Quantitative Characterization of the Binding of Fluorescently Labeled Colchicine to Tubulin in Vitro Using Fluorescence Correlation Spectroscopy†

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Received September 10, 1998; Revised Manuscript Received February 11, 1999

ABSTRACT: Fluorescence correlation spectroscopy (FCS) is a new technique that allows the determination of the diffusion constant of a fluorescent molecule in solution. Also, the binding of the fluorescent molecule to a target can be analyzed, if the difference in the diffusion coefficients of the free and bound ligand is sufficiently large. With FCS, the interaction between fluorescein−colchicine (FC) and tubulin has been studied in vitro. A fast and reversible binding is observed with an association constant at room temperature of $(3.9 \pm 0.1) \times 10^4\ M^{-1}$. No competition with colchicine is seen, indicating that FCS reveals the existence of a new binding site on tubulin. FCS is not able to show the binding of FC to the original colchicine binding site, even though it exists, because the fluorescence of FC is strongly quenched upon binding to this site. This quenching is evident in spectrofluorometry experiments, revealing a slow binding of FC to tubulin that is subject to competition with colchicine. FCS allows the determination of the diffusion coefficients of both free and bound fluorescent colchicine which were found to be $(2.6 \pm 0.2) \times 10^{-10}$ and $(2.0 \pm 0.2) \times 10^{-11}\ m^2\ s^{-1}$, respectively. It can be concluded that fluorescent labeling, especially of small molecules, can interfere considerably with the binding behavior that is being studied. Although general qualitative effects in vivo are similar for colchicine and its fluorescein derivative, this quantitative study of the binding to tubulin presents a nuanced view, and the existence of a second binding site for FC can even explain some conflicting indications in the literature.

The effects of the cytostatic colchicine on the polymerization behavior of tubulin are well-documented phenomena, both in vivo and in vitro (*1*, *2*). This organic molecule inhibits the polymerization of tubulin into microtubules, part of the cytoskeleton of living cells, through binding to the tubulin dimer. The aspects of the binding on which this laboratory has been focusing during the last several years are the following: a kinetic characterization of the binding process, mapping of the colchicine binding site on tubulin via competition studies between colchicine derivatives, and identification of the structural parts of colchicine that are important in the different steps of the interaction (*3*−*6*). These studies have been completed using stopped flow and spectrofluorometric techniques.

Recently, some indications have appeared in the literature (*7*) showing that the interaction kinetics change when the tubulin concentration is lowered below the micromolar level: association and dissociation rate constants rise and the affinity is decreased. It is more than probable that this behavior is caused by the partial dissociation of tubulin dimers into monomers.

The original aim of this work was therefore to study the colchicine−tubulin interaction at nanomolar protein concentrations to further explore this new aspect of the binding.

Since more conventional fluorescence techniques such as stopped flow and spectrofluorometry are not sensitive enough to be used at the nanomolar level, fluorescence correlation spectroscopy (FCS)[1] was a logical choice for performing the binding study. With this technique, the diffusion of fluorescent molecules in the excitation volume of a confocal microscope is quantitatively analyzed. When the fluorescent molecule binds to a target, the different diffusion behavior of the free and the bound fluorescent molecules can be used to quantify the two fractions and therefore the binding constant for binding of the ligand to the target.

A drawback of this technique in the present situation is, however, that when molecules are investigated that do not absorb at the laser excitation wavelength, covalent linking to a fluorescent marker prior to an FCS analysis is necessary. In our case, fluorescein−colchicine (FC) was used as a fluorescent substitute for colchicine itself (Figure 1). The influence of FC on living cells in comparison with that of colchicine has been investigated by several authors (*8*−*11*). Qualitatively, the two molecules exhibit comparable behavior, and competition for the same binding site on tubulin is observed. Under some experimental conditions, however, binding of FC to tubulin is still mentioned, even in the presence of high (preincubation) concentrations of colchicine

† The Fund for Scientific Research (FWO)-Flanders is gratefully acknowledged for financially supporting this research (Grant 9.0320.97). E.V.C. is a research assistant of the FWO. The instrument for sedimentation analysis was supported by the FWO (Grant 6.2182.94N).

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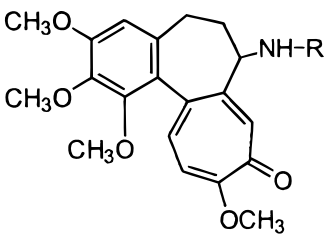
[1] Abbreviations: FCS, fluorescence correlation spectroscopy; FC, fluorescein−colchicine; MAPs, microtubule-associated proteins; MES, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine triphosphate; GDP, guanosine diphosphate; MTC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one.

