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α-Tubulin influences nucleotide binding to β-tubulin: An assay using picomoles of unpurified protein

(*in vitro* expression/GTP/magnesium/HPLC)

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ABSTRACT **Tubulin binds guanine nucleotides tightly within its β subunit. Whether the α subunit influences binding to this site has been unknown. This question was addressed by comparing the nucleotide binding properties of the free β subunit with those of the heterodimer. The free β subunit was obtained from an *in vitro* expression system and its nucleotide binding properties were determined by an assay that requires ≈100-fold less protein than conventional assays. This assay exploits the observation that the recovery of β-tubulin from Mono Q anion-exchange columns is dependent on added nucleotide. Our results demonstrate that the newly synthesized β subunit and the heterodimer bind nucleotides with similar specificity. We found that in the presence of magnesium the α subunit enhances GTP binding to the β subunit ≈4-fold. However, in the absence of magnesium the α subunit appears to specifically weaken GTP binding to the β subunit. Thus, nucleotide binding to the E site in the heterodimer may not be solely defined by the β subunit.**

Microtubules are cytoskeletal polymers assembled mainly from tubulin, a heterodimeric protein (≈100 kDa) composed of two homologous subunits—α- and β-tubulin. A variety of investigations have shown that the heterodimer binds two guanine nucleotides tightly: one exchangeably at a site in β-tubulin (E site) and one nonexchangeably at a site presumably in α-tubulin (1–4). Nucleotide binding to the E site plays an essential role in microtubule function (5, 6). For example, MgGTP in the E site stimulates microtubule assembly, whereas MgGDP destabilizes assembled microtubules. The competing effects of GTP and GDP give rise to a complex phenomenon at microtubule ends, termed "dynamic instability," thought to be essential for a variety of cellular processes, including kinetochore capture, chromosome movement, and division axis orientation (7–10).

Despite the importance of the E site for microtubule function, very little is known concerning the structure of this site. In addition, despite numerous studies of guanine nucleotide binding to the heterodimer, there has been no study of GTP or GDP binding to the E site in the isolated β-tubulin subunit. Thus, differences in nucleotide binding between the free β subunit and the heterodimer and what role the α subunit plays, if any, in nucleotide binding to the E site are unknown. In large part, this difficulty has arisen from an inability to isolate active β-tubulin monomer despite a reasonably high dissociation constant for the heterodimer (11, 12).

As an alternative approach, we have developed a system to express small amounts of functional β-tubulin subunits *in vitro* (13). In this system, very small quantities (<1.5 pmol per 50-μl reaction mixture) of highly radiolabeled β-tubulin are produced by the translation of β-tubulin mRNA in rabbit reticulocyte lysates (RRLs). Such miniscule amounts of protein preclude conventional biochemical assays for ligand binding. In this report, we describe an assay for nucleotide binding to the picomole amounts of β-tubulin produced in RRLs and begin to elucidate the role of the α subunit in nucleotide binding to the E site of the β subunit.

MATERIALS AND METHODS

Materials. GTP (IIS), GDP (type I), ITP (grade III), XTP (grade I), and ATP (A5384) were obtained from Sigma. The concentrations of the nucleotide stock solutions (≈0.1 M) were determined spectrophotometrically. Chemical purity was assessed by anion-exchange HPLC and by TLC on PEI-cellulose F plates (EM Science). GTP (IIS) contained ≈4% GDP and ≈1% GMP. In the Mg^{2+}-free study, GTP was further purified by anion-exchange chromatography to reduce GDP contamination to <1%.

***In Vitro* Translations.** Chicken β-tubulin mRNA (5–10 μg) was translated in micrococcal nuclease-treated RRLs (Promega) for 1 hr at 30°C and prepared for HPLC as described (13, 14). These lysates are estimated to contain 1 mM each ATP and GTP (Promega). Free nucleotide as well as unincorporated radiolabel were removed by sequential centrifugation through six 5-ml Sephadex G-25 spin columns (14). The effluents were kept on ice for subsequent analysis by HPLC.

