of the superimposed β isotypes were determined from the position of colchicine at the β subunit of the crystal structure.

Tubulin residues lying within 5 and 8 Å from the center of mass of the colchicine molecule were studied.

RESULTS AND DISCUSSION

Biphasic Kinetics of Colchicine Binding to Tubulin and the Role of Its C Termini. The C termini of tubulin are not only responsible for distinguishing the isotypes of tubulin but also profoundly influence other properties such as polymerization (*19*, *34*, *35*), colchicine binding (*21*), chaperone activity (*36*), bis-ANS binding, etc. (*37*). There are reports that the presence of the β_{III} isotype gives rise to the biphasic binding kinetics of the colchicine−tubulin interaction because the removal of this isotype produces a monophasic binding curve. Different combinations of tubulin isotypes, namely, ($\alpha\beta_{II} + \alpha\beta_{IV}$) exhibit a monophasic pattern, whereas biphasic kinetics were observed when a mixture of isotypes, i.e., ($\alpha\beta_{III} + \alpha\beta_{II}$) or ($\alpha\beta_{III} + \alpha\beta_{IV}$), were tested (*14*). The kinetics of colchicine binding with $\alpha\beta$, $\alpha\beta_s$, and $\alpha_s\beta_s$ tubulin were measured at 37 °C under pseudo-first-order conditions using colchicine fluorescence at 430 nm. The tubulin concentration was 3 μM, and the colchicine concentration was 50 μM in all cases. The kinetics of colchicine binding remained biphasic with $\alpha\beta_s$ tubulin (Figure 3). The apparent on-rate constants were 98.1 M^{-1} s^{-1} for the fast phase and 26.3 M^{-1} s^{-1} for the slower phase (Table 1). This biphasic nature of colchicine binding persists even when the C terminus of both α and β subunits were cleaved ($\alpha_s\beta_s$). The on-rate constants were 118 and 26.5 M^{-1} s^{-1} for the fast and slow phases, respectively. Because it has been previously demonstrated that the slow phase in the binding kinetics is due to the presence of the $\alpha\beta_{III}$ isotype (*14*), we studied the effect of subtilisin cleavage on the binding kinetics for the purified $\alpha\beta_{III}$ isotype. As shown in Figure 4, the kinetics of colchicine binding for the $\alpha\beta_{III}$ isotype does not change

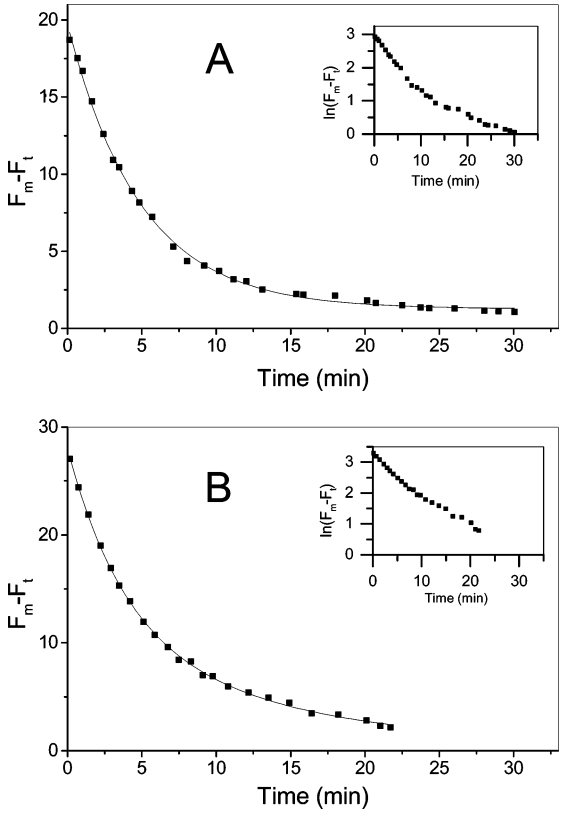

FIGURE 3: Kinetics of colchicine binding to $\alpha\beta$ tubulin. Tubulin (3 μM, mixture of all isotypes) was incubated with 50 μM colchicine at 37 °C, and the binding was monitored fluorometrically at 430 nm. The samples were excited at 380 nm to reduce the inner-filter effect because of the high drug concentration, and the emission was monitored at 437 nm. (A) $\alpha\beta$. (B) $\alpha\beta_s$ shows the plot of ($F_m - F_t$) versus time. Inset in each panel shows the plot of ln($F_m - F_t$) versus time.