10.1021/bi9821925 CCC: $18.00
Published on Web 03/31/1999



Colchicine

R = −CO-CH$_3$

Fluorescein-colchicine

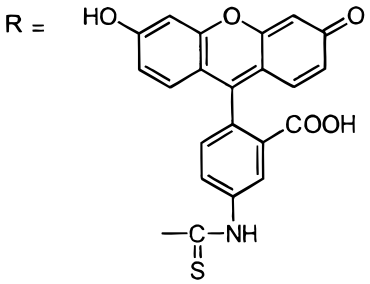


FIGURE 1: Structure of colchicine and its fluorescein-labeled derivative. Colchicine consists of a trimethoxybenzene and a tropolone ring, connected by a double bridge.

(*8*, *11*). In contrast to colchicine, FC also seems to bind to microtubules as well as to tubulin (*11*).

This quantitative in vitro study shows that results obtained with FC cannot be interpreted in terms of the interaction between tubulin and the colchicine moiety alone, because the presence of the fluorescein part strongly influences the binding. Moreover, the results presented here demonstrate the existence of a second binding site for FC on tubulin, different from the colchicine site.

The technique of FCS has certainly great potential for the study of binding phenomena because it can be used on small volumes, possibly even directly in living cells, and it allows for detection of binding phenomena even if binding is not accompanied by a fluorescence change. Moreover, separation of free and bound ligands is not necessary. Like every technique, it also has its limitations, and the major one is certainly the necessity for having a fluorescence signal with excitation in the 480−540 nm region. When the molecule being studied is not fluorescent (or not in the right excitation region), this creates the need for conjugation to fluorescent groups as in our case. This conjugation may alter the binding process. Even then, the technique can be successfully applied through the study of competition between a fluorescently labeled competitor and the molecules of interest. Our situation suffers from an additional problem, i.e., the creation of a new binding site for the conjugated molecule. Nevertheless, this second binding phenomenon can be used to probe the diffusion behavior of the target protein.

MATERIALS AND METHODS

Protein. Microtubule protein [tubulin and microtubule-associated proteins (MAPs)] was purified from pig brains according to the method of Shelanski et al. (*12*), modified by Engelborghs et al. (*13*). This protein solution was further purified as described by Weingarten et al. (*14*) with the following additional steps. After the tubulin fraction had been eluted from a phosphocellulose column equilibrated in MES buffer [50 mM MES, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM NaN_3, with the ionic strength adjusted to 0.1 M with NaCl (pH 6.4)], the protein was concentrated in Centricon tubes (Amicon, Beverly, MA) and then passed through a Sephadex 25 column (PD-10 columns, Pharmacia, Uppsala, Sweden). This was necessary to remove GTP and ATP remaining from the purification protocol and to change the solvent to phosphate buffer [50 mM KH_2PO_4 and 0.5 mM $MgCl_2$, with the ionic strength adjusted to 0.1 M with KCl (pH 7)]. The FCS measurements were taken at this pH to reduce the triplet decay of the fluorescein moiety, which complicates the data analysis.

Finally, the purified tubulin was concentrated in Centricon tubes to a final concentration of 70 μM and stored in liquid nitrogen. Prior to use, the protein solution was adjusted to 0.5 mM GDP to stabilize tubulin.

Chemicals. FC was purchased from Molecular Probes (Eugene, OR). Colchicine was from Janssen (Geel, Belgium). MES was from Acros. EGTA was from Sigma (St. Louis, MO). $MgCl_2$ and KH_2PO_4 were from Merck (Darmstadt, Germany). NaN_3 was from Riedel-De Haen (Seelze-Hannover, Germany). NaCl and KCl were from BDH Laboratory Supplies (Poole, England). Nucleotides were from Boehringer Mannheim. MTC was a kind gift from T. Fitzgerald (Tallahassee, FL). All chemicals were analytical grade.

Fluorescence Correlation Spectroscopy. Although the theoretical background of FCS was developed more than 20 years ago (*15*, *16*), it was experimentally only realized in the early 1990s (*17*, *18*).

