Energy-Transfer Studies of the Distance between the High-Affinity Metal Binding Site and the Colchicine and 8-Anilino-1-naphthalenesulfonic Acid Binding Sites on Calf Brain Tubulin†

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ABSTRACT: The high-affinity metal divalent cation Mg^{2+}, associated with the exchangeable guanosine 5′-triphosphate (GTP) binding site (E site) on purified tubulin, has been replaced by the transition metal ion Co^{2+} on tubulin as well as on the tubulin–colchicine, tubulin–allocolchicine, and tubulin–8-anilino-1-naphthalenesulfonic acid (tubulin–ANS) complexes. While pure native tubulin readily incorporated 0.8 atom of Co^{2+} per tubulin α–β dimer, incorporation was reduced to 0.4 atom of Co^{2+} per mole of tubulin when it was complexed with colchicine, indicating that the conformational change induced in tubulin by the binding of colchicine leads to a reduced accessibility of the divalent cation binding site linked to the E site without necessarily changing the intrinsic binding constant. The fluorescence emission spectra of tubulin-bound colchicine, allocolchicine, and ANS displayed a strong overlap with the Co^{2+} absorption spectrum, identifying these as adequate donor–acceptor pairs. Fluorescence energy-transfer measurements were carried out between tubulin-bound colchicine (or allocolchicine) and ANS as donors and tubulin-complexed Co^{2+} as acceptor. It was found that the distance between the ANS and the high-affinity divalent cation binding sites is greater than 28 Å, while that between the colchicine and the divalent cation binding sites is greater than 24 Å. Since the binding of colchicine to tubulin induces a conformational change in the tubulin molecule in the domain of the E-GTP binding site [Andreu, J. M., & Timasheff, S. N. (1982) *Biochemistry 21*, 6465–6476] and the tightly bound divalent cation is closely associated with the exchangeable GTP [Monasterio, O. (1987) *Biochemistry 26*, 6099–6106], the conformational change induced in tubulin by complexing with colchicine must be long-range in nature.

The microtubule protein tubulin is unusual in its propensity to polymerize into a variety of ordered structures, the extent and direction of this polymerization and the geometry of the polymers being strongly linked to the binding of different ligands (Timasheff & Grisham, 1980). A large number of compounds are known to interact with tubulin, the consequence of these interactions being a strong function of the nature of the ligand and the conditions employed.

Typical of such compounds are the antimitotic drugs vinblastine, taxol, and colchicine. Colchicine, at low concentrations, causes the substoichiometric poisoning of microtubule growth (Olmsted & Borisy, 1973; Margolis & Wilson, 1977; Sternlicht & Ringel, 1979; Margolis et al., 1980; Lambeir & Engelborghs, 1980) by presumably binding at the ends of microtubules (Margolis & Wilson, 1977). At higher concentrations, however, it can form stable complexes with tubulin (Andreu & Timasheff, 1982a) which polymerize into structures geometrically quite distinct from microtubules, although thermodynamically very similar, indicating the essential identity of intraprotein bonds formed (Andreu & Timasheff, 1982b; Andreu et al., 1983; Saltarelli & Pantaloni, 1982). Vinblastine behaves in a similar manner. Taxol, on the other hand, promotes the growth of microtubules with properties different from those formed in the absence of the drug (Schiff & Horwitz, 1981; Kumar, 1981). Podophyllotoxin (Cortese et al., 1977), rotenone (Marshall & Himes, 1978), and benzimidazole derivatives (Friedman & Platzer, 1978) have all been shown to compete with colchicine for binding to the tubulin dimer. Other compounds shown to bind to tubulin are the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS)[1] (Bhattacharyya & Wolff, 1974; Lee et al., 1975) and daunomycin (Na & Timasheff, 1977).

While none of the above compounds is involved in the regulation of tubulin assembly in vivo, tubulin does possess additional binding sites for compounds which strongly influence its assembly into microtubules, namely, GTP (Weisenberg et al., 1968), Ca^{2+} (Solomon, 1977; Grisham et al., 1980; Hayashi & Matsumura, 1975), and Mg^{2+} (Frigon & Timasheff, 1975b). The nucleotide GTP has two binding sites on the tubulin dimer, one characterized by very tight binding and nonexchangeability, while the GTP at the other site exchanges readily with free GTP (Weisenberg et al., 1968). The presence of Mg^{2+} favors microtubule polymerization (Lee & Timasheff, 1977) whereas Ca^{2+} causes their rapid disassembly (Weisenberg, 1972).

