N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)colcemid, a probe for different classes of colchicine-binding site on tubulin

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The nature of binding of 7-nitrobenz-2-oxa-1,3-diazol-4-yl-colcemid (NBD-colcemid), an environment-sensitive fluorescent analogue of colchicine, to tubulin was tested. This article reports the first fluorometric study where two types of binding site of a colchicine analogue on tubulin were detected. Binding of NBD-colcemid to one of these sites equilibrates slowly. NBD-colcemid competes with colchicine for this site. Binding of NBD-colcemid to this site also causes inhibition of tubulin self-assembly. In contrast, NBD-colcemid binding to the other site is characterised by rapid equilibration and lack of competition with colchicine. Nevertheless, binding to this site is highly specific for the cholchicine nucleus, as alkyl-NBD analogues have no significant binding activity. Fast-reaction-kinetic studies gave 1.76×10^5 M^{-1} s^{-1} for the association and 0.79 s^{-1} for the dissociation rate constants for the binding of NBD-colcemid to the fast site of tubulin. The association rate constants for the two phases of the slow site are 0.016×10^{-4} M^{-1} s^{-1} and 3.5×10^{-4} M^{-1} s^{-1}, respectively. These two sites may be related to the two sites of colchicine reported earlier, with binding characteristics altered by the increased hydrophobic nature of NBD-colcemid.

Tubulin, a 100 kDa protein of two subunits α and β, binds the plant alkaloid colchicine and its structural analogues with high affinity [1]. Binding of colchicine and its analogues to tubulin typically disrupts organized assembly of tubulin monomers into microtubules. The precise mechanism(s) by which colchicine disrupts tubulin polymerisation is unknown and is the topic of intense study [2—4]. Several features of colchicine/tubulin interaction are unusual. The association is a slow, poorly reversible process which is highly temperature dependent [5—7]. It has been argued that rings B and C of the colchicine molecule are responsible for such unusual properties [8]. The kinetics of colchicine/tubulin interaction were found to consist of two steps, as described by the following scheme [9]:

$$T + C \rightleftharpoons [TC] \rightarrow TC^*. \qquad (1)$$

This sequence of reaction was postulated to involve an initial rapid equilibration step between colchicine (C) and tubulin (T) to form a low-affinity tubulin/colchicine complex [TC], followed by a slow and essentially irreversible step forming the fluorescent complex TC*.

Recently, a fluorescent analogue of colcemid, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)colcemid (NBD-colcemid) has been synthesized and used for the study of cytoskeletal and dynamic cell structure in vivo [10]. It disrupts microtubules in vivo and is reported to bind tubulin with a stoichiometry of 1:1. NBD-colcemid has a higher quantum yield and is more hydrophobic, compared to colchicine and colcemid. However, its specificity of inhibition of tubulin self-assembly

in vitro, detailed binding properties with tubulin, specificity with respect to the colchicine-binding site and whether any special properties are generated upon introduction of a bulky hydrophobic group at the B ring of colchicine, remain unanswered. Our studies aim to answer these questions.

MATERIALS AND METHODS

Materials

NBD-colcemid was purchased from Molecular Probe (USA). Colchicine, colcemid, podophyllotoxin, GTP, EGTA, Pipes, polyester-bound TLC silica-gel plates were from Sigma Chemical Co. (USA). Phosphocellulose was from Whatman (UK) and [^{3}H]colchicine was from Amersham (UK). All other reagents were of analytical grade.

Tubulin isolation and assay

Tubulin was isolated from goat brains by two cycles of temperature-dependent assembly, followed by phosphocellulose column chromatography of microtubular proteins [11], except for the polymerisation study where three cycles were performed. The composition of the reassembly buffer was 0.05 M Pipes, pH 6.9, containing 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP. After column chromatography, the protein was concentrated in Amicon CF50A membrane cone. Purified tubulin was stored in aliquots at $-70°C$. Protein concentrations were determined by the method of Lowry et al. [12].

Pure tubulin (1.8 mg/ml) in Pipes buffer was polymerised at 37°C in the presence of 1 mM GTP. Polymerisation was initiated by 20 μM taxol and turbidity was measured by ab-

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Abbreviation. NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl.

sorbance at 380 nm rather than the usual 350 nm, as NBD-colcemid has an absorbance maximum near 350 nm. A Shimadzu UV-160 double-beam spectrophotometer, fitted with a temperature-controlled bath, was used for this purpose.

