Determination of Free and Bound Microtubular Protein and Guanine Nucleotide under Equilibrium Conditions†

Barry Zeeberg and Michael Caplow*

ABSTRACT: The dissociation constant for GDP binding to the E site of tubulin isolated by chromatography on Sepharose 6B is equal to 6.1×10^{-8} M, as determined by the Hummel–Dryer procedure. This is smaller than any previously reported value, and the discrepancy with earlier results is analyzed. By use of a recently described column centrifugation procedure [Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899], it was established that GDP and GTP bind to the same site. GTP is bound 2.8-fold tighter than GDP, and the dissociation constant is 2.2×10^{-8} M. A new method for the determination of dissociation constants for a protein-bound ligand, based on a quantitative analysis of the loss of ligand during exclusion chromatography, is presented. This has been used to determine that the dissociation constant for GDP bound to tubulin is equal to 5.5×10^{-8} M, in excellent agreement with that determined independently from the Hummel–Dryer method. A previous theoretical treatment [Dixon, H. B. F. (1976) *Biochem. J. 159*, 161–162] of ligand loss during exclusion chromatography is discussed.

It is important to know the dissociation constants for GDP and GTP binding to the exchangeable nucleotide binding site on tubulin (E site), in order to interpret mechanisms for the role of nucleotides in microtubule assembly (Carlier & Pantaloni, 1978; MacNeal & Purich, 1977, 1978; Penningroth & Kirschner, 1977; Weisenberg et al., 1976). A number of laboratories have attempted these determinations, and a wide range of values has been reported (see Discussion). In general, binding studies with tubulin are made technically difficult by the following. (a) Binding is tight so that very dilute solutions are required for any dissociation to occur. (b) Tubulin is isolated with an E site which is saturated with guanine nucleotide so that it is difficult to titrate the binding site by adding nucleotide.

In experiments reported here the dissociation constant for GDP was determined with particular emphasis on these problems. By use of the Hummel–Dryer procedure (Hummel & Dryer, 1962), it was found to be 6.1×10^{-8} M.

A new method for the determination of dissociation constants for a protein-bound ligand, based upon a quantitative analysis of the loss of ligand during exclusion chromatography, is presented.

Materials and Methods

Porcine tubulin was prepared by ATP-induced polymerization (Zeeberg et al., 1977) and depolymerized at 0 °C in RB (0.1 M 2-(*N*-morpholino)ethanesulfonic acid, 0.5 mM $MgCl_2$, and 1 mM EGTA, pH 6.8) or in quarter-strength RB when a phosphocellulose purification step (Weingarten et al., 1975) was included. Except where noted, the protein used was obtained after depolymerization in RB and fractionation on a Sepharose 6B column equilibrated with RB. The nonring protein was used. Protein concentrations were calculated from the absorbance at 278 nm and an extinction coefficient of 1.2 A/(mg/mL) (Jacobs et al., 1974). Concentrations of protein in the eluate in Hummel–Dryer studies were determined using a modification of the Bradford procedure (Bradford, 1976). In a 5-cm cuvette, 0.7 mL of sample was mixed with 0.7 mL of a solution made by mixing 50 mL of ethanol, 100 mL of 85% phosphoric acid, approximately 60 mg of Coomassie brilliant blue (Sigma), and water to a volume of 500 mL. The dye concentration was adjusted so that the absorbance of a blank at 595 nm was 1.6, in a 5-cm path length cell. The absorbance change, measured against a water blank, was linear with protein concentration in the range $(1.8–7.1) \times 10^{-8}$ M. The absorbance change when this assay is used is 11.88 A units/nmol.