A confocal microscope is used to focus laser excitation light into a solution of fluorescent molecules, creating an open excitation volume of approximately 1 fL. The diffusion of these molecules through the excitation volume generates fluorescence fluctuations. The average duration of these fluctuations reflects the average residence time of the fluorescent molecules in the excitation volume (diffusion time). Small and fast diffusing molecules will exhibit a very small diffusion time, in contrast to big and slow diffusing molecules. The intensity of the fluctuations is determined by the quantum yield of the fluorescent molecules and, in the case of complexes, by the number of fluorescent molecules bound together to the target.

The quantitative analysis of these fluctuations is carried out by calculating the so-called autocorrelation function. In this function, the fluorescence intensity at time t [$F(t)$] is compared to (multiplied with) the fluorescence intensity at time $t + \tau$, where τ is a variable interval. This product is averaged over all possible pairs of intensities that can be found in a long measurement session. When τ is very short, the intensities at t and $t + \tau$ will be correlated, while when τ is very long, no correlation will be found. The autocorrelation function therefore will vary between $\langle F(t)^2 \rangle$ at $\tau = 0$ and $\langle F(t) \rangle^2$ at $\tau = \infty$.

Mathematically, this leads to the following definition of the normalized correlation function $G(\tau)$ (*17*):

$$G(\tau) = \frac{\langle F(t) \rangle^2 + \langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \qquad (1)$$

where $\langle F(t)\rangle$ is the average fluorescence and δF the deviation from the average fluorescence at time t or a time interval τ later.

The autocorrelation function can be related to the diffusion time τ_d, concentration (N/excitation volume), and the diffusion coefficient D of the molecules of interest, by eqs 2 and 3 (*19*). The derivation of these relations can be found in detail in ref *15*.

$$G(\tau) = 1 + \frac{1}{N} f(\tau/\tau_d) \tag{2}$$

with

$$f(\tau/\tau_d) = \left(\frac{1}{1+\tau/\tau_d}\right)\left[\frac{1}{1+(\omega_1/\omega_2)^2\tau/\tau_d}\right]^{1/2} \tag{3}$$

where ω_1 and ω_2 are the half-axes of the excitation volume perpendicular to and along the laser beam, respectively, and N is the average number of fluorescent particles in the excitation volume.

The relation between τ_d and D is given in eq 4.

$$\tau_d = \frac{{\omega_1}^2}{4D} \tag{4}$$

When the fluorescent molecule also binds to a target with a sufficiently different diffusion coefficient (a factor of >2), the correlation function can be described as a sum of two fractions that diffuse independently (eq 5):

$$G(\tau) = 1 + \frac{1}{N}[(1-y) \times f(\tau/\tau_{free}) + y \times f(\tau/\tau_{bound})] \tag{5}$$

From this equation, the diffusion coefficient and concentration of both free and bound ligands can be determined with $1 - y$ and y being the free and bound fraction of the fluorescent ligand, respectively, and τ_{free} and τ_{bound} being the diffusion times of free and bound ligands, respectively (*19*).

FCS measurements were taken using a commercial Confo-Cor instrument (Zeiss, Jena, Germany). In this configuration, laser light from an Ar^+ laser (488 nm, 10 mW laser output attenuated by a neutral density filter with an optical density of 1.5) is focused into the sample solution via an objective lens (C-Apochromat 40×, 1.2 W). The emitted fluorescence passes through the same objective and is focused by means of a confocal setup on an avalanche photodiode. The signal is processed by a hardware correlator, and the resulting autocorrelation function is analyzed with the FCS ACCESS software package (EVOTEC, Hamburg, Germany). To determine the dimensions of the excitation volume (ω_1 and ω_2), a measurement was taken on a molecule with a known diffusion coefficient (rhodamine 6G with a D of 2.8×10^{-10} m^2 s^{-1}). From D and the measured diffusion time, ω_1 and ω_2 could be determined. The value of ω_1 and the ratio ω_2/ω_1 were treated as known parameters in all further experiments.

Measurements were taken in typical sample volumes of 120 μL. Concentrations of FC were between 6.5 and 22 nM, and concentrations of tubulin were in the micromolar range. Due to the large excess of tubulin, the exact absolute concentration of FC drops out of the binding equation (see the Results). In the competition experiments, tubulin (67.5 μM) was incubated prior to the measurement with 350 μM colchicine at room temperature for 30 min and subsequently separated from excess free colchicine on a Sephadex 25 column, yielding 0.8 mol of colchicine per mole of tubulin. All FCS measurements were taken at room temperature and in phosphate buffer [0.1 M ionic strength (pH 7), with 0.5 mM Mg^{2+} and 0.5 mM GDP]. The measuring time was 40 s, and all samples were measured 10 times.