The [³H]colchicine-binding assays were performed by the method of Banerjee and Bhattacharyya [13].

Fluorescence measurements and determination of binding parameters

Fluorescence spectra were recorded in a Hitachi F-3000 spectrofluorometer connected to a constant-temperature circulating-water bath. Unless mentioned otherwise, the excitation wavelength was 465 nm when less than 10 µM NBD-colcemid was used and 500 nm (to minimise inner filter effect) when more than 10 µM NBD-colcemid was used. Excitation wavelengths for colchicine and tryptophan of tubulin were 350 nm and 290 nm, respectively. Emission wavelengths were 530, 336 and 430 nm for NBD-colcemid, tryptophan and colchicine, respectively. 5-nm band pass was used for both excitation and emission.

All experiments were carried out in either Pipes or 10 mM phosphate, pH 6.9, containing 0.25 mM $MgCl_2$ and 0.5 mM EGTA (buffer A), unless stated otherwise. Stock solutions of NBD-colcemid were prepared in absolute ethanol and stored at $-20°C$. Dilute solutions, if required, were prepared in buffer A immediately before use. As NBD-colcemid is prone to photodecomposition, purity of NBD-colcemid was checked by TLC on silica-gel plates and by absorption spectra. Concentrations of NBD-colcemid were measured by absorbance at 470 nm using a molar absorption coefficient of 21 000 M^{-1} cm^{-1} [10].

The dissociation constants and stoichiometries of NBD-colcemid binding to tubulin were determined from conventional Scatchard plot [14]. Fluorescence enhancement (Q) was calculated from a double-reciprocal plot [15] using the equation

$$F_0/(F-F_0) = \{K_d/(Q-1)\}\ 1/P_T + 1/(Q-1),$$

where F_0 is the fluorescence of a fixed concentration of drug, F is its fluorescence at a particular concentration of the protein and P_T is the total concentration of the protein. The protein concentration used in reverse titration was always in excess over the drug concentration and an approximation was made that the total protein concentration is equal to the free protein concentration. Q at 530 nm was calculated by titrating a fixed amount (0.5 µM) of NBD-colcemid with increasing amounts of tubulin (1–10 µM). Stoichiometry and K_d of NBD-colcemid binding to tubulin was determined from a Scatchard plot using 2 µM tubulin and varying NBD-colcemid over 0.2–20 µM.

The protein-bound drug concentrations were calculated by the method of Mas and Colman [16] using the equation

$$[PD] = \{D_T/(Q-1)\}\ \{(F/F_0)-1\},$$

where [PD] is the concentration of the protein-bound drug, D_T is the total drug concentration, F is the fluorescence value at a fixed concentration of protein and a particular concentration of drug and F_0 is the fluorescence of the same concentration of drug without the protein. Q determined from the previous double-reciprocal plot was used. The value of protein-bound drug concentration thus obtained was used for Scatchard analysis. In these experiments excitation wavelength of 500 nm was used to avoid inner filter effect.

In case of Scatchard analysis of colchicine binding to tubulin, correction for inner filter effect was carried out using the following equation

$$F_{corrected} = F_{observed}\ [\text{antilog}\ \{(A_{ex} + A_{em})/2\}],$$

where A_{ex} is the absorbance at the excitation wavelength and A_{em} is the absorbance at the emission wavelength.

To follow the time dependence of NBD-colcemid binding to tubulin by quenching of tryptophan fluorescence, tryptophan fluorescence of 2 µM tubulin was measured at 336 nm upon excitation at 290 nm. This was followed for 2 h at 37°C. In another set, 2 µM fresh tubulin was mixed with 4 µM NBD-colcemid and kinetics followed for 2 h at 37°C. Absorbance of the two sets of solutions were measured at 290 nm and 336 nm, and corrections for inner filter effect were carried out using the equation given above. Volume corrections were performed for fluorescence values where addition of NBD-colcemid was made. The fluorescence values were normalised by dividing the fluorescence values obtained at different intervals by that obtained initially (before drug addition). The second set of values was then divided by the corresponding values of only tryptophan fluorescence, i. e. those of the first set to correct for the decay of colchicine-binding site. These values were then plotted against time.