For column centrifugation studies, guanine nucleotides, both radioactive (New England Nuclear) and nonradioactive (Sigma), were purified by chromatography on PEI-cellulose shortly before use. The materials were eluted with 1 M HCl, absorbed onto charcoal, and desorbed with NH_3–ethanol. After evaporation at 35 °C, the products were stored over a desiccant at −40 °C. The purification was necessary because a preparation of Sigma GDP contained 90.3% GDP, 2.3% GTP, and 7.5% GMP, as determined by thin-layer chromatographic analysis. A batch of [^{3}H]GDP (New England Nuclear) was 15.3% tritiated water, determined by counting aliquots of a distillate of an aqueous solution. Another sample from the same batch of [^{3}H]GDP lost 19% of its radioactivity when this material was evaporated to dryness at 35 °C. A sample of [^{3}H]GTP (New England Nuclear) had 27% of the tritium in the associated water. In the Hummel–Dryer binding studies and the experiment described in Figure 1, the commercial radioactive nucleotide solution was evaporated to dryness at 37 °C prior to use.

Binding of guanine nucleotides to tubulin was studied by using a column centrifugation method with Sephadex G-25 (Penefsky, 1977). Reactions were started by the addition of 50 μL of protein to a nucleotide mixture, and 50 μL of this solution was centrifuged within 1 min. The column centrifugation procedure using 1 cm^3 of packed Sephadex G-25 allows only 0.9% of unbound ligand to elute from the column, as determined from control experiments in the absence of protein. The protein yield is 60% when the tubulin concentration is in excess of 8 μM, determined by centrifugation into tared tubes and protein analysis of the eluate. Reproducibility between duplicate samples was within 5%. Binding studies were carried out at approximately 22 °C (room temperature). The reactions at different nucleotide concentrations were all carried out at the same time so that any minor decay in the tubulin would not influence the results. Also, in extensive control studies of tubulin polymerizability as a function of time

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Table I: Measurement of GDP Binding to Tubulin Using the Hummel-Dryer Technique[a]

gel filtration medium	column GDP (μM)	initial protein (μM)	peak protein (μM)	protein-bound GDP (μM)	GDP-bound/tubulin (mol/mol)
Sephadex G-25	1.49	4.25	0.53	0.52	0.98
	0.597	2.12	0.148	0.131	0.88
	0.224	0.743	0.062	0.0492	0.79
	0.0755	0.363	0.0226	0.0206	0.91
	0.0336	0.165	0	0	
Bio-Gel P-6	0.78	2.64	0.30	0.13	0.44
	0.195	0.88	0.067	0.04	0.59 } av = 0.49
	0.140	0.44	0.036	0.016	0.43
	0.0585	0.147	0.023	0.0075	0.32
	0.00492	0.147	0.043	0.0012	0.028
Bio-Gel P-6	0.00492	0.158	0.034	0.0018	0.053

[a] The nonring fraction obtained from Sepharose 6B chromatography was mixed with a quantity of concentrated [^{3}H]GDP so that the concentration of [^{3}H]GDP was equal to that in the column. For reactions using [^{3}H]GDP concentrations from 1.49 to 0.14 μM, 0.15 mL of protein was passed through a 1 × 12.5 cm column. At lower [^{3}H]GDP concentrations, 0.7 mL was passed through a 1.7 × 12 cm column. Columns were equilibrated with nucleotide solutions containing about 26 000 cpm/mL. The results are averages of duplicate determinations.

stored at 0 °C, it was found that this remains unchanged over the time during which the protein was stored. There is no release of bound nucleotide during column centrifugation, as evidenced by the observation that an identical stoichiometry is obtained when the length and volume of the column were varied by a factor of 2. Also, studies on the time dependence for nucleotide binding to the E site show that binding is complete at the time when column centrifugation is initiated (approximately 30 s at room temperature).

Results

GDP Binding to Tubulin. Tubulin isolated by cycles of polymerization and depolymerization and subsequent Sephadex G-25 chromatography is a mixture of ring aggregates and nonring tubulin protein. These can be separated on Sepharose 6B. The ring aggregates have no E site[1] and the nonring tubulin protein was used in the binding study described here.