Table 1: Comparison of the Colchicine-Binding Parameters for Tubulin, Digested Tubulin, and Purified $\alpha\beta_{III}$

different types of tubulin	kinetic pattern	k_{on} (fast) (M^{-1} s^{-1})	k_{on} (slow) (M^{-1} s^{-1})
$\alpha\beta$ tubulin	biphasic	78.1	16.03
$\alpha\beta_s$	biphasic	98.12	26.3
$\alpha_s\beta_s$	biphasic	118	26.5
$\alpha\beta_{III}$	monophasic	9.8	
subtilisin-digested $\alpha\beta_{(s)III}$[a]	monophasic	9.3	

[a] $\alpha\beta_{(s)III}$ indicates β-C-terminus-digested isotype $\alpha\beta_{III}$.

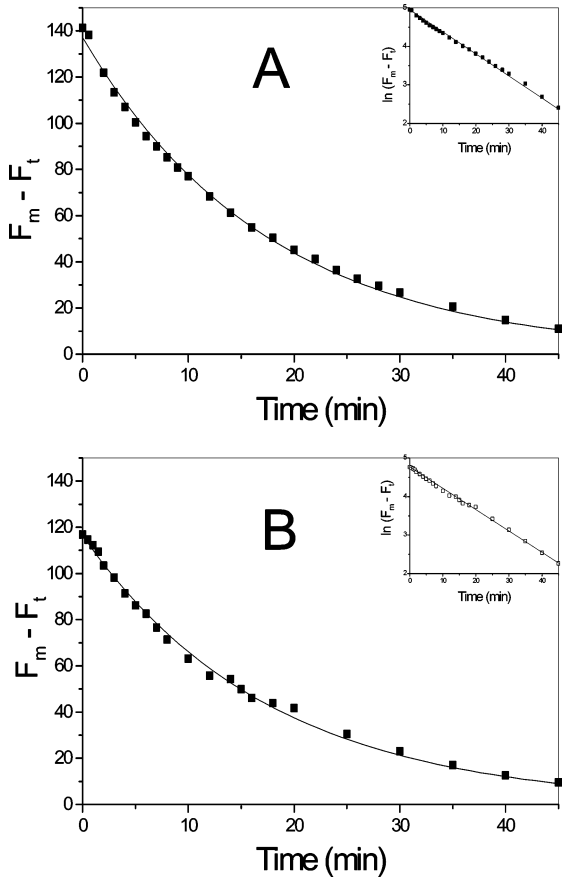

FIGURE 4: Kinetics of colchicine binding to purified β_{III} tubulin isotype. Purified isotype $\alpha\beta_{III}$ tubulin (3 μM) was incubated with 50 μM colchicine at 37 °C, and the binding was monitored fluorometrically at 430 nm. (A) $\alpha\beta_{III}$ isotype. (B) Subtilisin-digested $\alpha\beta_{III}$ isotype shows the plot of ($F_m - F_t$) versus time. Inset in each panel shows the plot of $\ln(F_m - F_t)$ versus time.

significantly upon removal of the C terminus from β_{III}. The on-rate constants were 9.8 and 9.3 M^{-1} s^{-1} for native and subtilisin-cleaved $\alpha\beta_{III}$, respectively. These results clearly indicate that the C terminus of β tubulin does not play any role in the interaction of colchicine with tubulin and that different tubulin isotypes must differ in other parts of the tubulin molecule. From the above observations, it can be concluded that kinetics of the tubulin−colchicine interaction is not a "tail" specific phenomenon of the β-tubulin isotype.

Mapping the Colchicine-Binding Site. Our experiments with C-termini-cleaved tubulin showed biphasic kinetics similar to native tubulin (uncleaved); therefore, it became clear that the C termini, which lies far away from the colchicine-binding site, plays no role in regulating colchicine−tubulin-binding kinetics. From a sequence alignment of the β subunit of the crystal structure and the β isotypes of tubulin (Figure 5), it has been found that the isotypes differ in many other regions of the sequence besides the C-terminal end. The amino acids differing in the case of the β isotypes and the β subunit of the crystal structure have been marked in bold. Amino acids lying within 5 Å from the bound colchicine molecule and which differs among different isotypes and the β subunit of the crystal structure have been marked in bold as well as underlined in Figure 5.