Statistical analysis

The time dependence of binding of NBD-colcemid to tubulin was analysed by a non-linear least-squares fit procedure. The obtained experimental values were fitted to a single exponential, single exponential with a zero time offset, double exponential and double exponential with a zero-time offset function. The parameters were systematically varied within a wide range (a coarse grid search followed by a fine grid search). The parameters that gave the best χ-square value were judged to be the best-fit parameters.

Fast-reaction kinetic studies

The fast phase of the kinetics of association of NBD-colcemid with tubulin was studied with a Union Giken RA-401 stopped-flow spectrofluorometer. Samples were excited at 445 nm with the help of a cutoff filter. The dead time of the instrument was determined to be 0.5 ms. The reacting solutions were maintained at constant temperature ($\pm 0.1°C$) by a Lauda circulating-water bath. Experiments were performed under pseudo-first-order kinetic conditions with 2.5 µM NBD-colcemid and 25–50 µM tubulin (final concentrations after mixing). The association rate constant, k_1 for the binding of NBD-colcemid was also determined by second-order plots of the increase of ligand fluorescence, when an equal concentration (70 µM) was mixed with tubulin (70 µM).

RESULTS

Inhibition of tubulin polymerisation

The structure of NBD-colcemid, which can decorate microtubules *in vivo*, is shown in Fig. 1a. Hiratsuka and Kato [10] reported that some microtubules are disrupted *in vivo* by this drug. We have tested its self-assembly inhibitory capacity *in vitro* using purified tubulin. Assembly of purified goat brain tubulin was initiated by using 10 µM taxol. As
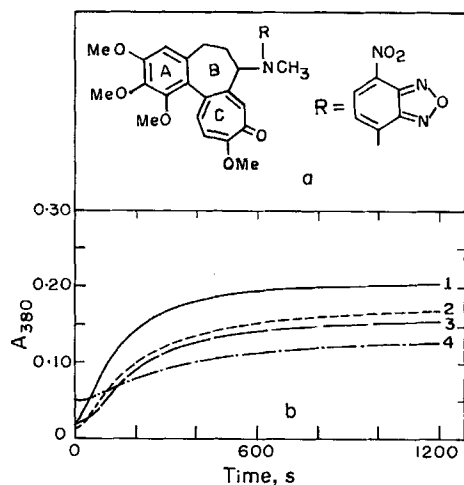
Fig. 1. Structure of NBD-colcemid (a) and inhibition of tubulin polymerisation by NBD-colcemid (b). 1.8 mg/ml tubulin was polymerised by 1 mM GTP and 20 µM taxol in 50 mM Pipes, pH 6.9, containing 0.5 mM $MgCl_2$ and 1 mM EGTA (1) without drug, (2) with 10 µM NBD-colcemid, (3) with 15 µM NBD-colcemid and (4) with 20 µM NBD-colcemid.

shown in Fig. 1 b, NBD-colcemid shows progressive concentration-dependent inhibition of assembly and 57% inhibition was obtained with 20 µM NBD-colcemid (the highest concentration tested) at protein/drug molar ratio of 1 : 1.1. This result indicates that substitution of NBD group in the B ring probably did not affect the biological activity of the colcemid molecule significantly.

Time dependence of NBD-colcemid binding

Colchicine is known for its slow and time-dependent binding to tubulin. Previously, it was observed that this time dependence is related to the presence of substituents in the B ring, especially the C=O group at C7 of the colchicine nucleus [8]. Colcemid, a colchicine analogue which lacks the C=O group, binds tubulin much faster than colchicine [13]. Time dependence of NBD-colcemid was tested by observing NBD-colcemid fluorescence as a function of time after mixing 3 µM tubulin with 5 µM NBD-colcemid at 37°C. The profile of fluorescence increase (shown in Fig. 2a) is characterised by a very rapid phase which is over within the time taken to mix the protein and the drug, and a slow phase which levels off after around 2 h. Interestingly, tubulin saturated with colchicine still binds NBD-colcemid, but in this case, the slow time-dependent part of the fluorescence increase is lost. These results indicate that there are two types of NBD-colcemid-binding sites on tubulin. Binding to one of these sites equilibrates slowly and colchicine competes with NBD-colcemid for this site. NBD-colcemid binding to the other site equilibrates very fast and colchicine does not compete effectively with NBD-colcemid for this site.