Although the Hummel–Dryer procedure is well suited for determination of the nucleotide dissociation constant, it is not most accurate for determining the maximal stoichiometry for binding under saturating conditions. This limitation primarily results from the fact that the very high levels of nucleotide with which the column is equilibrated for saturation studies produce a high background which must be subtracted from the protein–nucleotide peak. In contrast, the column centrifugation procedure (Penefsky, 1977) quantitatively separates protein-bound nucleotide from the unbound nucleotide (see Materials and Methods), so that there is a very low background of unbound nucleotide to be subtracted from the protein–nucleotide eluate. In stoichiometry measurements using column centrifugation with 25–50 μM [^{3}H]GTP and 6 μM tubulin, the stoichiometry for nucleotide binding was found to be 0.83 (range 0.79–0.89, three determinations with two different protein preparations). This nearly unitary stoichiometry shows that the protein we are studying in the following Hummel–Dryer experiments is fully active and the binding constant reflects that of tubulin and not a minor contaminant. The stoichiometries found in the Hummel–Dryer experiments (column 6, Table I) do not appear to be in perfect agreement with those obtained in column centrifugation studies. As discussed above, the column centrifugation values are more accurate. In addition to the high background in the Hummel–Dryer procedure, there are several other factors which account for the inaccuracy. (a) The need for large amounts of tritiated nucleotide for column equilibration did not allow the use of radioactive nucleotide purified by thin-layer chromatography, which gives a low yield. From thin-layer chromatographic analysis, the [^{3}H]GDP used was determined to be about 80% pure; at a later date analysis showed the material to be 70% pure. Results in Table I are not corrected for this. (b) Protein concentrations in the eluted peak are low and there is an approximately 15% error in the protein assay used. In the column centrifugation study, these determinations were much more accurate since the high concentrations of protein allowed determinations of the absorbance at 278 nm. (c) As a result of variability in the Sepharose 6B chromatography, there is some variation in the degree of contamination of the protein by tubulin ring aggregates, which lack an E site.[1] The only effect of the inert ring protein is to decrease the observed stoichiometry.

Hummel–Dryer experiments were done using Sephadex G-25 or Bio-Gel P-6. In experiments using Sephadex G-25 (Table I), the protein recovery is very poor when concentrations were below about 0.3 μM. In the protein concentration range where it is possible to use G-25, the GDP-bound/tubulin (Table I, column 6) did not vary with column GDP concentrations from 1.49 to 0.0755 μM. This means that the dissociation constant for the tubulin–GDP complex is less than 0.0755 μM.

In order to more accurately determine this very low dissociation constant, it was necessary to use Bio-Gel P-6 since better protein recovery was obtained at low protein concentrations. As in the Sephadex G-25 experiments it was found that the GDP-bound/tubulin (Table I, column 6) did not vary with column GDP concentrations from 0.78 to 0.0585 μM. With approximately 10-fold lower column GDP (0.00492 μM), extensive dissociation was observed.

In order to calculate a dissociation constant from this result, we utilized the equilibrium expression

$$K_D = \frac{(\text{tubulin})(\text{GDP})}{(\text{T–GDP})}$$

where (tubulin) is equal to the difference between the total tubulin which is able to bind GDP (at saturating concentrations of GDP) and that tubulin which has GDP bound under the given experimental conditions. The total tubulin which is able to bind GDP (at saturating concentrations of GDP) is equal to the total protein concentration (0.043 μM) times the average stoichiometry of 0.49 (column 6, Table I). Therefore

$$K_D = \frac{[(0.49)(0.043) - 0.0012](0.00492)}{(0.0012)}$$

The dissociation constant for GDP is calculated to be equal

[1] Manuscript submitted for publication.

Table II: Column Centrifugation Determination of the Relative Dissociation Constants for GTP and GDP by a Competitive Binding Assay

tubulin (μM)	endogenous GTP[a] (μM)	added GTP (μM)	total GTP (μM)	endogenous GDP[a] (μM)	added GDP (μM)	total GDP (μM)	tubulin-GTP[b] (μM)	tubulin-GDP[b] (μM)	(A) (free GTP)/(free GDP)	(B) tubulin-GTP/tubulin-GDP	B/A
20.69	5.17	0	5.17	15.52	0	15.52	3.70	7.04	0.17	0.53	3.1
17.74	4.44	0	4.44	13.31	43.04	56.35	1.39	8.05	0.063	0.17	2.7
14.61	3.65	0	3.65	10.96	88.60	99.56	0.674	7.36	0.0323	0.092	2.8
13.79	3.45	17.48	20.93	10.34	83.68	94.02	2.62	4.96	0.20	0.53	2.7