To study whether these changes occur at the colchicine-binding site or not, the three-dimensional models of individual β isotypes (β_I, β_{II}, β_{III}, and β_{IV}) were constructed using homology modeling. The result is presented in Table 2.

Distinguishing Isotypes on the Basis of Residues Surrounding the Bound Colchicine Molecule in Tubulin. It can be noted from Table 2 that amino acid residues lying within 5 and 8 Å from the colchicine molecule, in the crystal structure, are identical for β_I and β_{IV}. Isotypes β_{II} and β_{IV} differ at only position 318: isoleucine^{318} for β_{II} while valine^{318} for β_{IV}. However, isotype β_{III} has three changes: serine^{242}, threonine^{317}, and valine^{353} in place of leucine^{242}, alanine^{317}, and threonine^{353} for β_{IV}. Despite having a single difference in amino acid residue, a combination of ($\alpha\beta_{II}$ + $\alpha\beta_{IV}$) tubulin exhibits monophasic kinetics for colchicine−tubulin interaction. Addition of the $\alpha\beta_{III}$ isotype to either $\alpha\beta_{II}$ or $\alpha\beta_{IV}$ to form ($\alpha\beta_{III}$ + $\alpha\beta_{II}$) or ($\alpha\beta_{III}$ + $\alpha\beta_{IV}$) changes the binding kinetics from monophasic to biphasic (*14*) (with the appearance of the slow phase of binding). Thus, it seems that $\alpha\beta_{III}$ is responsible for the biphasic kinetic pattern. From Table 2, we see that $\alpha\beta_{III}$ has a much wider variation in the nature of amino acid residues (two within 5 Å and in addition one within 8 Å) at the colchicine-binding site compared to that of $\alpha\beta_{II}$ and $\alpha\beta_{IV}$. These differences in the nature of amino acids may explain the difference in the association rates as well as the affinity constant values for the $\alpha\beta_{III}$−colchicine interaction.

Previously, an analysis was done to define a part of the colchicine-binding site by correlating the experimentally determined association constants with some available primary sequences (*38*). Primary β-tubulin sequences of some organisms with known binding affinity constants were compared, and it was suggested that the relative affinities of different tubulins for colchicine depend on residues in the immediate vicinity of β^{316}. It has been reported from a comparison of residues with the experimental association constants that tubulin with isoleucine^{316} in β_I (*38*) must bind colchicine significantly more weakly than with valine^{316} in β_{II} (*38*) but more strongly than methionine^{316} in *Caenorhabditis elegans* or phenylalanine^{316} in the *Saccharomyces cerevisiae* system (*38*). An examination of the residues lying at the colchicine-binding site for $\alpha\beta_{III}$, $\alpha\beta_{II}$, and $\alpha\beta_{IV}$ revealed that there are differences around β^{316}. Besides this, there are also differences in other regions surrounding the colchicine molecule. β_{II} has an isoleucine residue at position 318, whereas β_{IV} has a valine residue at the same position. The colchicine-binding affinity constant for $\alpha\beta_{IV}$ is about 14-fold greater than that of $\alpha\beta_{II}$, whereas the corresponding rate constant for $\alpha\beta_{IV}$ is twice that of $\alpha\beta_{II}$. Indeed, the side chains of isoleucine^{318} and valine^{318} differ only in the presence or absence of an ethyl group. It is clear from Figure 6A, that the distance between the carbon of the 3-methoxy group of ring A and the side-chain methyl carbon of valine^{318} in β_{IV} is 4.21 Å. On the other hand, the distance between the