Structural similarity of NBD-colcemid with colchicine and the inhibition of self-assembly caused by NBD-colcemid suggested that NBD-colcemid may bind tubulin at the colchicine site. To test this hypothesis, NBD-colcemid was allowed to compete with [³H]colchicine for tubulin binding. As shown in Fig. 3, 63.5% inhibition of [³H]colchicine binding was observed in the presence of 40 µM NBD-colcemid. No significant [³H]colchicine binding was observed with tubulin after incubation with excess NBD-colcemid (data not
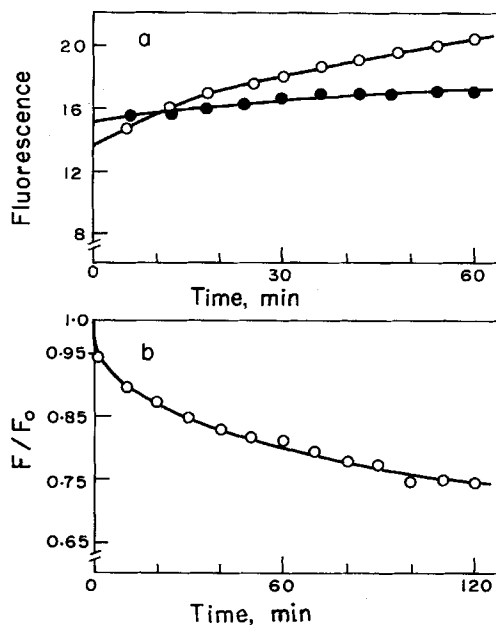

Fig. 2. Time-dependence of binding (a) and tryptophan fluorescence quenching (b) of NBD-colcemid binding to tubulin. (a) NBD-colcemid (final concentration 5 µM) was added to either tubulin (○) or tubulin/colchicine complex (●) in buffer A. Kinetics of binding were followed for 1 h at 37°C by measuring the fluorescence in each case. Excitation and emission wavelengths were 465 nm and 530 nm respectively. (b) Solution conditions are same as in (a). Excitation and emission wavelengths were at 290 nm and 336 nm respectively. To 2 µM tubulin, 4 µM NBD-colcemid was added and fluorescence at 336 nm was followed for 2 h (○).
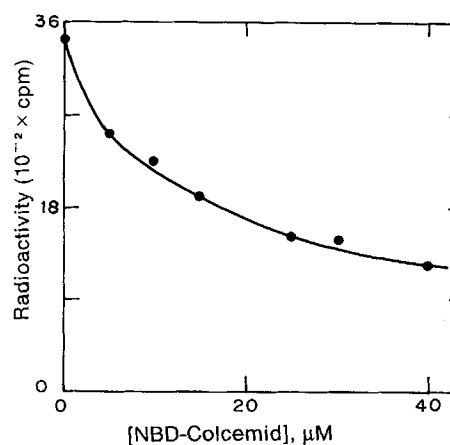
Fig. 3. Effect of NBD-colcemid on [³H]colchicine binding to tubulin. Tubulin (3 µM) was added to a reaction mixture containing a fixed concentration of colchicine (3 µM) in buffer A, and NBD-colcemid was added to give the final concentrations indicated in the figure and incubated for 1 h at 37°C. The binding assay was performed according to Banerjee and Bhattacharyya [17].

shown). This suggests that NBD-colcemid and [³H]colchicine compete for the same binding site on tubulin molecule.