Analytical Boundary Sedimentation. Sedimentation coefficients were determined using analytical boundary sedimentation in a Beckman Optima XL-A analytical ultracentrifuge. At regular time intervals, absorption profiles were recorded at 280 nm, first at 20 000 rpm to sediment possible high-molecular mass particles and finally at 40 000 rpm to sediment tubulin dimers. All measurements were carried out at 23 °C, and the data were processed as described elsewhere (*20*).

Spectrofluorometry. Time-based fluorescence measurements were taken on a SPEX spectrofluorometer (Fluorolog 1691). Samples were excited at 488 nm, and the emitted fluorescence was recorded at 520 nm (the excitation and emission spectral bandwidths were 7.2 and 3.6 nm, respectively). The measurements were taken in the same buffer as the FCS measurements at 23 °C.

RESULTS

Fluorescence Correlation Spectroscopy

FCS with Pure FC. Before a study of the binding of FC on tubulin was performed, FC was characterized separately in a FCS experiment. A measurement on a FC solution with a nanomolar concentration typically yielded a diffusion time between 70 and 80 μs. Since this value is dependent on the excitation volume which varies slightly from day to day, the diffusion time of pure FC was determined separately before each set of measurements with tubulin was taken. The best results were obtained when this τ_{free} was then introduced as a known parameter into the fittings of the mixtures of FC and the protein.

Characteristics of FC Binding to Tubulin. When FC (nanomolar concentration) was mixed with tubulin (micromolar concentration), a typical noisy fluorescence signal was observed. The autocorrelation function of this signal can be analyzed in terms of the presence of two diffusing species (Figure 2). The fractions of free and bound FC are graphically seen as the amplitude fractions of the fast and slow decay, respectively, and the diffusion times of both species are represented by the midpoints of both decays.

To show the reversibility of the FC−tubulin interaction, the binding was studied in two different ways (Figure 3). In a first experiment, FC (concentration between 6.5 and 22 nM) was added to different concentrations (micromolar range) of tubulin and the fractions of free and bound FC were determined. Second, FC (80.5 μM) was, prior to the measurement, incubated with tubulin (14 μM) for 30 min at room temperature, and the complex was separated from the excess FC on a Sephadex 25 column (the sample eluted clearly in two separate bands from the column). In the FCS experiment, this compound was diluted to a nanomolar concentration range in different (micromolar) concentrations of tubulin and assessed as described above.

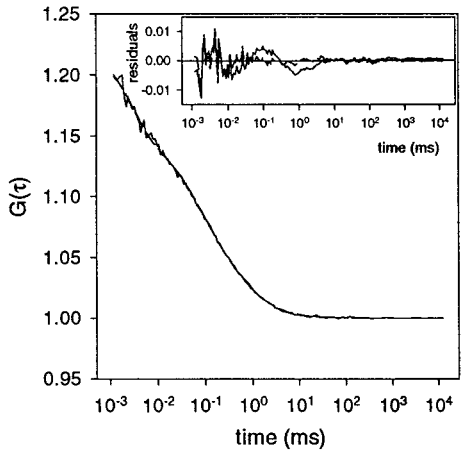


FIGURE 2: Autocorrelation function (solid line) of a mixture of free FC and FC bound to tubulin (70 μM). The dashed line represents a two-component fit yielding 73% free FC and 27% bound FC. The diffusion time of FC was measured in a previous experiment and fixed here at the determined value of 73.7 μs. The diffusion time of bound FC (and so the diffusion time of tubulin) was 721.3 μs. The inset shows the residuals for both a one-component fit (solid line) and a two-component fit (dashed line).

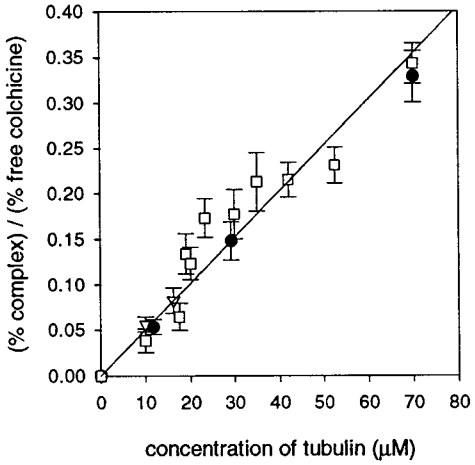


FIGURE 3: Interaction between FC and tubulin as determined by FCS. FC was (●) added as a free molecule to tubulin, (□) complexed with tubulin and diluted to the nanomolar concentration range in the different tubulin solutions, and (▽) added as a free molecule to tubulin that was complexed with unlabeled colchicine prior to the FCS measurement.