[a] Determined from the titration of phosphocellulose-purified protein (Zeeberg & Caplow, 1978) which shows that there is 1 mol of exchangeable nucleotide per mol of tubulin and that 75% of the exchangeable nucleotide is GDP and 25% is GTP. [b] The amount of bound nucleotide was determined in the column effluent following column centrifugation of a 50-μL aliquot. The values given are averages of duplicate determinations, which agreed within about 5%. The amount of tubulin-GDP in the reactions with 14.61 and 13.79 μM protein was not experimentally determined but was calculated by subtracting the observed tubulin-[^{3}H]GTP concentration from the concentration of the tubulin E site (55% of the total tubulin determined by column centrifugation studies with an excess of added [^{3}H]GDP or [^{3}H]GTP).[1]

to 8.1×10^{-8} M. From the result in the last experiment in Table I, a K_D equal to 4.1×10^{-8} M is calculated; the average from these determinations equals 6.1×10^{-8} M.

Do GDP and GTP Bind to the Same Site? The following two lines of evidence indicate that GDP and GTP bind to the same site; that is, there is no tubulin species which binds GDP or GTP exclusively.

(1) Tubulin purified by phosphocellulose chromatography was mixed with varying amounts of either [^{3}H]GDP, [^{3}H]-GTP, or GDP and [^{3}H]GTP and then analyzed by column centrifugation. In each case it was found that the ratio of (bound GTP)/(bound GDP) was proportional to the ratio of (free GTP)/(free GDP) (Table II, right-hand column). This result is in accord with a model in which GTP and GDP bind competitively, for which the binding equation derived from the two equilibrium expressions for GDP and GTP binding is

$$\frac{\text{bound GTP}}{\text{bound GDP}} = \frac{K_{GDP}}{K_{GTP}} \frac{\text{free GTP}}{\text{free GDP}}$$

These ratios are given in the three right-hand columns of Table II, and the ratio of the dissociation constants for GDP and GTP is equal to 2.8; the dissociation constant for GTP is equal to 2.2×10^{-8} M.

(2) Radioactive guanine nucleotide binding was also studied by using Sephadex G-25 columns (1 × 12 cm) where all of the fractions were collected for radioactivity measurement and the amount of bound nucleotide was determined from the void-volume radioactivity. The stoichiometries for GTP and GDP binding were both equal to 0.5–0.6 mol/mol of phosphocellulose-purified tubulin under saturating conditions (200–500 μM added nucleotide with 25 μM tubulin). When tubulin was incubated with an equimolar mixture (230 μM each) of saturating amounts of GDP and GTP of identical specific activity, the total amount of nucleotide bound was the same as when either of the nucleotides was incubated alone (by using 460 μM nucleotide). This indicates that GDP and GTP bind to the same site; otherwise, an increased amount of bound nucleotide would be expected with the mixture.

Determination of the GDP Dissociation Constant by Exclusion Chromatography. The amount of nucleotide remaining associated with tubulin after passage through a Sephadex G-25 column is theoretically a function of the dissociation constant and other parameters which are either directly measurable or controllable. Such parameters are column geometry, ratio of gel void volume and inner bead volume, protein dilution during passage, and initial concentrations of protein and nucleotide.

An equation (eq 1, Appendix) was developed which allows the calculation of the amount of nucleotide remaining associated with the protein, upon movement through each successive increment of the column, as a function of the dissociation constant. This equation and its derivation are described in the Appendix. Successive evaluations of this equation by a programmable calculator permit a large number of increments to be taken.