To characterise further the fast and slow binding of NBD-colcemid to tubulin, the effect of NBD-colcemid on tryptophan fluorescence of tubulin was studied. Absorption spectra of NBD-colcemid overlaps well with the emission spectra of tryptophan. Thus, the binding of NBD-colcemid to tubulin may lead to quenching of its tryptophan fluorescence if it
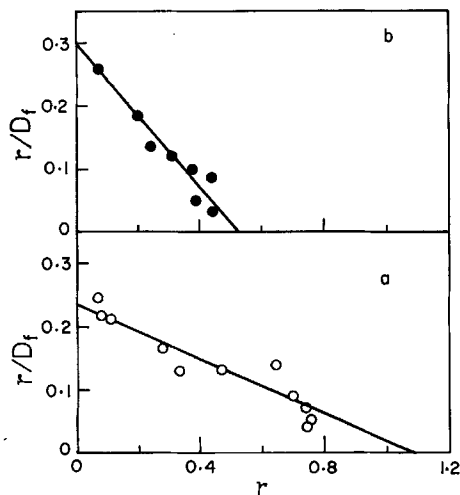
Fig. 4. Scatchard plot of NBD-colcemid binding to tubulin. (a) 2 μM tubulin was titrated with 0.2−20 μM NBD-colcemid at 37 °C. Solution conditions are the same as in Fig. 2a. (b) 55 μM tubulin was incubated with 455 μM colchicine for 1 h, and from that 2 μM complex was titrated with 0.2−20 μM NBD-colcemid. Solution conditions are the same as in Fig. 2a, except that 400 μM excess colchicine was added to it. In both cases, fluorescence was seen after 1 h at 530 nm upon excitation at 500 nm.

binds to a site at or near which one or more tryptophans are present. When tryptophan fluorescence was followed as a function of time after mixing tubulin and NBD-colcemid, it was observed that NBD-colcemid causes an instantaneous quenching, and, subsequently, the quenching increases with time, attaining saturation after about 2 h (Fig. 2 b). The kinetics of tryptophan quenching of tubulin upon NBD-colcemid binding is very similar to that observed for enhancement of NBD-colcemid fluorescence (Fig. 2 a).

Equilibrium and kinetic properties

From the results presented above, it is clear that NBD-colcemid has at least two types of binding site on tubulin. To obtain more quantitative information on the two NBD-colcemid-binding sites on tubulin, the stoichiometries, dissociation constants and on-rate constants were determined.

The fluorescence of free NBD-colcemid was enhanced 13-fold upon binding to tubulin. A fluorescence titration of the binding at 37 °C was analysed by a Scatchard plot (Fig. 4 a), yielding a dissociation constant of 5 μM. The intercept on the abscissa is 1.1, indicating the stoichiometry of the NBD-colcemid/tubulin interaction. Under identical conditions, and with the same tubulin preparation, the stoichiometry of colchicine binding to tubulin (measured for the purpose of comparison) was found to be 0.6 mol/mol tubulin (data not shown). This is in accordance with the published literature values of 0.6−0.8 [5, 17]. It is clear from Fig. 2 that there are at least two NBD-colcemid-binding sites on tubulin, one of which equilibrates very fast compared to the other. Moreover, prior incubation of tubulin with colchicine abolishes binding at the slow binding site, whereas the fast NBD-colcemid-binding site remains unaffected. We carried out Scatchard analysis of NBD-colcemid binding to tubulin which already had colchicine bound to it. The result of such an experiment is presented in Fig. 4b. As the bound colchicine affects the slow binding site, the result of this Scatchard plot indicates the binding parameters of the fast site. The
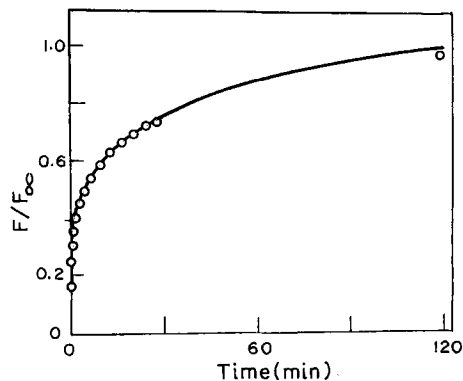
Fig. 5. Kinetics of NBD-colcemid binding to tubulin under pseudo-first-order conditions. 1 μM tubulin was mixed with 30 μM NBD-colcemid at 20 °C and fluorescence recorded frequently. Solution conditions are the same as in Fig. 2a. Excitation and emission wavelengths were 465 nm and 530 nm, respectively.
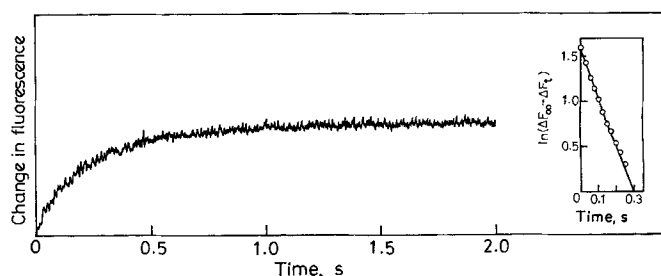