No difference can be seen between the results from both measurements, indicating a fast reversibility of the binding of FC to tubulin. Moreover, the measured fractions did not change during the time needed for the experiments (several minutes) or in control measurements after up to 1 h, meaning that the system equilibrates very rapidly.

The data were fitted to an equation derived from the expression of the apparent equilibrium association constant (K_{app}) for the binding of FC to tubulin (eq 6).

$$K_{app} = \frac{[\text{FC}-\text{tubulin}]}{[\text{FC}_{free}][\text{tubulin}_{free}]} \tag{6}$$

Since the concentration of FC is very low in comparison to the concentration of tubulin, the free concentration of tubulin at equilibrium can be considered equal to the total concentration of protein. Furthermore, the ratio of the concentrations of complex versus free FC is equal to the ratio of the fractions of both particles. The exact concentration of FC is in this way no longer present in the equation (eq 7):

$$K_{app}[\text{tubulin}_{total}] = \frac{\text{fraction of complexed FC}}{\text{fraction of free FC}} \tag{7}$$

When the fraction of complexed FC is estimated from the amplitude fractions of the autocorrelation function, an underestimation of the value is likely because a reduction of the fluorescence signal per molecule was immediately observed upon the addition of FC to tubulin. This would lead to a value of the apparent association constant that is too low. Normally, this problem can be corrected for when the signal per molecule for free and bound ligand is known (see eqs 8 and 9; personal communication with B. Hecks at EVOTEC).

$$K_{app}[\text{tubulin}_{total}] = \left(\frac{y}{1-y}\right)\frac{1}{\alpha^2} \tag{8}$$

with

$$\alpha = \frac{(\text{fluorescence per molecule})_{bound-FC}}{(\text{fluorescence per molecule})_{free-FC}} \tag{9}$$

where y and $1 - y$ are the apparent amplitude fractions of bound and free FC as measured by FCS, respectively, and α is the correction factor.

In this case, however, the concentration of tubulin could not be increased enough to saturate FC completely, so the signal per molecule of bound FC could not be determined. Therefore, an extrapolation of the fluorescence per particle to high tubulin concentrations was carried out, by fitting the fluorescence per particle to a saturation function (eq 10).

$$\text{fpm} = \frac{\text{fpm}_0 + \text{fpm}_\infty K_{app}[\text{tubulin}]}{1 + K_{app}[\text{tubulin}]} \tag{10}$$

where fpm is the average fluorescence signal per molecule and fpm_0 and fpm_∞ are the fluorescence per molecule of free and bound FC, respectively. The parameter fpm_∞ allowed the calculation of an α value of 0.36. When this correction was considered, K_{app} was determined to be $(3.9 \pm 0.1) \times 10^4\ \text{M}^{-1}$. The association equilibrium constant in the fitting of the counts per molecule (eq 10), determined at $3.2 \times 10^4\ \text{M}^{-1}$, is in good agreement with the corrected equilibrium constant obtained from the fraction measurements (eq 8). Since the binding of FC to tubulin is therefore one with low affinity, the original aim of this study, to analyze the colchicine−tubulin interaction at nanomolar concentrations, proved to be impossible. The behavior of fluorescein−colchicine in its interaction with tubulin addresses a more general point of interest, namely, the effect of fluorescent probes on the system being studied.

Competition between FC and Colchicine. Nonlabeled colchicine is known to interact with tubulin in a slow and practically irreversible way, exhibiting an association equilibrium constant of $1.2 \times 10^7\ \text{M}^{-1}$ (5), which is quite different from the results obtained here with FC. Therefore, a competition experiment between both derivatives was carried out. After tubulin was complexed with colchicine as described in Materials and Methods, a FCS experiment was performed (with uncomplexed FC), again with FC at a

Table 1: Diffusion Coefficients of Tubulin and Fluorescein−Colchicine As Determined with Fluorescence Correlation Spectroscopy in Comparison with Literature Values

species	diffusion coefficient ($m^2\ s^{-1}$)
FC	$(2.6 \pm 0.2) \times 10^{-10}$
tubulin	$(2.0 \pm 0.2) \times 10^{-11}$
tubulin dimer[a]	5×10^{-11}
tubulin ring[a]	10^{-12}

[a] Diffusion coefficient of tubulin dimers and rings determined by quasi elastic light scattering (*24*).

nanomolar concentration (14.7−17.3 nM) and varying micromolar concentrations of the tubulin−colchicine complex. In Figure 3, the fractions of free and bound FC are compared to the measurements with noncomplexed tubulin. As the results match perfectly, it may be concluded, under these experimental conditions, that binding of colchicine in its binding site does not eliminate binding of FC.