The interactions of tubulin with this variety of compounds at presumably different binding sites raise the question of the spatial distribution of these sites on the tubulin dimer, not only with respect to each other but also with respect to tubulin–tubulin contacts in the various tubulin polymers. As an approach to this problem, we have initiated measurements of the

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; GTP, guanosine 5′-triphosphate; CD, circular dichroism; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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distances between pairs of sites using the techniques of NMR (Monasterio, 1987) and of fluorscence energy transfer. The purpose of this paper is to report results of such studies by the fluorescence technique.

Fluorescence energy transfer has been used successfully (Stryer, 1978) to measure the distance between sites on protein molecules by using the theory developed by Förster (1948) and validated by Stryer and Haugland (1967). In the present study, an attempt has been made to measure the distance between the high-affinity metal binding site on tubulin and the colchicine and ANS binding sites, respectively. Recently, we have shown in an NMR study (Monasterio & Timasheff, 1987; Monasterio, 1987) that the high-affinity metal binding site on tubulin is associated with GTP at the exchangeable GTP site, confirming the reported linkage between GTP and divalent metal ion binding (Jemiolo & Grisham, 1982; Croom et al., 1985). In the present study, we have replaced the high-affinity Mg^{2+} ion by the transition metal ion cobalt which has appropriate absorption properties in the visible region for spectral overlap between cobalt absorption and colchicine and ANS fluorescence. The substitution of the colored metal ion Co^{2+} for metal ions with unsuitable absorption characteristics had been used successfully (Vallee et al., 1971) to probe metal ion binding sites on proteins. For example, cobalt has been used as an acceptor in energy-transfer studies to measure the distance between the metal ion binding site and dansylated substrates in carboxypeptidase A (Latt et al., 1972) and between the terbium and cobalt binding sites in thermolysin (Horrocks et al., 1975).

Materials and Methods

Chemicals. GTP (type II), colchicine, and glycerol were Sigma products. Ultrapure guanidine hydrochloride was obtained from Heico. Sephadex G-25 and Chelex 100 chelating resin were obtained from Pharmacia and Bio-Rad, respectively. 8-Anilino-1-naphthalenesulfonic acid (ANS) was purchased from Molecular Probes and used without further purification. Cobalt atomic absorption standard was from Fisher Scientific Corp. Allocolchicine was prepared according to the method of Fitzgerald (1976) and Deinum et al. (1981).

Preparation of Tubulin and Stable Tubulin–Colchicine Complex. Calf brain tubulin was isolated from freshly slaughtered animals by the modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970; Lee et al., 1973) utilizing the small procedural changes described by Prakash and Timasheff (1983). The concentration of native tubulin was routinely measured by its absorbance at 275 nm in 6 M guanidine hydrochloride utilizing a molar extinction coefficient of 1.03 (Na & Timasheff, 1982). The stable tubulin–colchicine complex was prepared by the method described by Andreu and Timasheff (1982a). The concentration of the complex was measured by using either a molar absorption coefficient of 1.16 in 6 M guanidine hydrochloride at 275 nm or of 1.23 at 276 nm in neutral buffer (Andreu & Timasheff, 1982a).

Preparation of the Tubulin–Cobalt Complex. The tubulin–cobalt complex was prepared by initially following the same procedure as that used routinely for the equilibration of tubulin with the desired experimental buffer (Na & Timasheff, 1982); 2×10^{-4} M $CoCl_2$ was included in the buffer that was used for equilibration of the final Sephadex G-25 column. After elution from the column, the protein was allowed to stand on ice for 30 min and then passed through a Chelex 100 chelating resin in order to remove any weakly bound cobalt.

Visible Spectroscopy. All spectra of tubulin–cobalt complexes were recorded on a Perkin-Elmer Lambda 3B UV–visible spectrophotometer. After background correction, spectra of the tubulin–cobalt complex were read against an equivalent concentration of tubulin in the reference compartment. All preparations of tubulin and tubulin–cobalt were filtered through 0.22-μm filters before the spectra were recorded, in order to avoid interference due to light scattering by large particles.

Fluorescence Spectroscopy. Fluorescence measurements were performed on a Perkin-Elmer 650-40 spectrofluorometer using the ratio mode and 5-nm excitation and emission slits. All spectra were corrected by subtracting background, any instrumental variation as a function of wavelength being taken into account by generating correction factors with rhodamine B and a light diffuser.