Fig. 6. Stopped-flow trace for the interaction of NBD-colcemid and tubulin at 20 °C. NBD-colcemid (5 μM) and tubulin (50 μM) were mixed in the cell (2 mm path length) of the stopped-flow instrument, and the fluorescence change was recorded keeping a constant time of 1 ms. The samples were excited at 445 nm and emission recorded above 490 nm. Solution conditions were the same as in Fig. 2a (inset).

dissociation constant for the fast site is 1.75 μM with a stoichiometry of 0.55 mol NBD-colcemid/mol tubulin.

Kinetic studies

The kinetics of the association reaction were studied under pseudo-first-order conditions by monitoring the increase of ligand-emission intensity as a function of time. The NBD-colcemid data was then fitted to a single exponential, single exponential with a zero-time offset, double exponential and double exponential with zero-time offset. By far the best fit was obtained with a bi-exponential function with a zero-time offset. The best χ-square value obtained with functions of this nature was two orders of magnitude better than the best value obtained with only the double-exponential function. This indicates that the kinetic data can be best explained by a rapid binding phase followed by slow binding step(s). The two rate constants for the slow phase are most probably due to differences in binding of the isotypes of tubulin preparation, as reported earlier [18]. Under identical conditions, colchicine binding to tubulin gave a best fit to a bi-exponential function. A typical kinetic profile is shown in Fig. 5. The on-rate constants for the two slower phases are given by 0.016×10^{-4} M^{-1} s^{-1} and 3.5×10^{-4} M^{-1} s^{-1} respectively. The on-rate constant for the rapid phase was measured by stopped-flow method, as described below.
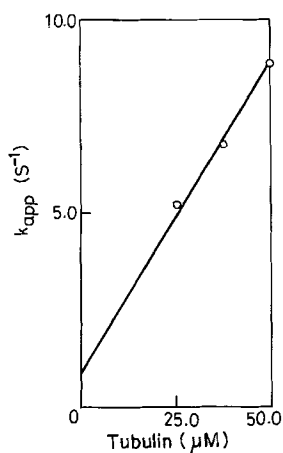
Fig. 7. Determination of the rate constant for the association of NBD-colcemid to tubulin at 20 °C. K_{app} values determined were plotted as a function of tubulin concentration after mixing. The resultant concentration of NBD-colcemid was 2.5 μM. The slope and intercept of the straight line gave k_1 and k_{-1}, respectively.

Fast-reaction-kinetic studies

Equilibrium fluorescence titration revealed a large change in the fluorescence of NBD-colcemid upon binding to tubulin, providing a convenient means of studying the kinetics of this interaction. A representative stopped-flow fluorescence trace for the association process is shown in Fig. 6. The approach of fluorescence to its final value was essentially a monophasic first-order process. The rate constant for the first-order approximation K_{app} was obtained by fitting the data to a single exponential function as 5.22 s⁻¹. A natural-log plot of the change in fluorescence viz ln $(F_\infty - F_t)$ versus time t, where F_∞ and F_t are the fluorescence values at infinity and at time t, respectively, also gave K_{app} as 5.23 s⁻¹ (inset of Fig. 6).

The kinetic data under pseudo-first-order conditions were interpreted in terms of a single-step reaction

$$T + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} TC, \qquad (1)$$

where T and C refer to tubulin and NBD-colcemid, and k_1 and k_{-1} are the association and dissociation rate constants, respectively. Eqn 2 relates the observed rate constant (K_{app}) for the approach of the fluorescence to its final value, to the first-order rate constant for the formation of the complex (k_1), the rate constant for the dissociation of the complex (k_{-1}) and the concentration of the component in excess [T]

$$K_{app} = k_1[T] + k_{-1}. \qquad (2)$$

The values of k_1 and k_{-1} were determined from the slope and the intercept, respectively, of the linear plots of K_{app} versus [T] (Fig. 7). The values of k_1 and k_{-1} thus determined were 1.76×10⁵ M⁻¹ s⁻¹ and 0.79 s⁻¹, respectively. The value of the dissociation rate constant could not be determined directly due to the lack of a displacing ligand, as neither colchicine nor any of its derivatives were able to displace NBD-colcemid bound to tubulin at the fast site.