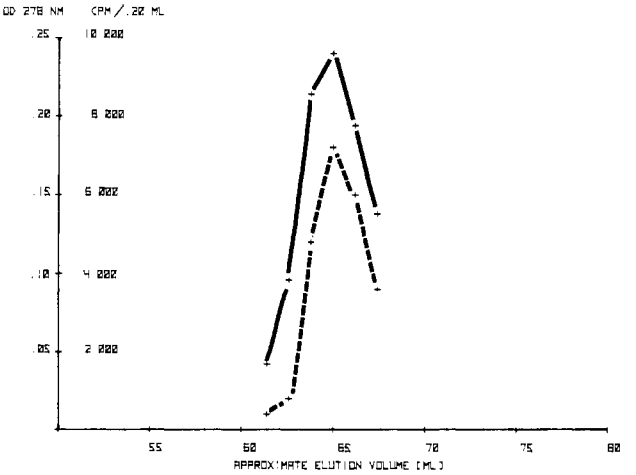

FIGURE 1: Distribution of protein (—) and radioactivity (- - -) eluting in the void volume of a Sephadex G-25 column (80 × 1.7 cm).

A solution (2 mL) containing 6.36 nmol of tubulin (obtained by Sepharose 6B chromatography in RB) and a trace quantity of [^{3}H]GDP was chromatographed at 5 °C on a Sephadex G-25 column in RB (80 × 1.7 cm), with a flow rate of approximately 1 mL/min. Figure 1 shows the distribution of protein and radioactivity eluted in the void volume. The fraction eluting at 64 mL is considered the best fraction for the application of the theoretical treatment, since it is preceded by a fraction containing only 20% as much nucleotide; the theoretical treatment makes the assumption that there is no significant nucleotide left behind by fractions in front of the one upon which the analysis is performed.

Figure 2 summarizes the results of the theoretical calculation of the final ratio of moles of GDP per mole of tubulin as a function of the dissociation constant. Additional calculations with 1272 increments demonstrate that there is no significant change in the final result of a typical calculation when the number of increments is doubled. This shows that the results here reflect those which would be obtained for an infinite number of increments. The penultimate values for GDP/tubulin have been calculated and represent the amount of GDP which would have been associated with the protein had the void volume of the column been 2.0 mL less than that used for Figure 2. There are relatively small differences between any two corresponding values, so that the calculation is not

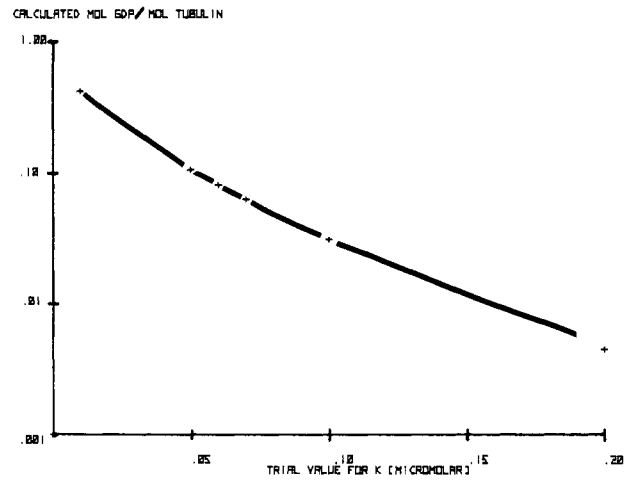

FIGURE 2: Results of a theoretical calculation for moles of GDP per mole of tubulin eluting from a Sephadex G-25 column (80 × 1.7 cm) as a function of trial values for K, the dissociation constant for the tubulin–GDP complex.

subject to unusually large errors as a result of small errors in the determination of the gel volume.

Comparison of the experimental data (Figure 1) and the data in Figure 2 indicates that an interpolated value for K_D of approximately 5.5×10^{-8} M would yield a calculated GDP/tubulin ratio similar to the experimental value of 0.090. This value for K_D is in excellent agreement with that obtained by the Hummel–Dryer procedure.

Discussion

Theoretical and Practical Considerations Using the Hummel–Dryer Procedure with Tubulin for Determining K_D for GDP. The Hummel–Dryer procedure permits the relatively rapid determination of dissociation constants under equilibrium conditions. Alternate procedures such as equilibrium dialysis are too slow or involve exposure of the protein–ligand complex to nonequilibrium conditions, as in the filtering and washing phases of filter binding assays. The following theoretical and practical considerations should be taken into account when the Hummel–Dryer procedure is used.