Anion-Exchange HPLC/Nucleotide Binding Assay. Effluents (80 μl) were analyzed on a Mono Q anion-exchange HPLC column (Pharmacia LKB). The samples were eluted at ambient temperature with a linear 40-min gradient of 0–1 M NaCl formed from buffers A (20 mM sodium phosphate, pH 6.8) and B (20 mM sodium phosphate, pH 6.8/1 M NaCl) at a flow rate of 1 ml/min. Under these conditions, we typically recovered ≈5% and ≈12% of the β_{II} and β_{III} forms, respectively. However, when the running buffers were supplemented with high concentrations of GTP and magnesium, we typically recovered ≈30% of either β_{II} or β_{III} (13).

To assay for nucleotide binding based on enhanced protein recoveries buffers A and B were supplemented with various fixed concentrations of nucleotide with 0.5 mM $MgCl_2$. The samples in Fig. 1*B* were also supplemented with nucleotide before injection. However, control studies established that such additions were unnecessary. Binding studies were typically performed starting with low concentrations of nucleotides in the running buffers and proceeding to progressively higher concentrations. Control studies established that the apparent K_d values obtained for nucleotide binding were independent of the order of nucleotide addition to the running buffers.

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Abbreviation: RRL, rabbit reticulocyte lysate.

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Mg^{2+}-Free Nucleotide Binding Studies. In the Mg^{2+}-free studies, magnesium was omitted from the HPLC running buffers. Mg^{2+} contamination in these buffers was determined by atomic absorption spectrophotometry (AA/AE spectrophotometer 357, Instrumentation Laboratory, Lexington, MA). Buffer A contained no detectable Mg^{2+} contaminant, whereas buffer B contained ≈3 μM Mg^{2+}. The buffers were supplemented to 1 mM EDTA to complex this contaminant as well as the small amounts of Mg^{2+} introduced with the samples. Based on an effective Mg^{2+} complexation constant of 1 μM at pH 6.8, this concentration of EDTA should bind essentially all of the magnesium (15, 16).

RESULTS

***In Vitro* Expression of β-Tubulin.** We have previously shown (13) that when β-tubulin mRNA is translated in RRLs in the presence of [^{35}S]methionine, picomole amounts of highly radiolabeled functional β-tubulin are produced. Under these conditions, >80% of total radiolabeled protein are full-length β-tubulin polypeptides (13). These nascent polypeptides exist in three distinct molecular forms separable by either anion-exchange or size-exclusion chromatography: an ≈180-kDa noncovalent complex containing β-tubulin and other lysate protein(s) (β_I), the ≈50-kDa newly synthesized monomeric β subunit (β_{II}), and an ≈100-kDa heterodimeric form (β_{III}) arising from association of the translated β subunits with residual rabbit α-tubulin subunits in the lysate (13).

Nucleotide Binding Assay. ^{35}S-labeled β-tubulin was synthesized in RRLs and processed as described. Aliquots (80 μl) were loaded on a Mono Q anion-exchange HPLC column with various fixed concentrations of GTP in the running buffers. GTP binds tightly to the anion-exchange resin at salt concentrations <0.35 M NaCl (Fig. 1*A*). However, above 0.35 M NaCl—i.e, within the salt concentrations used to elute the β-tubulin forms (0.42–0.62 M NaCl)—GTP does not interact with the column resin and the free GTP concentration in the column is identical to the GTP concentration in the running buffer (Fig. 1*A*). Fig. 1*B* shows typical recoveries of the β_I, β_{II}, and β_{III} forms as a function of various fixed GTP concentrations (0–500 nM) with 0.5 mM Mg^{2+} in the running buffers. As the concentration of GTP in the buffers is increased, one observes a progressive increase in the area under the β_{II} and β_{III} peaks (Fig. 1*B*). This increase in radiolabel recovery was shown to be specific to full-length β-tubulin by SDS/PAGE fluorography (data not shown). In contrast to our findings for β_{II} and β_{III}, no consistent or systematic increase in the recovery of the β_I form was observed.