The association rate constant was also determined from a second-order plots for the approach of the fluorescence to its final value upon mixing of equivalent concentration of T and C. Under such a situation, the dependence of fluo-
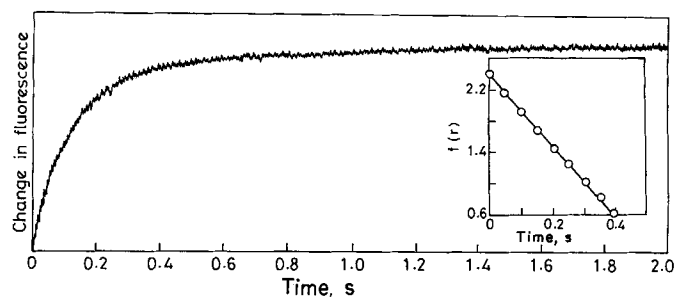
Fig. 8. Evaluation of k_1 from the second-order approach to equilibrium for formation of tubulin/NBD-colcemid complex. Tubulin and NBD-colcemid were mixed at equimolar concentration (70 μM each) in the stopped-flow cell at 20 °C. The second-order plot for fluorescence change is shown in the inset. Eqn 3 was used to calculate the value of k_1 from the slope of the second-order plot. The excitation and emission wavelengths used were 445 nm and 490 nm, respectively. Solution conditions were the same as in Fig. 2 a.

Table 1. Enhancement of NBD-colcemid fluorescence in the presence of different proteins.

Protein	Fluorescence enhancement
	%
BSA	2
Lysozyme	<2
Papain	<2
Hexokinase	16
λ repressor	11
Tubulin	100

Solution conditions were the same as in Fig. 2a, and protein concentrations used were 5 μM each. Fluorescence was measured (530 nm excitation) at 465 nm after a 5-min incubation at 25 °C. BSA, bovine serum albumin. The fluorescence increase for tubulin was considered as 100%.

rescence is given by Eqn 3 for a single reversible bimolecular reaction:

$$f(R) = \ln \{R/\bar{R} \, (R - \bar{R}) + R_m^2)\},$$

or

$$f(R) = \frac{-k_1 \, (R_m^2 - \bar{R}^2)}{R \Delta F_m} t + \ln \frac{\bar{R}}{R_m^2}, \qquad (3)$$

where R is the difference between the fluorescence at infinity and at a given time t, $\bar{R}$ is the value of R at $t = 0$, R_m is the value of R if the reaction was irreversible and ΔF_m is the maximum change in fluorescence. The value of k_1 was obtained from a linear plot of $f(R)$ versus time as shown in the inset of Fig. 8. k_1 thus obtained, for the interaction of NBD-colcemid with the fast site, was 1.33×10⁵ M⁻¹ s⁻¹, which is in good agreement with the value of k_1 obtained from pseudo-first-order kinetic plots.

Specificity of the fast NBD-colcemid site

The binding of NBD-colcemid to the fast site is not due to the presence of the NBD group only, as we observed that 2-mercaptoethanol conjugate of 4-(N-iodoacetoxyethyl-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole did not bind to tubulin. NBD-colcemid, by virtue of its possession of several aromatic rings, may act as a non-specific hydrophobic fluo-

rescent probe. Such non-specific hydrophobic probes, e. g. 1,8 anilino naphthalene sulphonic acid, often shows selective binding. They are, however, in general, not protein specific and bind to many proteins with similar affinity. To rule out the possibility that the fast binding site of NBD-colcemid is due to non-specific hydrophobic interactions, binding of NBD-colcemid to a large number of proteins was tested. The results with a few of these proteins are shown in Table 1. A lack of significant binding to several other proteins indicates the specific nature of NBD-colcemid binding to tubulin.

Urea and guanidine hydrochloride are often used as destroyers of the tertiary structure of proteins. The ligand binding specificity, in most cases, is a function of the tertiary structure of proteins. Thus, a decrease in binding and consequent fall in ligand fluorescence, upon incubation with urea or guanidine hydrochloride, would indicate the specific nature of drug binding. Both the fast and slow NBD-colcemid-binding sites are sensitive towards these denaturing agents (data not shown). This also indicates the specific nature of NBD-colcemid binding to tubulin.