(1) The fraction of the protein which has nucleotide bound is independent of the protein concentration and is only dependent upon the GDP concentration in the column. This can be seen from consideration of the equilibrium expression

$$K = \frac{(\text{GDP})(\text{tubulin})}{(\text{tubulin–GDP})}$$

Since (GDP) remains constant throughout the column, this equation can be rewritten as (GDP)/K = (tubulin–GDP)/(tubulin). Therefore, the ratio of bound to free tubulin is a function only of (GDP). As a result, the progressive protein dilution which occurs during column passage is without consequence. In contrast, in the absence of the infinite reservoir of (GDP) provided by the column

$$K = \frac{(\text{GDP})_{\text{initial}} - (\text{tubulin–GDP})(\text{tubulin})}{(\text{tubulin–GDP})}$$

and the fraction of the protein which has nucleotide bound is no longer independent of protein concentration.

(2) The nucleotide concentration in the column must not significantly be decreased as a result of binding to eluted protein, so that (1) will remain valid. This requires that the total amount of GDP in the column be very large relative to the amount of eluted bound GDP. The total column nucleotide/protein ratio must be high when the nucleotide concentration exceeds K_D and the eluted protein is saturated with nucleotide but can be low when this concentration is below K_D and the protein is only partially saturated.

The amount of nucleotide removed from the column buffer by binding to the protein in the experiments reported here (Table I) is not a significant fraction of the total column nucleotide, even at the lowest column nucleotide concentrations. For example, in the experiment using 0.00492 μM column nucleotide and 0.158 μM protein, the total column GDP was 0.107 nmol [(27.2 mL of column bed volume × 0.8 mL of buffer) per (mL of column volume × 0.00492 nmol/mL)]. The amount of nucleotide eluting with the protein was equal to (0.158 nmol of tubulin per mL)(0.7 mL of tubulin applied to the column)(0.53 nmol of GDP eluted per nmol of tubulin) = 0.0059 nmol of GDP. This is only 5.5% of the total column nucleotide.

(3) Tubulin isolated without added guanine nucleotide contains a significant amount of endogenous exchangeably bound GDP, whose presence can cause experimental errors: the protein solution applied to the Hummel–Dryer column had high specific activity [^{3}H]GDP added so that the counts per minute per milliliter was equal to that for the nucleotide solution with which the column was equilibrated (if the specific activity of the nucleotide in the protein solution had been adjusted to be equal to that of the column nucleotide, then there would have been more counts per minute per milliliter associated with the protein than with the column buffer, so that an artifactual peak of radioactivity above the background eluting with the protein could result). However, this resulted in different nucleotide specific activities for column nucleotide as compared with endogenous protein nucleotide. This difference could pose a problem, since it is necessary for the original specific activity of the GDP in the lower portion of the column to remain unchanged, because this is the nucleotide which coelutes with the tubulin, is in equilibrium with the bound GDP, and whose specific activity is used to calculate K_D. In fact, the specific activity in the lower portion of the column is unchanged in the experiments where the column nucleotide concentration was less than the subsequently determined K_D and the protein concentration was also low, since the upper portion of the column was shown to deplete the protein of endogenous exchangeably bound GDP. A solution (0.7 mL) containing 0.158 μM tubulin and a trace quantity of [^{3}H]GDP was applied to a 1.7 × 6 cm Bio-Gel P-6 column (half the length of the columns used in the Hummel–Dryer binding studies) equilibrated with RB. The [^{3}H]GDP per nanomoles of eluted tubulin was less than 5% of the initial value. It can be concluded that the initial endogenous exchangeably bound GDP does not significantly mix with nucleotide in the lower portion of the column. However, in experiments where column nucleotide and protein concentrations are high, loss of endogenous protein nucleotide in the top half of the column is less efficient, so that the total amount of column nucleotide has to be in sufficient excess to withstand the diluting effect of the lower specific activity endogenous protein nucleotide.