Atomic Absorption Spectroscopy. The amount of tightly bound cobalt associated with tubulin after preparation of the tubulin–cobalt complex was determined by atomic absorption spectroscopic analysis using a Perkin-Elmer Model 303 spectrometer.

Binding of ANS to Tubulin. The binding of ANS to tubulin was measured from the enhancement of ANS fluorescence on binding to tubulin (Bhattacharyya & Wolff, 1974; Lee et al., 1975); ANS was excited at 380 nm, and emission was measured at 480 nm. The association constant, K_a, was calculated as described by Lehrer and Fasman (1966):

$$K_a = \frac{\beta}{1-\beta}\frac{1}{[L_f]} \quad (1)$$

where $\beta = (Fl - Fl_p)/(Fl_{pl} - Fl_p)$ and $[L_f] = [L] - \beta[C]$; Fl_p, Fl_{pl}, and Fl are the fluorescence intensities of unliganded protein, fully liganded protein, and of the experimental mixture, respectively, $[L_f]$ and [L] are the free and total concentrations of ligand, respectively, and [C] is the concentration of binding sites on tubulin, which was assumed to be 1 (Bhattacharyya & Wolff, 1975; Lee et al., 1975). The fluorescence of totally liganded tubulin was calculated by extrapolation of a plot of $1/(Fl - Fl_p)$ vs $1/[C]$ to $1/[C] = 0$, and K_a was then calculated from the slope of $\beta/(1 - \beta)$ vs $[L_f]$.

Measurement of the Quantum Yields of the Tubulin–ANS and Tubulin–Colchicine Complexes. The quantum yields of tubulin–ANS, tubulin–colchicine, and tubulin–allocolchicine were obtained by comparing the integrated fluorescence emission spectrum of each complex with that of a standard using the relation described by Parker and Rees (1960). The standard used was quinine sulfate in 1.0 N sulfuric acid at 25 °C using an excitation wavelength of 365 nm (Demas & Crosby, 1971; Melhuish, 1961).

In the calculation of the quantum yield of the tubulin–ANS complex, it was not possible to utilize a concentration of ANS sufficient to saturate the ANS binding site without having an absorbance at the excitation wavelength where inner filter effects become significant. Consequently, two procedures were used to calculate the tubulin–ANS quantum yield: (1) by combining the calculated quantum yield of free ANS with the fluorescent enhancement on binding of ANS to tubulin; (2) by utilizing the binding constant of the tubulin–ANS interaction to calculate the concentrations of free and bound ANS at known total concentrations of tubulin and ANS, and combining the result with the calculated quantum yield of free ANS.

Determination of Fluorescent Energy Transfer. Distances between the bound cobalt and the colchicine and ANS binding sites were measured by fluorescence energy-transfer experiments with the application of the theory developed by Förster

(1948), which relates the efficiency of energy transfer to the distance between donor and acceptor. The relationship between energy-transfer efficiency, E, and the distance between donor and acceptor sites, R, is described by

$$R = R_0(E^{-1} - 1)^{1/6} \quad (2)$$

where R_0 is the distance at which the energy-transfer efficiency is 50%. The value of R_0 is a function of the properties of the donor and acceptor pairs and can be calculated directly from the relation:

$$R_0 = (JK^2Q_DN^{-4})^{1/6}(9.7 \times 10^3 \text{ Å}) \quad (3)$$

where K^2 is the orientation factor for a dipole–dipole interaction between donor and acceptor, J is the spectral overlap integral of donor fluorescence and acceptor absorption, Q_D is the quantum yield of the donor in the absence of acceptor, and N is the refractive index of the medium between donor and acceptor, taken as that of water, 1.4 (Stryer, 1978).

In our experiments, the donor was ANS, colchicine, or allocolchicine; the acceptor was cobalt. The value of the orientation factor, K^2, was taken as $^2/_3$ which is its value when donor and acceptor rotate freely in a time short relative to the excited-state lifetime of the donor (Förster, 1948). Although Co^{2+} appears to be bound rigidly to the protein, this assumption is valid in this case, as Co^{2+} has electronic transitions along all three perpendicular directions (Luk, 1971; Latt et al., 1972; Horrocks et al., 1975) and, thus, is nearly an isotropic acceptor. The values of J were calculated by numerical integration, as described by Jacobson and Colman (1983).