The nucleotide-dependent enhancement was analyzed by double-reciprocal plots of the increase in cpm vs. GTP concentration (Fig. 1*B Inset*) (see *Discussion*). The linearity of the plots for β_{II} and β_{III} suggests that the β-tubulin polypeptides interact with GTP at a single site over the range of GTP concentrations used. When the slopes of the least-squares fit lines were divided by the *y* intercepts, apparent K_d values for MgGTP binding of 37 nM for β_{II} and 10 nM for β_{III} were obtained (Fig. 1*B Inset*). The apparent K_d for β_{III}, the heterodimer, is in excellent agreement with the K_d for MgGTP binding to the E site of bovine tubulin dimer measured by a variety of conventional binding assays (Table 1).

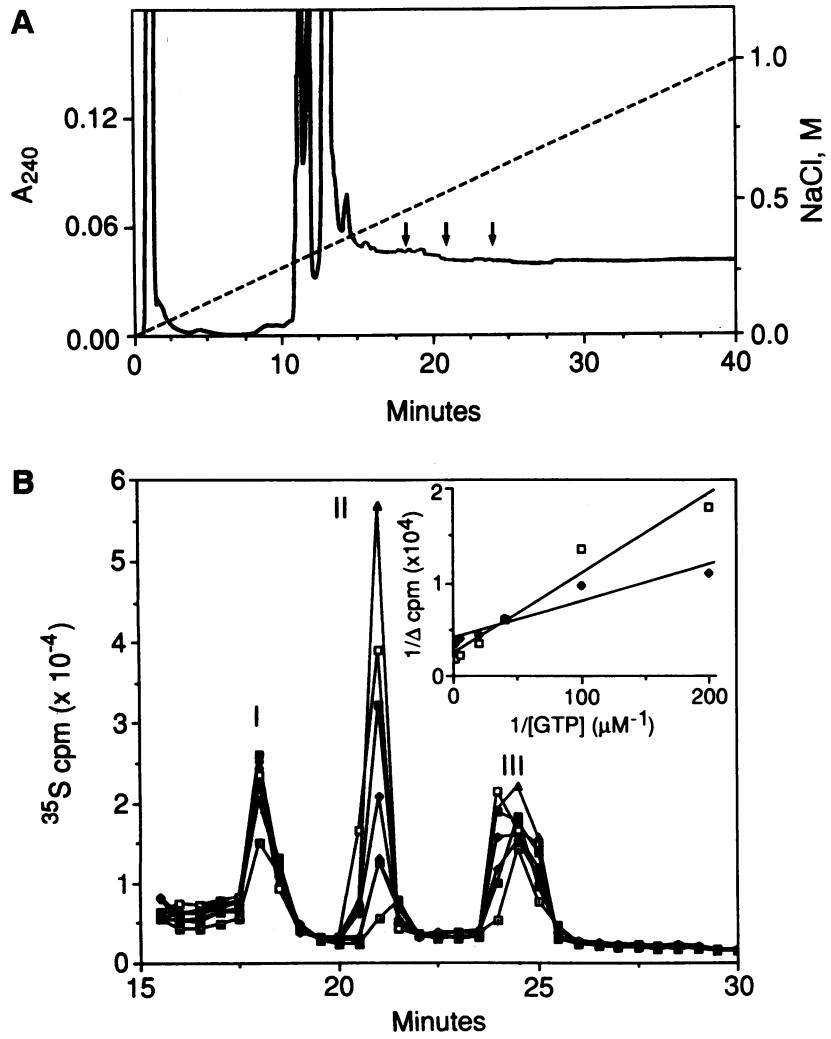

FIG. 1. Nucleotide binding assay based on enhanced recoveries. ^{35}S-labeled translation mixtures were prepared and treated as described, loaded on a Mono Q anion-exchange HPLC column, and eluted with a linear gradient (---) of 0–1 M NaCl in 20 mM sodium phosphate (pH 6.8) buffer. (*A*) GTP concentration is constant during elution of the β-tubulin polypeptides. In this study, the column buffers were supplemented to 10 μM GTP and 0.5 mM $MgCl_2$. GTP binds to the anion-exchange resin and is released at 0.35 M NaCl (large peak at ≈14 min). At higher salt concentration, the GTP concentration in the column is constant, as evidenced by the elevated background absorbance, and equal to 10 μM. The β-tubulin forms (arrows) elute in this constant GTP region. (*B*) Elution of the ^{35}S-labeled β-tubulin forms as a function of GTP concentration. Column running buffers were supplemented to 0.5 mM $MgCl_2$ and various fixed concentrations of GTP as follows: □, 0 nM; ◆, 5 nM; ■, 10 nM; ◆, 25 nM; ■, 50 nM; □, 200 nM; ▲, 500 nM. Fractions were collected at 0.5-min intervals and 400-μl aliquots were removed from each fraction for liquid scintillation counting. This figure shows the progressive increase in the recovery of radiolabel at the β_{II} and β_{III} positions with increasing GTP concentrations for a single experiment. (*Inset*) Double-reciprocal analysis of this increase provides estimates of the apparent dissociation constants (K_d) for GTP binding to β_{II} (□) and β_{III} (◆) where $1/\Delta cpm = (K_d/\Delta cpm_{max})(1/[GTP]) + 1/\Delta cpm_{max}$, or apparent K_d = slope/*y* intercept (see *Discussion*). Δcpm denotes the increase in radiolabel relative to the 0 GTP control; Δcpm_{max}, the corresponding increase in radiolabel at "infinite" concentrations of GTP.