1SA0B	MREIVHIQAGQCGNQIGAKFWEVISDEHGIDP**T**G**S**YHGDSDLQLERINVYYNEA**AGN**KYV	60
β_{I}	MREIVHIQAGQCGNQIGAKFWEVISDEHGIDP**T**G**T**YHGDSDLQLDRI**S**VYYNEA**TGG**KYV	60
β_{II}	MREIVHIQAGQCGNQIGAKFWEVISDEHGIDP**T**G**S**YHGDSDLQLERINVYYNEA**AGN**KYV	60
β_{III}	MREIVHIQAGQCGNQIGAKFWEVISDEHGIDP**S**G**N**YVGDSDLQLERINVYYNEA**SSH**KYV	60
β_{IV}	MREIVHIQAGQCGNQIGAKFWEVISDEHGIDP**T**G**T**YHGDSDLQLERINVYYNEA**TGG**KYV	60
β_{kidney}	MREIVHLQAGQCGNQIGAKFWEVISDEHGIDP**T**G**T**YHGDSDLQLERINVYYNEA**TGG**KYV	60
1SA0B	PRA**I**LVDLEPGTMDSVRSG**P**FG**QI**FRPDNF**V**FGQSGAGNNWAKGHYTEGAELVDSVLD**V**V	120
β_{I}	PRA**I**LVDLEPGTMDSVRSG**P**FG**QI**FRPDNF**V**FGQSGAGNNWAKGHYTEGAELVDSVLD-V	119
β_{II}	PRA**I**LVDLEPGTMDSVRSG**P**FG**QI**FRPDNF**V**FGQSGAGNNWAKGHYTEGAELVDSVLD-V	119
β_{III}	PRA**I**LVDLEPGTMDSVRSG**A**FG**HL**FRPDNF**I**FGQSGAGNNWAKGHYTEGAELVDSVLD-V	119
β_{IV}	PRA**V**LVDLEPGTMDSVRSG**P**FG**QI**FRPDNF**V**FGQSGAGNNWAKGHYTEGAELVDSVLD-V	119
β_{kidney}	PRA**V**LVDLEPGTMDSVRSG**P**FG**QI**FRPDNF**V**FGQSGAGNNWAKGHYTEGAELVDSVLD**V**V	120
1SA0B	RKE**S**E**S**CDCLQGFQLTHSLGGGTGSGMGTLLISK**I**REEYPDRIMNTFSV**V**PSPKVSDTVV	180
β_{I}	RKE**A**E**S**CDCLQGFQLTHSLGGGTGSGMGTLLISK**I**REEYPDRIMNTFSV**V**PSPKVSDTVV	179
β_{II}	RKE**A**E**S**CDCLQGFQLTHSLGGGTGSGMGTLLISK**I**REEYPDRIMNTFSV**M**PSPKVSDTVV	179
β_{III}	RKE**C**E**N**CDCLQGFQLTHSLGGGTGSGMGTLLISK**V**REEYPDRIMNTFSV**V**PSPKVSDTVV	179
β_{IV}	RKE**A**E**S**CDCLQGFQLTHSLGGGTGSGMGTLLISK**I**REEYPDRIMNTFSV**V**PSPKVSDTVV	179
β_{kidney}	RKE**A**E**S**CDCLQGFQLTHSLGGGTGSGMGTLLISK**I**REEYPDRIMNTFSV**V**PSPKVSDTVV	180
1SA0B	EPYNAT**L**S**V**HQLVENTDETY**C**IDNEALYDICFRTLKL**T**TPTYGDLNHLVS**A**TMSGVTTC**L**	240
β_{I}	EPYNAT**L**S**V**HQLVENTDETY**C**IDNEALYDICFRTLKL**T**TPTYGDLNHLVS**G**TMSGVTTC**L**	239
β_{II}	EPYNAT**V**S**V**HQLVENTDETY**S**IDNEALYDICFRTLKL**T**TPTYGDLNHLVS**A**TMSGVTTC**L**	239
β_{III}	EPYNAT**L**S**I**HQLVENTDETY**C**IDNEALYDICFRTLKL**A**TPTYGDLNHLVS**A**TMSGVTTC**S**	239
β_{IV}	EPYNAT**L**S**V**HQLVENTDETY**C**IDNEALYDICFRTLKL**T**TPTYGDLNHLVS**G**TMSGVTTC**L**	239
β_{kidney}	EPYNAT**L**S**V**HQLVENTDETY**C**IDNEALYDICFRTLKL**T**TPTYGDLNHLVS**A**TMSGVTTC**L**	240
1SA0B	RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLT**S**RGSQQYRALTVPELTQQMFD**A**KNMM	300
β_{I}	RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLT**S**RGSQQYRALTVPDLTQQVFD**A**KNMM	299
β_{II}	RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLT**S**RGSQQYRALTVPELTQQMFD**S**KNMM	299
β_{III}	RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLT**A**RGSQQYRALTVPELTQQMFD**A**KNMM	299
β_{IV}	RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLT**S**RGSQQYRALTVPELTQQMFD**A**KNMM	299
β_{kidney}	RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLT**S**RGSQQYRALTVPELTQQMFD**A**KNMM	300
1SA0B	AACDPRHGRYLTVA**AV**FRGRMSMKEVDEQML**NV**Q**N**KNSSYFVEWIPNNVK**T**AVCDIPPRG	360
β_{I}	AACDPRHGRYLTVA**AV**FRGRMSMKEVDEQML**NV**Q**N**KNSSYFVEWIPNNVK**T**AVCDIPPRG	359
β_{II}	AACDPRHGRYLTVA**AI**FRGRMSMKEVDEQML**NV**Q**N**KNSSYFVEWIPNNVK**T**AVCDIPPRG	359
β_{III}	AACDPRHGRYLTVA**TV**FRGRMSMKEVDEQML**AI**Q**S**KNSSYFVEWIPNNVK**V**AVCDIPPRG	359
β_{IV}	AACDPRHGRYLTVA**AV**FRGRMSMKEVDEQML**NV**Q**N**KNSSYFVEWIPNNVK**T**AVCDIPPRG	359
β_{kidney}	AACDPRHGRYLTVA**AV**FRGRMSMKEVDEQML**NV**Q**N**KNSSYFVEWIPNNVK**T**AVCDIPPRG	360
1SA0B	LKM-**SA**TFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLV	419
β_{I}	LKM-**AV**TFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLV	418
β_{II}	LKM-**SA**TFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLV	418
β_{III}	LKM-**SS**TFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLV	418
β_{IV}	LKMS**AV**TFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLV	419
β_{kidney}	LKM-**SA**TFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLV	419
1SA0B	SEYQQYQDATA**D**E**QGEFEEEGEEDEA**-----	445
β_{I}	SEYQQYQDATA**EEEEDFGEEAEEEA**------	443
β_{II}	SEYQQYQDATA**D**E**QGEFEEEEGEDEA**-----	444
β_{III}	SEYQQYQDATA**EEEGEMY**E**DDEEESESQGPK**	449
β_{IV}	SEYQQYQDATA**EEEGEFEEEAEEEVA**-----	445
β_{kidney}	SEYQQYQDATA**EEEGEFEEEAEEEVA**-----	445