Results

Incorporation into Tubulin of Tightly Bound Cobalt. The tight binding of cobalt by tubulin was monitored by atomic absorption spectrophotometry. When tubulin was incubated with 2×10^{-4} M $CoCl_2$ in 20 mM Pipes and 1×10^{-4} M GTP, pH 7.0, buffer, 0.8 atom of cobalt was incorporated per tubulin dimer. This result is similar to that reported by Himes et al. (1982) of 0.9–1.3 mol of tightly bound cobalt per mole of tubulin dimer. The consistent incorporation of 0.8 mol of cobalt per mole of tubulin was observed only if the incubation was carried out by passage through a Sephadex G-25 column equilibrated with the appropriate buffer as described under Materials and Methods. Direct addition of a small aliquot of concentrated cobalt chloride to a tubulin solution resulted in some precipitation of the protein due to the locally high concentration of cobalt chloride, with resulting inconsistencies in the deduced binding stoichiometries.

In contrast to pure tubulin, when the tubulin–colchicine complex was incubated with cobalt at the above conditions, only 0.4 mol of cobalt was incorporated per mole of tubulin dimer. Incubation for 1 h at 37 °C in 20 mM Pipes, 3.4 M glycerol, and 2×10^{-4} M $CoCl_2$, pH 7.0, to increase the rate of exchange, resulted in an increase in the incorporation to only 0.4–0.6 mol of cobalt. However, incubation of tubulin with cobalt prior to complexation with colchicine resulted in a tubulin–colchicine complex with 0.8 mol of cobalt incorporated per mole of the protein.

In the case of the tubulin–ANS system, the cobalt incorporation was carried out in the presence of 1 M sucrose. This is due to the observation that incubation of tubulin with ANS at 20 °C was accompanied by a significant increase with time of the fluorescence emission of ANS, due to the known instability of tubulin (Prakash & Timasheff, 1982). To stabilize the tubulin, 1 M sucrose was included in all the buffer systems (Frigon & Lee, 1972; Andreu & Timasheff, 1982a) used in the ANS studies. Under these conditions, the fluorescence emission of the tubulin–ANS complex remained constant over the time period of the experiment. In the presence of 1 M sucrose, however, the high-affinity metal binding site exchanged to a lesser extent than in its absence, with only 0.4–0.6 mol of cobalt being incorporated per mole of tubulin dimer.

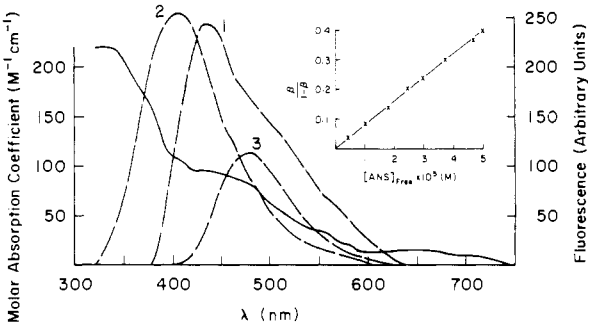

FIGURE 1: Overlap of the absorption spectrum of the tubulin–cobalt complex (—) with the fluorescence emission spectra of the tubulin–colchicine (curve 1), tubulin–allocolchicine (curve 2), and tubulin–ANS (curve 3) complexes. The spectra were recorded in 20 mM Pipes and 1×10^{-4} M GTP, pH 7.0 at 20 °C. The absorption spectrum of the tubulin–cobalt complex was recorded with an identical concentration of tubulin in the reference compartment. The fluorescence emission spectra were obtained by utilizing 5-nm excitation and emission slits. The excitation wavelengths for the three complexes were 350 nm for tubulin–colchicine, 320 nm for tubulin–allocolchicine, and 380 nm for tubulin–ANS. The relative magnitudes of the fluorescence emission spectra drawn have no significance, since the units are arbitrary; the sole purpose of this figure is to demonstrate the extent of overlap between these emission spectra and the absorption spectrum of the tubulin–cobalt complex. Inset: Fluorometric titration of tubulin (5×10^{-6} M) with ANS in 20 mM Pipes and 0.1 mM GTP, pH 7.0 at 20 °C. From the slope, K_a was evaluated to be 8×10^3 M^{-1}.