Nucleotide Specificity. To test for nucleotide specificity, translation mixtures were chromatographed on the Mono Q column in the presence of various concentrations of GDP, ITP, XTP, ATP, or CTP (Fig. 2). The quality of the resulting elution profiles for GDP, ITP, and XTP was comparable to that shown in Fig. 1 for GTP, and these profiles were analyzed as discussed above. As shown in Table 1, the apparent K_d values for GTP and GDP binding to tubulin dimer (β_{III}) are in excellent agreement with published values derived from standard nucleotide binding assays. Furthermore, the apparent K_d values for XTP, ITP, and ATP (Table 1) are significantly larger than the values determined for GTP and GDP, consistent with the fact that tubulin binds guanine nucleotides preferentially. In the case of CTP, no recovery enhancement was observed even at nucleotide concentrations as high as 0.5 mM (Fig. 2*E*). This finding, which implied a lack of CTP binding, is consistent with the finding by Duanmu *et al.* (20) that pyrimidine nucleotides, in contrast with purine nucleotides, are incapable of inducing microtubule assembly. These results taken together argue that our assay has both the sensitivity and specificity necessary to determine binding to the E site of tubulin dimer.

Table 1 also shows the apparent dissociation constants for GTP, GDP, ITP, XTP, ATP, and CTP binding to the newly synthesized free β subunit, β_{II}, determined simultaneously with those of the tubulin heterodimer, β_{III}. These apparent K_d values were consistently 1.7- to 7-fold larger than those measured for the heterodimer and maintained the same order of nucleotide affinity. It therefore appears that the enhanced recovery of β_{II}, like β_{III}, occurs through nucleotide binding to the E site. Furthermore, the presence of the subunit seems to somehow increase the affinity of the E site in β-tubulin for purine nucleotides.

Effect of Mg^{2+}. Huang *et al.* (3) and Correia *et al.* (4) reported that the binding of GTP, and not GDP, to the E site of tubulin dimer is strongly dependent on Mg^{2+}. We therefore used our assay to investigate GTP and GDP binding to β_{II} and β_{III} in the absence of magnesium. Free magnesium was removed by the addition of EDTA to the running buffers. The results of the investigation for both the free β subunit and the heterodimer are shown in Table 2. In the absence of Mg^{2+}, the apparent K_d values for GTP and GDP binding to the heterodimer are in close agreement with the values determined by Correia *et al.* (4, 22), further supporting the conclusion that recovery of β-tubulin from the anion-exchange column is dependent on nucleotide binding to the E site.