FIGURE 5: Sequence alignment of β isotypes of tubulin. 1SA0B, tubulin β subunit of the tubulin−colchicine−stathmin domain complex (crystal structure); β_{I}, vertebrate β isotype I; β_{II}, vertebrate β isotype II; β_{III}, vertebrate β isotype III; β_{IV}, vertebrate β isotype IV; β_{kidney}, kidney β-tubulin sequence (GenBank accession number P05217). The amino acids differing in case of β isotypes and the β subunit of the crystal structure have been marked in bold. The amino acids within a 5 Å distance from the bound colchicine molecule, which are different among different isotypes, and the β subunit of the crystal structure have been marked in bold as well as underlined.

Table 2: Residues of Different β-Tubulin Isotypes Lying in Close Proximity to the Colchicine Molecule

crystal structure[a]	isotype 1[b] β_I	isotype 2[b] β_{II}	isotype 3[b] β_{III}	isotype 4[b] β_{IV}
GLN (133)				
ASN (167)				
GLU (200)				
TYR (202)				
GLY (237)				
VAL (238)				
THR (239)				
THR (240)				
CYS (241)				
LEU (242)			**SER**	
ARG (243)				
GLN (247)				
LEU (248)				
ASN (249)				
ALA (250)				
ASP (251)				
LEU (252)				
ARG (253)				
LYS (254)				
LEU (255)				
ALA (256)				
VAL (257)				
ASN (258)				
MET (259)				
VAL (260)				
PHE (268)				
LEU (313)				
THR (314)				
VAL (315)				
ALA (316)				
ALA (317)			**THR**	
VAL (318)		**ILE**		
ILE (347)				
PRO (348)				
ASN (349)				
ASN (350)				
VAL (351)				
LYS (352)				
THR (353)			VAL	
ALA (354)				
THR (376)				
PHE (377)				
ILE (378)				
GLY (379)				
ASN (380)				

[a] Residues in the crystal structure, lying within 8 Å (normal font) and 5 Å (bold front) of colchicine are shown. The residue numbering follows the convention used in the crystal structure (1SA0). The residue numbering in the crystal structure differs from the sequence numbering (see Figure 5) because the crystal structure omits residue numbers 45−46 and 361−368 to suit proper superimposition of the α and β chain in 1SA0. [b] For the isotypes, a residue is shown only if it differs from the equivalent residue in the crystal structure.