Energy Transfer between Colchicine and Cobalt. Figure 1, curve 1, shows the fluorescence emission spectrum of the tubulin–colchicine complex. The absorption spectrum of the tubulin–cobalt complex is shown by the solid line. There is obviously sufficient overlap to render this donor–acceptor pair suitable for fluorescence energy-transfer experiments. The spectral overlap integral, J, was calculated by numerical integration over 5-nm intervals and was found to be 3.4×10^{-16} M^{-1} cm^3. The quantum yield of tubulin–colchicine was calculated to be 0.023, which is in good agreement with the value of 0.03 reported by Bhattacharyya and Wolff (1974). It should be noted that the observed efficiency of energy transfer, E_0, is dependent on the fractional occupancy of cobalt, f_{Co}, and is related to the actual efficiency, E, by

$$E = E_0/f_{Co} \quad (4)$$

The variation of cobalt incorporation from 0.4 to 0.8 mol/mol of tubulin–colchicine was found to have no effect on the quantum yield of the tubulin–colchicine complex. This lack of a decrease in colchicine fluorescence in the presence of bound cobalt indicates that the two sites are separated by a distance large enough to preclude efficient energy transfer. Assumption of a 5% possible error in the measurement of fluorescence intensities leads to the conclusion that the cobalt and colchicine binding sites on tubulin are separated by a distance greater than 17 Å, estimated from the calculated value of R_0 of 10.3 Å.

In an attempt to increase the efficiency of energy transfer between the colchicine and cobalt binding sites, a closely related analogue of colchicine, allocolchicine, was used since its fluorescence quantum yield is considerably greater than that of colchicine. Allocolchicine behaves similarly to colchicine in its ability to poison microtubule assembly substoichiometrically (Fitzgerald, 1976). A stable tubulin–allocolchicine complex was prepared in a manner similar to that of the

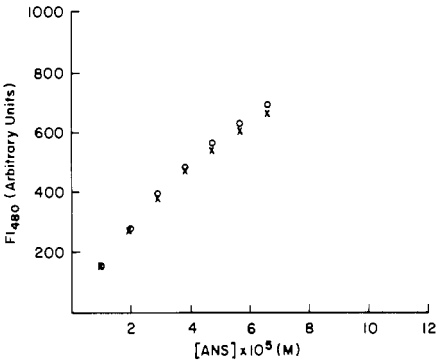

FIGURE 2: Dependence of fluorescence emission of tubulin (×) and tubulin–cobalt (○) on ANS concentration. The tubulin–cobalt assay mixture contained 0.46 mol of cobalt per mole of tubulin dimer. The experiment was performed in 20 mM Pipes, 1 M sucrose, and 0.1 mM GTP, pH 7.0, and the concentrations of both tubulin and tubulin–cobalt were 1 mg/mL.

tubulin–colchicine complex with only minor modifications. The very low solubility of allocolchicine in aqueous solution precluded incubation in the presence of 1 mM allocolchicine. For this reason, allocolchicine was added in small aliquots to tubulin or to the tubulin–cobalt complex, each aliquot being allowed to react for a short time to permit binding prior to the further addition of allocolchicine. The allocolchicine was added at room temperature over a 30-min period to a final concentration of 5×10^{-4} M. Any unbound allocolchicine was removed by the same procedures as used to remove unbound colchicine. The spectral overlap of the allocolchicine fluorescence emission and the cobalt absorption spectra, shown in Figure 1, curve 2, is sufficient for energy-transfer studies. The spectral overlap integral in this case was calculated to be 3.3×10^{-16} M^{-1} cm^3 and the quantum yield of allocolchicine to be 0.2 when excited at 320 nm. This corresponds to R_0 = 14.7 Å. As was the case for tubulin–colchicine, incorporation of cobalt by tubulin had no effect on the quantum yield of the tubulin–allocolchicine complex. This result leads to the conclusion that the allocolchicine fluorophore and the cobalt binding site are separated by a distance exceeding 24 Å, if one assumes a 5% error in fluorescence measurements.

Tubulin–ANS–Cobalt System. (A) Binding of ANS to Tubulin. The results of the fluorometric binding study of the ANS interaction with tubulin are presented in the inset of Figure 1. The slope of the $\beta/(1 - \beta)$ vs $[ANS]_F$ plot gives a value of 8×10^3 M^{-1} for the binding equilibrium constant. This compares well with the value of 1×10^4 M^{-1} reported by Lee et al. (1975) for the same interaction. When the ANS concentrations were sufficiently high for inner filter effects to become significant, correction factors were applied by the preparation of a calibration curve from a plot of the fluorescence of free ANS as a function of concentration (Ward, 1985). Any fluorescence due to free ANS was also subtracted.