Table 1. Apparent K_d values of the free β subunit (β_{II}) and tubulin heterodimer (β_{III}) for various nucleotides

Nucleotide	Apparent K_d for the β subunit (β_{II}), nM	Apparent K_d for tubulin heterodimer (β_{III}), nM	Published K_d for tubulin heterodimer, nM
GTP	39 ± 5	10 ± 1	18,* 17,† 10–16‡
GDP	68 ± 10	40 ± 4	61,* 83,† 37–40‡
ITP	9,000 ± 3,100	1600 ± 400	10,000§ 30,000¶
XTP	12,000 ± 2,000	1700 ± 500	300,000¶
ATP	≈4 × 10^6	>10^6	600,000¶ 200,000‖
CTP	NDB	NDB	NDB

The apparent dissociation constants for β_{II} and β_{III} were calculated from their recovery off an anion-exchange column as described in the text and Fig. 1. The apparent dissociation constants are shown ± SD where $n = 3$. NDB, no detectable binding (see Fig. 2*E*). Dissociation constants for the tubulin heterodimer were obtained from the following sources: *, ref. 17; †, ref. 18; ‡, ref. 4; §, by polymerization assay, ref. 19; ¶, by polymerization assay, ref. 20; ‖, ref. 21.

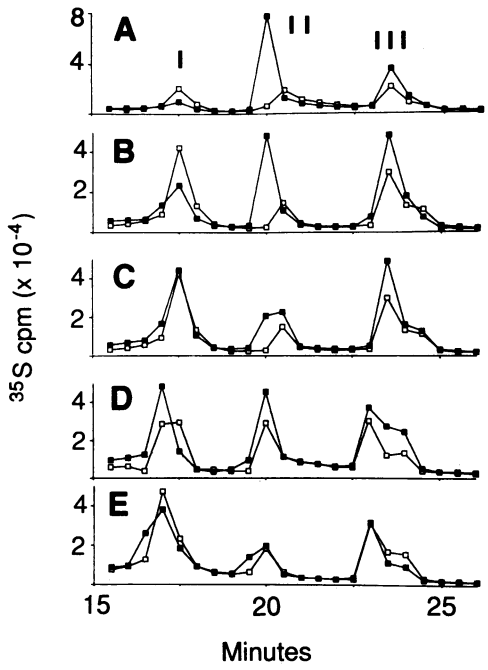

FIG. 2. The nucleotide-dependent recovery enhancements show specificity for guanine nucleotides. The effect of various purine and pyrimidine nucleotides on the recovery of β_{II} and β_{III} were determined as described in Fig. 1. (*A*) 0.5 μM GDP. (*B*) 100 μM ITP. (*C*) 100 μM XTP. (*D*) 500 μM ATP. (*E*) 500 μM CTP. □, Elution in the absence of nucleotide; ■, elution in the presence of nucleotide. The enhanced recoveries shown for GDP, ITP, and XTP correspond to 80–90% of the total enhancement possible with these nucleotides. No enhancement was observed in the presence of CTP.

Surprisingly, the Mg^{2+} dependence of GTP binding to the free β subunit was considerably less than that for the heterodimer. In the absence of Mg^{2+}, the apparent affinity of the heterodimer for GTP was reduced 800-fold, whereas the apparent affinity of the free β subunit for GTP was reduced only 40-fold (Table 2). In fact, in the absence of magnesium, the β_{II} form actually binds GTP ≈5-fold tighter than the heterodimer. In contrast, GDP binding to both β_{II} and β_{III} was relatively unaffected by the absence of Mg^{2+} (Table 2). Thus, α-tubulin association with the β subunit appears to specifically enhance the magnesium dependence of GTP binding to the E site 20-fold, yet it leaves the magnesium dependence of GDP binding essentially unaffected. Whether this represents a direct or indirect effect of the α subunit on the conformation of the E site in β-tubulin is not known.