It was questioned whether the binding of FC is due to nonspecific binding to tubulin. Fluorescein itself, however, does not show any affinity for tubulin in FCS measurements, nor does FC show any interaction with other proteins, e.g., lysozyme (results not shown).

Determination of Diffusion Coefficients. From the diffusion times calculated in the FCS fittings, the diffusion coefficients of free FC and FC coupled to tubulin could be derived. Since FC is small compared with the protein, the diffusion coefficient of bound FC represents the diffusion coefficient of tubulin itself. In Table 1, the results are given and in the case of tubulin compared with literature values. The value measured here for tubulin resembles mostly the reported value for the dimer, but since there is still a discrepancy between both results, the size of the FC−tubulin complex was measured independently using sedimentation analysis.

Analytical Ultracentrifugation

Determination of Sedimentation Coefficients. A protein sample containing 10 μM tubulin was analyzed in a boundary sedimentation experiment. The buffer composition was the same as in the FCS experiments with the exception of a lowered GDP concentration (40 μM instead of 0.5 mM) which minimized the background absorption at 280 nm. The measurement showed that the protein composition of the sample consisted mainly (90−95%) of particles with a sedimentation coefficient of 6.7 S, which is close to the reported value for tubulin dimers (*21*). A small (5−10%) fraction of protein aggregates with a sedimentation coefficient of 30 S was also found.

Spectrofluorometry

Binding of FC to the Colchicine Site. In the literature, a slow binding of FC to the colchicine site on tubulin with an association equilibrium constant on the order of $10^5\ M^{-1}$ has been independently demonstrated by two different laboratories (*9*, *11*). These findings can be reconciled with our results when the possibility that the FC fluorescence is strongly quenched upon binding to the original site is considered.

Fluorescein is known to occur both in a strongly fluorescent alkaline form and a practically nonfluorescent acid form (pK = 6.5; see also the Discussion). Assuming that the monoanionic acid form binds to the colchicine site with high affinity and slow kinetics, whereas the alkaline dianionic form interacts immediately but with lower affinity with a different binding site, one would observe a slow decrease in the fluorescein fluorescence during binding on tubulin. This is explained by the fact that the difference in the affinities of the two binding sites would shift the ionization equilibrium of FC to the low-fluorescent acid form.

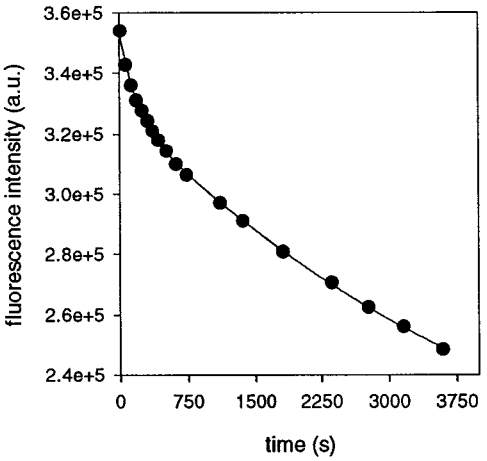


FIGURE 4: Interaction between FC and tubulin as measured in spectrofluorometry experiments. FC (480 nM) was added to 20.0 μM tubulin. The fast decay shows an observed rate constant of $(5.6 \pm 0.6) \times 10^{-3}\ s^{-1}$ with an amplitude of $(2.6 \pm 0.1) \times 10^4$ arbitrary units; the slow phase is characterized by an observed rate constant of $(2.0 \pm 0.3) \times 10^{-4}\ s^{-1}$ with an amplitude of $(1.6 \pm 0.2) \times 10^5$ arbitrary units.

This was effectively demonstrated in a spectrofluorometry experiment. A biexponential decay was observed when FC (concentration in the range of 10^{-7} M) was added to different micromolar concentrations of tubulin (Figure 4).