(B) Energy Transfer between ANS and Cobalt. The suitability of ANS and cobalt as a donor–acceptor pair for energy-transfer experiments is demonstrated by Figure 1, curve 3, which shows the large overlap between the fluorescence emission of tubulin-bound ANS and the absorption of tubulin-bound cobalt. The overlap integral, J, for this donor–acceptor pair was calculated to be 3.76×10^{-16} M^{-1} cm^3. The value of Q_D, the quantum yield of tubulin-bound ANS, was found to be 0.46, which is in good agreement with the value of 0.48 reported by Bhattacharyya and Wolff (1975). The efficiency of energy transfer between ANS and cobalt was measured by titrating a known concentration of tubulin or tubulin–cobalt with ANS and monitoring the fluorescence

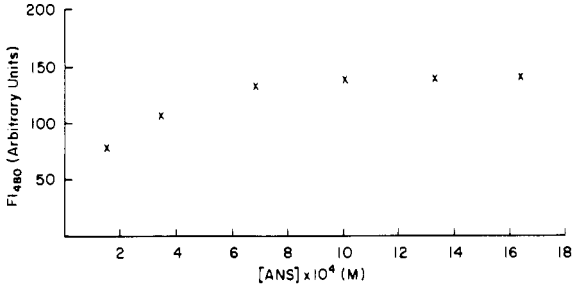

FIGURE 3: Dependence of the fluorescence of ANS on concentration in 20 mM Pipes, 1 M sucrose, and 1×10^{-4} M GTP, pH 7.0, at 20 °C using a front surface accessory. Excitation was performed at 348 nm using 5-nm excitation and emission slits.

emission at 480 nm as a function of the concentration of ANS. The results, presented in Figure 2, indicate that there is no detectable change in quantum yield of ANS due to energy transfer to cobalt, as the results for tubulin and tubulin–cobalt are superimposable. The curvature observed in the plots of fluorescence at 480 nm versus the concentration of ANS is caused predominantly by inner filter effects, as the binding site for ANS is far from saturated at the concentrations of ANS used in this experiment. No effort was made to correct for inner filter effects (Ward, 1985), since any correction would be identical for both the tubulin and tubulin–cobalt assay mixtures, leaving unchanged the ratio of tubulin–ANS fluorescence to that of tubulin–ANS–cobalt.

In these experiments, the detection of small changes in quantum yield due to energy transfer between donor and acceptor is complicated by inner filter effects which become greater as the concentration of ANS is increased. This obviously decreases the sensitivity of the experiment and correspondingly increases the error in determining any small changes that may be occurring via energy transfer. In an attempt to detect such small changes in fluorescence at high concentrations of ANS, where the ANS binding site is essentially saturated, studies were performed using front surface illumination since this eliminates the complicating inner filter effects. With front surface illumination at high absorbance at the excitation wavelength, fluorescence becomes independent of concentration and is directly proportional to the quantum yield of the fluorophore (Eisinger & Flores, 1979). Figure 3 shows the dependence of the fluorescence of free ANS on concentration. As can be seen, the fluorescence is independent of ANS concentration above 1×10^{-3} M. Addition of tubulin increases the observed fluorescence due to the 150-fold enhancement in ANS fluorescence on binding to this protein. Under these conditions, the change in fluorescence on addition of tubulin to 1.6×10^{-3} M ANS was found to be directly proportional to the concentration of tubulin. Energy transfer between ANS and cobalt would be expected to decrease the quantum yield and, thus, the observed fluorescence intensity. This was not observed, the dependence of the ANS fluorescence intensity of tubulin–cobalt on concentration being superimposable on that of tubulin. This would indicate that the ANS binding site is at a distance greater than 28 Å from the cobalt binding site, for an energy-transfer efficiency of less than 5%. This was calculated on the basis of a value of R_0 = 17.3 Å.