DISCUSSION

We have developed a nucleotide binding assay based on the enhanced recovery of proteins from a Mono Q anion-exchange column. The manner by which nucleotide binding at the E site affects the recovery of β-tubulin polypeptides seems to result from differential loss of β polypeptides on the column (loss of the nucleotide-free polypeptides > loss of nucleotide-containing polypeptides). This differential loss presumably reflects the fact that tubulin dimer can exist in two different conformations depending on whether or not the E site is occupied by nucleotide (23–25). We hypothesize that the column can be divided into "equilibrium plates" (the number of these plates being equal to the number of times tubulin and nucleotide come to equilibrium during chromatography) and model the column recovery in terms of a four-step process occurring at each of these plates (Fig. 3): 1,

Table 2. Apparent K_d values of the isolated β subunit (β_{II}) and tubulin heterodimer (β_{III}) for GTP and GDP in the absence and presence of magnesium (0.5 mM)

Nucleotide	Apparent K_d for the β subunit (β_{II}), nM	Apparent K_d for tubulin heterodimer (β_{III}), nM	Published K_d for tubulin heterodimer, nM
GTP			
+ Mg^{2+}	39	10	11–16*
− Mg^{2+}	1600 ± 400	8100 ± 600	2000–4500†
GDP			
+ Mg^{2+}	68	40	37–40*
− Mg^{2+}	140 ± 30	81 ± 10	62*

The apparent dissociation constants for β_{II} and β_{III} were calculated from their recovery off an anion-exchange column as described in the text and Fig. 1. EDTA (1 mM) was added to the column running buffers to complex magnesium. The apparent dissociation constants in the absence of magnesium are shown ± SD where $n = 3$. Dissociation contants for the tubulin heterodimer were obtained from the following sources: *, ref. 4; †, ref. 22.

entry of equilibrated mixture of tubulin and nucleotide; 2, protein interaction with the resin; 3, protein release from the resin; 4, reequilibration of nucleotide with the E site.

A variety of observations suggest that nucleotide in the running buffers establishes a dynamic equilibrium with the E site during protein elution: (*i*) the apparent K_d values are independent of elution rates (40 or 60 min 0–1 M salt gradients) (data not shown); (*ii*) the translation mixtures need not be supplemented with nucleotide before injection; and (*iii*) identical apparent K_d values are obtained irrespective of initial nucleotide concentration in the sample (less than ≈5 mM) (unpublished data). Furthermore, Caplow *et al.* (26) measured the half-life for nucleotide dissociation from the heterodimer as ≈25 sec for GTP and ≈5 sec for GDP, and their data suggest that the half-life for tubulin association with nucleotide is also rapid (≤25 sec) for nucleotide concentrations ≥10 nM. Thus the protein and nucleotide have more than sufficient time to establish a dynamic equilibrium on the column during the 7–10 min of protein elution in the presence of nucleotide (≥15-fold longer than the half-lives quoted above). This dynamic process generates two populations of β-tubulin polypeptides (either containing or lacking nucleotide) whose relative proportions are dictated by the K_d for nucleotide binding and the concentration of nucleotide in the running buffers (cf. Fig. 3 *A* vs. *B*).

Based on this model, the fraction of total protein recovered from the column for each β-tubulin form is given by

$$\text{fraction recovered} = [(1 - f_o)X_F + (1 - f_g)X_B]^N, \quad [1]$$

where f_o and f_g denote, respectively, the fraction of nucleotide-free protein and nucleotide-containing protein lost per equilibrium plate and N denotes the number of equilibrium plates [estimated to be ≤6 based on four half-lives for the association and dissociation of tubulin and nucleotide (26)]. X_F and X_B denote the corresponding fractions of nucleotide-free protein and nucleotide-containing protein, which are determined solely by the K_d of the protein for nucleotide and