From the relation between the observed association rate constant and the protein concentration, the association and dissociation rate constants for both the fast and slow phases could be determined (eq 11 and Figure 5).

$$k_{obs} = k_{diss} + k_{ass}[\text{tubulin}] \qquad (11)$$

In both cases, the association equilibrium constant, calculated from the rate constants, was in the range of $10^4\ M^{-1}$ [$(1.2 \pm 0.2) \times 10^4\ M^{-1}$ for the fast decay and $(4.5 \pm 1.0) \times 10^4\ M^{-1}$ for the slow decay].

This biexponential behavior could be due to a fast binding to a low-affinity site also demonstrated in the ConfoCor, parallel to a slow binding to the colchicine site, or to the fact that FC shows, as does colchicine, biexponential kinetics for the slow binding. To discriminate between these two possibilities, the experiment was repeated with tubulin (24 μM) previously incubated for 5 min with 112 μM MTC, a fast binding analogue of colchicine. The result of this measurement is shown in Figure 6. Since MTC binds to the colchicine site on tubulin, only the FC binding to its different binding site can be seen. As the fast decay is still visible with the same rate constant and a slightly higher amplitude as in a control experiment using tubulin that has not been previously incubated, it can be concluded that the biexponential decay is due to binding of FC to two different binding sites with different kinetics.

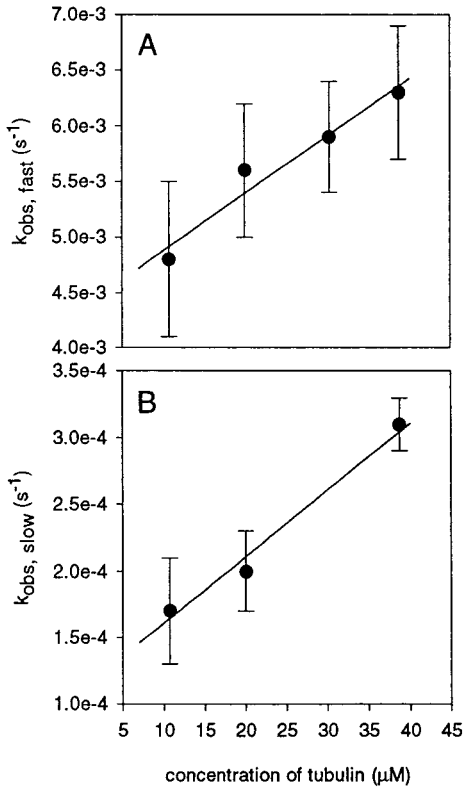


FIGURE 5: Determination of association and dissociation rate constants for the fast (A) and slow (B) decay. (A) The association rate constant is $(5.1 \pm 0.8) \times 10^1$ M^{-1} s^{-1} and the dissociation rate constant $(4.4 \pm 0.2) \times 10^{-3}$ s^{-1}, yielding an equilibrium association constant of $(1.2 \pm 0.2) \times 10^4$ M^{-1}. (B) The association rate constant is (5.0 ± 0.7) M^{-1} s^{-1} and the dissociation rate constant $(1.1 \pm 0.2) \times 10^{-4}$ s^{-1}, yielding an equilibrium association constant of $(4.5 \pm 1.0) \times 10^4$ M^{-1}.

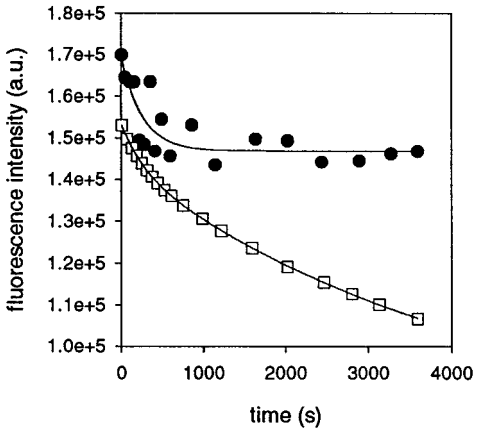


FIGURE 6: Competition of FC binding with MTC in the spectrofluorometer. (□) Interaction of FC (480 nM) with 24 μM tubulin. The association rate constant and amplitude of the fast phase are $(3.4 \pm 0.3) \times 10^{-3}$ s^{-1} and $(1.02 \pm 0.06) \times 10^4$ arbitrary units, respectively, and of the slow phase are $(2.1 \pm 0.2) \times 10^{-4}$ s^{-1} and $(6.8 \pm 0.4) \times 10^4$ arbitrary units, respectively. (●) Interaction of 480 nM FC with 24 μM tubulin that was incubated for 5 min with 112 μM MTC prior to the experiment. The association rate constant and amplitude of the only exponential phase left are $(3.9 \pm 1.4) \times 10^{-3}$ s^{-1} and $(2.2 \pm 0.4) \times 10^4$ arbitrary units, respectively, which coincides with the fast exponential decay of the interaction of FC with noncomplexed tubulin.