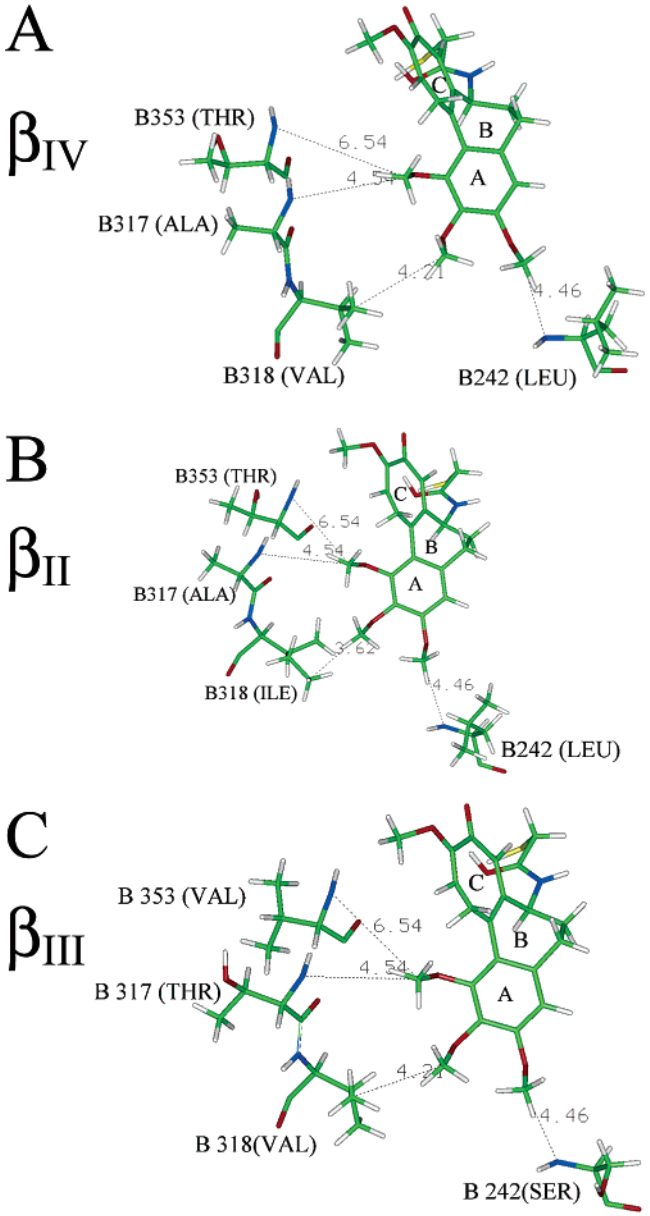

FIGURE 6: Residues of different β-tubulin isotypes lying in close proximity to the colchicine molecule. The colchicine molecule occupies the upper right-hand side in each panel. The numbers over the dotted line indicate the corresponding distance in angstroms between the atoms of the colchicine molecule and the nearby amino acid residues. (A) Residues of β_{IV} denoted by B353, B317, B318, and B242. (B) Residues of β_{II} denoted by B353, B317, B318, and B242. (C) Residues of β_{III} denoted by B353, B317, B318, and B242.

hydrogen of the 3-methoxy group of ring A and the side-chain ethyl carbon of isoleucine[318] in β_{II} is 3.62 Å (Figure 6B). The ethyl group, being a bulkier group than a methyl group, can enter into a van der Waals repulsive interaction with the 3-methoxy group of ring A of colchicine, which results in the lowering of the affinity constant and rate constant of β_{II} relative to β_{IV}. The affinity constant for colchicine binding for $\alpha\beta_{IV}$ is about 28-fold higher than that of $\alpha\beta_{III}$, and the rate constant is about 8 times greater. When β_{III} is compared to β_{IV}, there are two changes within 5 Å of the colchicine molecule. Leucine[242] and alanine[317] of $\alpha\beta_{IV}$ have been replaced by serine[242] and threonine[317], respectively, in $\alpha\beta_{III}$ (Figure 6C). Thus, we find that there is a shift from a hydrophobic to a hydrophilic environment. Because there is evidence that colchicine binding involves a hydrophobic interaction, we can conclude that these two alterations lower the K_a (affinity constant) of colchicine binding as well as the on-rate constant of $\alpha\beta_{III}$ relative to $\alpha\beta_{IV}$. In the case of kidney β tubulin, which lacks the β_{III} isotype (*12*), the apparent on-rate constant of binding is very close to that of the faster binding component ($\alpha\beta_{IV}$) of brain tubulin.