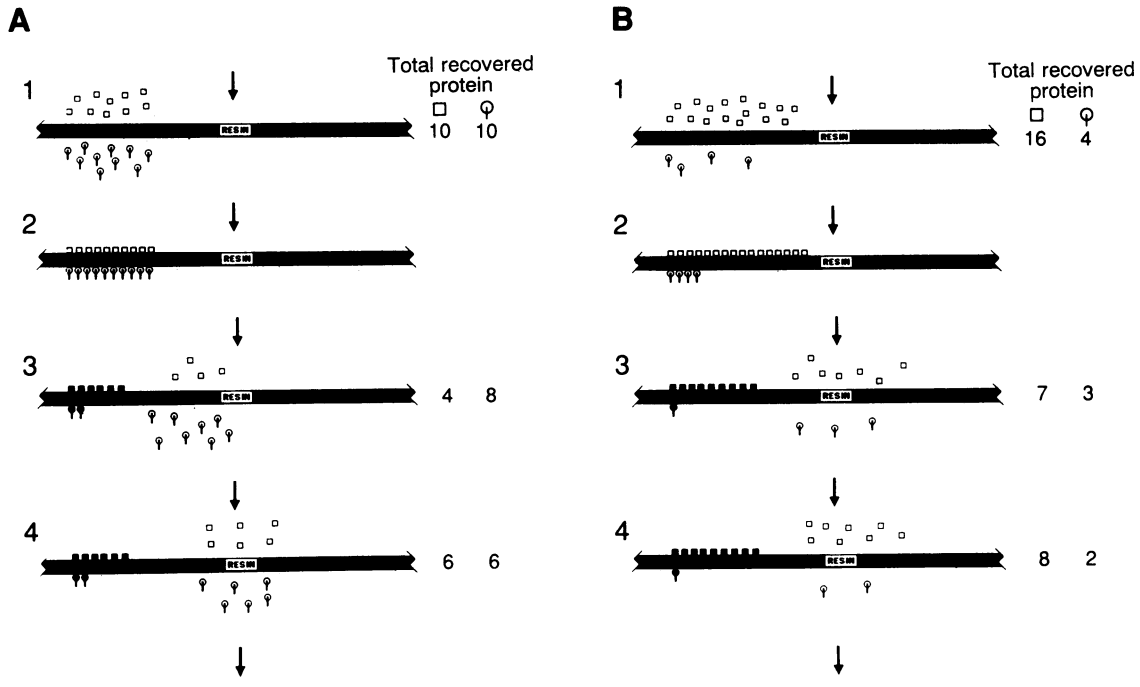

FIG. 3. A model for the nucleotide binding assay relating protein recoveries to nucleotide binding affinities. Total recovery from the anion-exchange column is given in Eq. 1 for N equilibrium plates. In this illustration, based on a single equilibrium plate, we examine the differential recoveries for 20 protein molecules (♀, nucleotide bound; □, nucleotide free) eluting in the presence of two different nucleotide concentrations: [nucleotide] = K_d (*A*) or [nucleotide] = 0.25 K_d (*B*), where K_d is the protein–nucleotide dissociation constant. 1, Entering proteins are initially in equilibrium with the nucleotide in the running buffers. 2, The proteins bind to the ion-exchange resin. 3, Sixty percent of the nucleotide-free ($f_o = 0.60$; Eq. 1) and 20% of the nucleotide-bound proteins ($f_g = 0.20$; Eq. 1) are irreversibly lost. 4, The released proteins reequilibrate with nucleotide in the running buffers before proceeding to the next equilibrium plate. For nucleotide concentrations equal to 0.25 K_d (*B*), K_d (*A*), or 10 K_d (data not shown), 10, 12, and 15 of the initial 20 protein molecules are recovered, respectively. When the "background" recovery (8 of 20; [nucleotide] = 0) is subtracted from these values, the double-reciprocal plot of the corrected recoveries vs. nucleotide concentration is linear and the calculated apparent K_d based on the slope and y intercept equals K_d. Furthermore, if more than one equilibrium plate is used in the analysis, linear double-reciprocal plots are again obtained with apparent $K_d \approx K_d$ for $N < 4$. This linearity, however, is not restricted to small N if f_g and f_o are sufficiently small. In this illustration, we have arbitrarily set f_g and f_o equal to 0.2 and 0.6 per equilibrium plate, respectively, and do not mean to imply that these are the actual losses encountered on the column.