Discussion

Tubulin–Cobalt Complex. Cobalt can form a strong complex with tubulin (Himes et al., 1982; Jemiolo & Grisham, 1982; Buttlaire et al., 1980; Eagle et al., 1983), replacing the Mg^{2+} at the exchangeable GTP site (Jemiolo & Grisham, 1982). The present studies show that the ability of cobalt to

be incorporated depends on the conditions employed and the state of liganding of tubulin. Under standard conditions, using a 20 mM Pipes and 0.1 mM GTP buffer, 0.8 mol of cobalt was routinely incorporated per mole of tubulin. If, however, the stable tubulin–colchicine complex was used in place of pure tubulin, only 0.4 mol of cobalt was incorporated per mole of tubulin dimer. This decreased exchangeability of the tightly bound metal ion in the tubulin–colchicine complex can be understood in terms of the conformational change induced in tubulin by the binding of colchicine, as has been inferred from kinetic studies (Garland, 1978; Lambeir & Engelborghs, 1981) and shown by circular dichroism (Andreu & Timasheff, 1982a) and the induction of GTPase activity in tubulin (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981). The present results indicate that this induced conformational change must occur in the region of the exchangeable GTP binding site which is also the locus of binding of the tightly bound divalent metal cation (Monasterio, 1987). Conversely, since the tight binding of cobalt does not interfere with the formation of the tubulin–colchicine complex, the colchicine binding site is independent of the nature of the divalent cation bound, and the conformational change which accompanies colchicine binding (Andreu & Timasheff, 1982a) is not affected by the divalent cation. Thus, the binding of colchicine only affects the accessibility to solvent of the divalent cation, but not necessarily the strength of the binding, i.e., the intrinsic binding constant. Concentrated sucrose also decreases the exchangeability of the high-affinity metal ion binding site. Since sucrose, in stabilizing proteins by its preferential exclusion from their surface, favors the more compact and stable conformations (Lee & Timasheff, 1981), this result indicates that the particular isomeric state of tubulin favored in the presence of sucrose also has a decreased access to contact with solvent of the metal binding site. This is consistent with the observation that concentrated glycerol greatly decreases the rate of hydrolysis by alkaline phosphatase of the exchangeable site GTP on tubulin (Seckler and Timasheff, unpublished results).

The visible absorption spectrum of cobalt is ideal for energy-transfer experiments, with ANS and colchicine or allocolchicine serving as fluorescent donors, since it overlaps almost completely with the fluorescence emission spectra of these ligands. Cobalt has been used extensively as a spectroscopic probe of metal ion binding sites in proteins (Latt & Vallee, 1971; Drum & Vallee, 1970; Mcmillin et al., 1974; Lindskog & Ehrenberg, 1967; Rosenberg et al., 1973). The absorption characteristics of cobalt chelation compounds in the visible region of the spectrum are indicative of the chelation state of the complex (Carlin, 1965) and of protein metal ion binding sites (Vallee, 1973), permitting one to define changes in coordination geometry on binding of substrates to metalloenzymes (Latt & Vallee, 1971). The absorption spectrum (Figure 1) of the tubulin-bound cobalt is characterized by a band centered at 330 nm in the near UV with weaker bands in the visible region of the spectrum which give rise to shoulders at 470 and 650 nm. The bands in the visible region can be attributed to d–d transitions within the cobalt atom (Cotton & Wilkinson, 1980), whereas the stronger band in the near UV can be most easily explained in terms of a charge transfer band between a chelating ligand and the cobalt itself. A band centered at 350 nm has been ascribed to a charge transfer transition in the UV–visible spectrum of cobalt bound to stellacyanin (Mcmillin et al., 1974). It should be noted that the stable Co–ATP complex (Cleland & Mildvan, 1979) has an absorption band in the near UV which cannot be ascribed to a d–d transition normally associated with the transition metal ion cobalt. Thus, this band in tubulin-linked cobalt could conceivably be due to the interaction between cobalt and the oxygen ligands provided by GTP, which is consistent with the NMR characterization of the tubulin–Mn^{2+} complex (Monasterio, 1987). The overlap of the absorption bands due to d–d transitions with the higher energy band in the near UV makes any interpretation of the visible region bands difficult. It is clear, however, that one cannot explain them on the grounds of purely tetrahedral or octahedral geometry normally associated with cobalt complexes. Coordination compounds involving cobalt in tetrahedral geometry are usually characterized by absorption bands in the region of 600–700 nm with molar extinction coefficients in the range 500–600 M^{-1} cm^{-1}. Octahedral coordination geometries involving cobalt usually possess absorption bands in the region 500–600 nm with molar absorption coefficients of about 10 M^{-1} cm^{-1} (Carlin, 1965). One must, therefore, conclude that the tubulin-bound cobalt possesses a distorted geometry which is a common occurrence for metal ion coordination states in proteins (Vallee, 1973).