In the FCS experiments, the slow fluorescence decrease in time was visible as an increase in the fraction of FC molecules in the triplet state, accompanied by a time-dependent decrease in the fluorescence signal per molecule. The effects were, however, too small and too obscured by random noise to be quantified by FCS.

DISCUSSION

The binding of FC to tubulin in vitro has been demonstrated to be quite different from the interaction of colchicine with the protein, since in addition to the original binding site, a second site has been discovered.

Binding of FC to the colchicine site exhibits slow kinetics, as does colchicine itself. In a spectrofluorometry experiment where MTC was added during binding of FC (not shown), the fluorescence decay immediately stopped but no reversal into an increase in the fluorescence was visible, indicating a very slow dissociation, which is again comparable to that of colchicine. An association equilibrium constant of $(4.5 \pm 1.0) \times 10^4$ M^{-1} was determined. When the limited accuracy with which especially the dissociation rate constant can be determined from eq 11 is taken into account, our results are in good agreement with the values in the literature (*9*, *11*; 2×10^5 and 7.1×10^4 M^{-1}, respectively). In these experiments, the influence on the binding of radioactively labeled colchicine by preincubation with FC was followed.

In our FCS measurements, a fast and lower-affinity binding of FC to another site on tubulin was shown for the first time and confirmed by spectrofluorometry experiments. Binding to the original site was invisible with FCS because of the strong quenching of fluorescein upon binding. The transition between the anionic and the far more fluorescent dianionic form shows a pK_a of 6.5 (*22*). Therefore, in our FCS experiments, performed at pH 7, the fluorescent alkaline form dominated and binding of this species to tubulin was seen. [Indeed, the fluorescence signal in preliminary experiments at pH 6.4 (not shown) was much lower than at pH 7.] The competition experiments in the literature, on the other hand, performed at pH 6.5, made use of radiolabel detection rather than fluorescence. Therefore, in these measurements, a high-affinity binding of the monoanionic form in competition with colchicine was observed, whereas the FCS experiments revealed a low-affinity binding of the dianionic form to a site different from the colchicine site.

This interpretation of FC binding to tubulin could also explain the fact that in some cases in the literature, depending on the concentrations and probably also the cell lines used, binding to a site different from the colchicine site is seen (*8*, *11*), and in other cases not (*9*, *10*, *23*).

Since with FCS no binding of fluorescein itself to tubulin was measured, the drastic change in the binding properties must originate either from a structural change in the colchicine or the fluorescein moiety due to covalent binding of one to the other or from the combined presence of fluorescein and colchicine in one molecule (bidentate effect). The discrimination between these three possibilities is beyond the scope of this paper.

A quantitative in vitro binding analysis, as presented here, shows that results obtained with a fluorescent derivative of colchicine do not necessarily represent those of colchicine itself. Moreover, we showed the existence of a second lower-affinity binding site of FC in addition to the colchicine site. In view of the general use of fluorescent derivatives of organic ligands in pharmaceutical research, this is a rather disturbing conclusion.

FC binding can be used to determine the diffusion coefficient of the tubulin complex at a value of $(2.0 \pm 0.2) \times 10^{-11}$ m^2 s^{-1}. Although this value is lower than the value obtained in previously reported measurements with quasi elastic light scattering (*24*, *25*), analytical boundary sedimentation shows that the solution consists of more than 90−95% of 6.7 S dimers. The diffusion coefficient measured in FCS is independent of the concentration of tubulin between 10 and 70 μM. The reason for the deviation between the diffusion coefficients measured by the two methods is currently under investigation.

ACKNOWLEDGMENT

We thank Dr. T. Aerts and Prof. Dr. J. Clauwaert (Biophysics Research Group, Department of Biochemistry, University of Antwerp, Antwerp, Belgium) for the analytical boundary sedimentation experiments. We also thank Dr. T. Fitzgerald for his gift of MTC.

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BI9821925