DISCUSSION

The observed association rate constant is several orders of magnitude slower than the diffusion-controlled reactions, and the kinetics of the fastest phase of the binding of NBD-colcemid to tubulin is qualitatively consistent with a single-step binding mechanism. In general, when the second-order rate constants for ligand binding to a protein differ by several orders of magnitude from that expected for a diffusion-controlled reaction, the binding is presumed to proceed through the formation of an intermidiate (TC_1) as shown in the equations below:

$$T + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} TC_1 \underset{k_{-2}}{\overset{k_1}{\rightleftharpoons}} TC^* ;$$
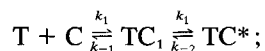
$$K_I = k_1/k_{-1} ; K_{II} = k_2/k_{-2} ;$$

where TC_1 and TC^* are the initial and the final complexes, respectively. Formation of the intermediate complex occur much faster than the ultimate complex. Our failure to observe the TC_1 complex could be due to an unobservable signal change in the faster step. If the fast step is always kinetically uncoupled from the second step, viz. $TC_1 \rightarrow TC^*$, which is the rate-determining event ($k_{-1} \gg k_2$), then k_{app} should progress from first-order dependence to a zero-order dependence as the concentration of the excess component, in this case T, increases from a value much lower than $1/K_I$ to $T \gg 1/K_I$. Since we have been able to examine the dependence of K_{app} on tubulin concentration to the extent of 50 μM, the association constant for the first step (K_I) has to be lower than $20000 M^{-1}$. Alternatively steric factors may be responsible for the low bimolecular association constant. Such steric effects have indeed been observed for the binding of dyes to cyclodextrins [19] and colchicine to tubulin [20]. Likewise, the binding of NBD-colcemid to the second site is also slow, and, since this site is also competed effectively by colchicine, it is likely that binding of NBD-colcemid to both sites are sterically limited.

Irrespective of the exact mechanism of the association of NBD-colcemid with tubulin, results presented so far had clearly established that there are two NBD-colcemid-binding sites to native tubulin. One site is fast equilibrating, where NBD-colcemid binds instantaneously, and the other a slow-

equilibrating site, binding to which needs more than 2 h for equilibration at 37 °C. These two types of binding are clearly evident from the time course of fluorescence enhancement, the time course of tryptophan quenching and the Scatchard analysis. Both these sites are specific for the colchicine nucleus, as shown by the lack of binding of NBD-colcemid to many other proteins and inability of a simple alkyl-NBD analogue to bind to tubulin. It is quite likely that the slow site is identical to that of the high-affinity colchicine-binding site. The binding kinetics, competition experiments and stoichiometries all point to this conclusion.

The relationship of the fast NBD-colcemid site with colchicine-binding sites is not clear at this moment. A second colchicine-binding site is not well characterized, although many studies have indicated its existence [21−23]. The second binding site of colchicine and its analogues on tubulin is detected at high drug concentrations, indicating a lower affinity ($K_d > 50$ μM) [24] compared to that of the primary binding site ($K_d < 10$ μM) [5]. No such difference in the affinity (2 μM vs 5 μM) between the fast and the slow site is observed in the present study. The high-affinity colchicine-binding site on tubulin is competitively inhibited by structural analogues, whereas it is not known whether its lower-affinity site is equally competable by its analogues.

In the case of colcemid/tubulin interaction, the lower-affinity colcemid-binding site on tubulin does not recognise colchicine, whereas the higher-affinity site on tubulin is competed by its analogues [25]. NBD-colcemid behaves similar to colcemid in this respect. The fast NBD-colcemid-binding site remains unaffected by prior incubation of tubulin with colchicine or colcemid (data not shown), whereas the slow site is competed by both colchicine and colcemid. The unique features of this fast NBD-colcemid binding are its high association rate and the high association constant. This site is detected when the NBD group and colcemid are covalently linked as NBD-colcemid, and neither colcemid nor simple NBD analogues can separately bind to such a site. The NBD group has considerable hydrophobic character and may provide excess binding energy through hydrophobic interactions, thus strengthening the binding. The covalently linked NBD group may also have entropic advantages (like a chelate effect) towards binding over the colchicine nucleus alone. The significance of this site and its relation with known functions of tubulin/microtubule system would be interesting to study.

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