the concentration of nucleotide in the running buffers. Thus, this simple model demonstrates how a seemingly irreversible process—i.e., the nucleotide-dependent recovery phenomenon—is a direct reflection of the reversible process of nucleotide binding to the E site. Furthermore, this model explicitly predicts a linear dependence of column recovery on nucleotide concentration in double-reciprocal plots, which mimics a binding equilibrium between ligand and protein (unpublished studies; see also Fig. 3). While this model is attractive, further studies are required to fully test its validity.

GTP binding to the heterodimer requires Mg^{2+}, whereas GDP binding does not (3, 4, 22, 27). Similarly, we found that GTP binding to the free subunit requires Mg^{2+}, whereas GDP binding does not. However, in this case the Mg^{2+} dependence was less pronounced (Table 2). At present, the molecular basis for this difference in the magnesium dependence is unknown. Interestingly, we have recently shown that the newly synthesized β subunit undergoes a conformational change upon association with α-tubulin (14). Thus, the differences in the nucleotide binding of β_{II} and β_{III} (Tables 1 and 2) may reflect this conformational change.

We also investigated the binding of XTP, ITP, and ATP to the β_{II} and β_{III} forms (Table 1). Our measured apparent binding constants for XTP and ITP were very similar, whereas Duanmu *et al.* (20) proposed that ITP binds 10-fold more effectively than XTP. However, these authors used a polymerization-based functional assay to obtain "threshold concentrations" rather than actual binding constants *per se*. Whether the differences between their values and ours reflect limitations of either assay is not yet apparent. One possible source for this discrepancy could be guanine nucleotide impurities (>0.1%) in our XTP stocks. However, careful chromatographic analysis failed to reveal any such contamination.

We found that ATP also enhanced the recovery of β_{II} and β_{III} from the anion-exchange column. However, double-reciprocal plots of the data showed considerable scatter for β_{III}. Our estimated apparent K_d value of $\geq$1 mM for ATP binding to the tubulin heterodimer falls quite close to the values reported by both Duanmu *et al.* (0.6 mM) (20) and Zabrecky and Cole (0.2 mM) (21, 28). Zabrecky and Cole (28), however, concluded that ATP binds to a third nucleotide site present on the α subunit, whereas we found that the free β subunit had a similar binding constant for ATP as the tubulin heterodimer. This latter finding is more consistent with the proposal of Duanmu *et al.* (20) that ATP binds weakly to the E site on the β subunit.

Our assay has several significant advantages over conventional ligand binding assays. It is highly sensitive and capable of measuring binding to <1 nM tubulin. This represents at least an $\approx$100-fold increase in sensitivity relative to conventional assays, such as the Hummel–Dryer assay (17). Furthermore, our assay permitted accurate measurement of nucleotide binding to β-tubulin without significant purification. The RRL has a number of nucleotide binding proteins involved in translation that could potentially compete with tubulin for nucleotide. However, since only β-tubulin is radiolabeled in the *in vitro* translation system its recovery can be specifically monitored despite the presence of these "contaminating" nucleotide binding proteins.

This assay may be adaptable to other ligand/receptor systems providing at least three criteria are fulfilled: (*i*) the receptor should exist in two distinct conformations dependent on whether or not the ligand binding site is occupied. (*ii*) The ligand concentration in the column buffers should be constant through the region where the receptor protein elutes. (*iii*) The ligand must be in sufficient excess to the receptor protein to permit the maintenance of a constant free-ligand concentration on the column (the assay works best with low concentrations of proteins). Interestingly, *in vitro* expression systems are ideally suited for the production of small quantities of highly radiolabeled receptor that would meet this last condition.

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