(4) The percentage rise of radioactivity over base-line level is inversely proportional to the ratio of column GDP concentration to the eluted protein concentration, so that within the limitations of the above three considerations, it is necessary to minimize the column GDP concentration.

By use of the Hummel–Dryer procedure with the above considerations taken into account, a K_D equal to 6.1×10^{-8} M has been determined for GDP (Table I). From the ob-

servation that GTP binds 2.8-fold more tightly than GDP (Table II), the dissociation constant for GTP is calculated to be 2.2×10^{-8} M.

Do GDP and GTP Bind to the Same Site? Earlier studies (Arai et al., 1975; Jacobs & Caplow, 1976; Levi et al., 1976; Maccioni & Seeds, 1977; MacNeal et al., 1977; Karr & Purich, 1978) have not established whether GDP and GTP bind to the same site or whether there is a tubulin subspecies which binds GDP or GTP only. Results obtained when the column centrifugation technique was used (Table II) show that GDP and GTP bind to the same site and there is no subspecies which has a specificity for one of the nucleotides. This is concluded from the observation that the ratio of (bound GTP)/(bound GDP) is proportional to the ratio of (free GTP)/(free GDP). The same conclusion is derived from the observation that an identical amount of radioactivity is found associated with the protein when the protein was chromatographed on Sephadex G-25 after exposure to an equimolar mixture (230 μM each) of GDP and GTP of identical specific activity as when either of the radioactive nucleotides was incubated alone (by using 460 μM nucleotide).

Determination of the GDP Dissociation Constant by Exclusion Chromatography. An equation (eq 1, Appendix) has been derived which allows the calculation of the amount of ligand remaining associated with a protein, upon movement through successive increments of a gel filtration column, as a function of the protein–ligand dissociation constant. By use of the results from an experiment in which tubulin was mixed with [^{3}H]GDP and chromatographed through an 80×1.7 cm Sephadex G-25 column (Figure 1), a K_D equal to 5.5×10^{-8} M is estimated from Figure 2. This value is in excellent agreement with that obtained by the Hummel–Dryer procedure. This agreement is taken to indicate the general utility for use of eq 1 for determining dissociation constants from gel filtration results.

The relationship between the ligand dissociation constant and ligand loss accompanying exclusion chromatography has previously been theoretically analyzed (Dixon, 1976). However, this analysis does not account for the progressive dilution of the protein during column passage.

Also, the derivation appears to be incorrect. The basic problem seems to stem from an inconsistency in the way that y is defined in the two expressions: $-ca\delta y = [L]\delta v$ and $[L] = Ky/(1 - y)$. In the first expression δy refers to the change in the total amount of ligand not yet left behind, and δy, therefore, should refer to bound and free ligand. However, in the second expression, y refers to the amount of bound ligand, not total ligand.

In general, one cannot equate the decrement in the amount of bound ligand with the decrement in the amount of total ligand. For example, in a hypothetical solution containing 5 μM total protein and an initial 5 μM total ligand, which has a dissociation constant equal to 1 μM, the calculated[2] protein–ligand concentration is 3.2087 μM and the calculated free ligand concentration is 1.7913 μM. When the total ligand concentration is decreased by 1%, to 4.95 μM (simulating a small decrement in a gel filtration experiment), the protein–ligand concentration is 3.1890 μM and the free ligand concentration is 1.7610 μM. The decrement in the total ligand concentration is 0.0500 μM, in the bound ligand concentration is 0.0197 μM, and in the free ligand concentration is 0.0303 μM. Similar calculations show that an even larger fraction of the decrement in total ligand concentration is constituted of the decrement in the free ligand concentration when the total ligand concentration has been substantially reduced (simulating a later point in the gel filtration process). These calculations show that it is incorrect to use the decrement in bound ligand as an approximation for the decrement in total ligand.