Distances between Sites. The energy-transfer experiments have shown that there is no detectable radiationless energy transfer between the ANS–cobalt and the allocolchicine– or colchicine–cobalt donor–acceptor pairs. This indicates that the ANS and cobalt binding sites are separated by at least 28 Å, while the distance of separation between the allocolchicine (or colchicine) and cobalt sites is greater than 24 Å. Thus, although cobalt is an ideal energy acceptor in that its spectrum overlaps near to completely the fluorescence emission spectra of both ANS and colchicine (or allocolchicine), its absorption is not strong enough to allow it to interact with fluorophores over the entire topography of a large protein, such as tubulin. These results, however, lead to a significant conclusion concerning the relative positions of the binding sites on the tubulin dimer. The binding of colchicine to tubulin is known to affect the properties of the exchangeable GTP site, in that it induces a GTPase activity in the tubulin dimer, which, normally, is present only when tubulin is incorporated into the microtubule (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981). The binding of colchicine to tubulin is accompanied by only a minor change in the CD spectrum. Therefore, the change in conformation could be expected to be minor (Andreu & Timasheff, 1982a). This would be consistent with close positioning of the colchicine and GTP sites on the tubulin dimer. The present results do not confirm this line of reasoning but, in fact, indicate a long-range conformational effect of colchicine on tubulin. What is the relation of these distances to the dimensions of the tubulin dimer? Sedimentation velocity studies (Frigon & Timasheff, 1975a,b) and X-ray scattering studies of tubulin (Bordas et al., 1983) have shown that the shape of the tubulin dimer in solution is not spherical but elongated. Bordas et al. (1983) have estimated the intersubunit distance to be 4.9 nm. Such a value exceeds the minimum distances of separation between the cobalt and ANS sites (28 Å) and the cobalt and colchicine binding sites (24 Å) measured in this study. This precludes an answer to the question of whether these sites lie on the same or different subunits of α–β-tubulin.

Neither is any clear answer to this question given by the various chemical and genetic studies aimed at its resolution. The position of the exchangeable GTP site, and thus of the high-affinity metal ion binding site, has been identified as being on the β subunit by the use of the photoaffinity analogue 8-azido-GTP (Geahlen & Haley, 1977, 1979) and UV cross-linking of GTP to tubulin (Nath et al., 1985; Hesse et

al., 1985). On the other hand, Maccioni and Seeds (1983), who used other GTP analogues, have found equal labeling of both α and β subunits. The location of the colchicine binding site is also complicated by contradictory reports. Luduena and Roach (1981) have found that colchicine inhibited the reaction of a bifunctional sulfhydryl reagent with tubulin. The same reagent failed to react with another variant of tubulin (Luduena et al., 1982) in which the cysteine in position 203 of the β subunit had been replaced by a serine (Little, 1979). The assumption that colchicine protects against this reagent by covering the sulfhydryl group (Ponstingl et al., 1984) has led to the conclusion that the binding site is located on the β subunit. Contrary to this, Serrano et al. (1984), who subjected tubulin to limited proteolysis by trypsin, have reported the localization of the colchicine binding site on a 16000 molecular weight fragment at the carboxyl terminal of the α chain.

Genetic evidence to date is also seemingly contradictory. Mutant tubulins from the fungus *Aspergillus nidulans*, with mutations in the β chain, have altered binding affinities for the benzimidazole antimitotic drugs (Morris et al., 1979). Similarly, in Chinese hamster ovary cells, mutations in the β chain have resulted in resistance to colchicine (Cabral et al., 1980). Contrary to this, Keates et al. (1981) identified an α-chain mutation with increased resistance to colcemid. All these seemingly contradictory results could be reconciled if colchicine bound at the subunit interface. This would be consistent with the stabilization by colchicine of the tubulin dimer against dissociation into subunits at low protein concentrations, reported by Detrich et al. (1982). An alternative explanation would be that mutations in either subunit affect the tertiary structure of both, with the result that colchicine binding would be altered no matter in which subunit the mutation took place.

Registry No. ANS, 82-76-8; Mg, 7439-95-4; Co, 7440-48-4; colchicine, 64-86-8; allocolchicine, 641-28-1.

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