When we compare colchicine-binding kinetics of $\alpha\beta_{II}$ and $\alpha\beta_{III}$, we find that the rate constant of $\alpha\beta_{II}$ is about 4 times that of $\alpha\beta_{III}$, whereas the corresponding affinity constant for colchicine binding is about 2 times greater. Isoleucine[318], leucine[242], and alanine[317] in $\alpha\beta_{II}$ have been replaced by valine[318], serine[242], and threonine[317] in $\alpha\beta_{III}$. Thus, the

hydrophobic residues (leucine and alanine) in the case of isotype $\alpha\beta_{II}$ have been replaced by hydrophilic residues (serine and threonine) in the case of $\alpha\beta_{III}$. This difference in the nature of residues might be responsible for the differential binding kinetics as well as the difference in affinity constant between β_{II} and β_{III}. Therefore, the variation in the nature of residues encompassing the colchicine molecule influences the kinetics and affinity constant of the colchicine−tubulin interaction. Interestingly, we found that the variation in the nature of residues (in a proximal region of 5 Å) lies around the A ring of colchicine only, as discussed later.

Effect of the Colchicine Structure on Kinetics of the Colchicine−Tubulin Interaction. The kinetics of the colchicine−tubulin interaction is mainly controlled by the B ring and the C-7 side chain of colchicine. Thus, AC (Figure 1D; having only the A and C ring of colchicine) binding to tubulin is instantaneous and reversible, whereas colchicine binding is slow and poorly reversible (*39*, *40*). However, the biphasic kinetics of colchicine binding to unfractionated tubulin indicates that the rate of the colchicine−tubulin interaction is also controlled at the protein level. The binding of desacetamidocolchicine (DAAC) (Figure 1C) to tubulin follows biphasic kinetics similar to those of colchicine. Moreover, like colchicine, the affinity constant of DAAC for $\alpha\beta_{III}$ is much less than that for $\alpha\beta_{II}$ and $\alpha\beta_{IV}$ (*15*). Therefore, it is obvious that the B-ring substituent does not distinguish different β isotypes. Again, there is a report that AC [2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone] (Figure 1D), which is devoid of the B ring, also exhibits biphasic kinetics in its binding to tubulin (*16*). Therefore, at this point, it is clear that the kinetics of binding of colchicine to tubulin may be a feature of rings A and C. Indeed, there are reports that colchicine analogues with a modified C ring such as colchicide (Figure 1B) and MD [2-methoxy-5-(2′,4′-dimethoxyphenyl)-2,4,6-cycloheptatriene-1-one] (Figure 1E) with a modified A ring do not recognize tubulin isotypes (*24*, *41*). Colchicide has a modified C ring, where the C-10 methoxy group of the ring C has been replaced by a hydrogen atom. This analogue does not show any fluorescence upon tubulin binding nor can it distinguish kinetically among different tubulin isotypes (*24*). MD, a fast binding colchicine analogue like AC (without the B ring), possesses a modified A ring, where the 3′ methoxy group in the A ring is replaced by a hydrogen (*41*). Like colchicide, MD is unable to differentiate among tubulin isotypes. One thing is common to both analogues that the replacement of a bulky methoxy group by a hydrogen atom abolished completely their ability to recognize different tubulin isotypes. Therefore, the presence of a methoxy group is the crucial determinant for this type of recognition. Because a larger methoxy group is replaced by a smaller hydrogen atom, the methoxy group of those analogues may play some role in generating biphasic kinetics of the colchicine−tubulin interaction. From Figure 6B, it appears that the 3-methoxy group of ring A of colchicine suffers steric repulsion with residue isoleucine^{318} in β_{II}. Such an interaction is absent in the case of β_{IV} (Figure 6A) as well as in the case of β_{III} (Figure 6C) because both of them have a valine residue at the same position and isoleucine happens to be much bulkier than valine.

ACKNOWLEDGMENT

We are grateful to Prof. Richard F. Ludueña of the Department of Biochemistry, University of Texas Health Science Center, TX, and Dr. Pradeep Parrack of the Biochemistry Department of Bose Institute, Calcutta, India, for their critical reading of the manuscript.

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BI050599L