Analysis of Earlier Determinations of Tubulin–Nucleotide Dissociation Constants. (a) Arai et al. (1975) used a nitrocellulose filter binding assay to quantitate the amount of [^{3}H]GTP or [^{3}H]GDP bound to tubulin in reaction mixtures containing a fixed nucleotide to tubulin ratio equal to 10, at varying dilutions. The recovery of bound radioactive nucleotide was very low (43% for GTP and 19% for GDP), necessitating a large correction. The amount of [^{3}H]GTP which was bound did not vary significantly at the different protein concentrations reported, making the dissociation constant difficult to calculate. More important, for the conditions used, we derive the analytical expression[3] for the dissociation constant:

$$K_T = \frac{0.1(\text{GTP})^2}{(\text{tubulin–GTP})} - (\text{GTP})$$

However, Arai et al. (1975) incorrectly calculated the dissociation constants from a plot of $[(\text{tubulin–GTP})(\text{volume})]^{-1}$ vs. $(\text{GTP})^{-1}$. This equation cannot be rearranged into the form of a linear double-reciprocal plot.

(b) In a study using the Hummel–Dryer method (Jacobs & Caplow, 1976), the calculated GTP binding constant is incorrect because the results at low nucleotide concentrations were obtained under conditions where the amount of nucleotide bound to the eluted protein was sufficiently great so as to deplete the column nucleotide to a significant extent. Also, the apparent extent of nucleotide binding was influenced by poor recovery of tubulin, as a result of its binding to the Sephadex G-25, which was most significant at lower protein concentrations.

(c) In another study using the Hummel–Dryer method a dissociation constant for GTP equal to 0.4 μM was reported (Levi et al., 1974). Since virtually no decrease in the amount of GTP bound was seen over the very limited (0.5–2.0 μM) concentration range used, it is not clear as to how a dissociation constant may be calculated from the results.

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[2] These values are calculated from the equilibrium expression

$$K = \frac{(\text{free protein})(\text{free ligand})}{(\text{protein–ligand})} = \frac{(\text{total protein} - \text{protein–ligand})(\text{total ligand} - \text{protein–ligand})}{(\text{protein–ligand})}$$

[3] Under conditions where a 10-fold excess of GTP is added to tubulin and varying dilutions of this mixture are made, the conservation equations given in (a) and (b) hold.

$$(\text{T})_{\text{total}} = (\text{T}) + (\text{T–GTP}) = 0.1(\text{GTP})_{\text{total}} \quad \text{(a)}$$

$$(\text{GTP})_{\text{total}} = (\text{GTP})_{\text{free}} \quad \text{(b)}$$

In eq b the contribution of T·GTP to the $(\text{GTP})_{\text{total}}$ is assumed to be negligible, since a 10-fold excess of GTP is maintained. Substituting into the equilibrium expression

$$K = \frac{(\text{T})(\text{GTP})_{\text{free}}}{(\text{T–GTP})} = \frac{[(\text{T})_{\text{total}} - (\text{T–GTP})](\text{GTP})_{\text{total}}}{(\text{T–GTP})}$$

$$K = \frac{(\text{T})_{\text{total}}(\text{GTP})_{\text{total}} - (\text{T–GTP})(\text{GTP})_{\text{total}}}{(\text{T–GTP})}$$

$$K = \frac{(\text{T})_{\text{total}}(\text{GTP})_{\text{total}}}{(\text{T–GTP})} - (\text{GTP})_{\text{total}}$$

Substituting from eq a yields

$$K = -(\text{GTP})_{\text{total}} + \frac{0.1(\text{GTP})_{\text{total}}(\text{GTP})_{\text{total}}}{(\text{T–GTP})}$$

(d) The initial rate of polymerization of 51 μM tubulin with 10–2000 μM GTP was reported to closely follow Michaelis–Menten type kinetics (Maccioni & Seeds, 1977). However, the Michaelis–Menten analysis requires a large excess of substrate over protein at all substrate concentrations used, which was not the case here. Furthermore, the concentration of GTP necessary for half-maximal velocity (87 μM) was incorrectly assumed to be equal to the dissociation constant; a kinetically determined K_m is only equal to a dissociation constant under special conditions (Fersht, 1977).

(e) It has been reported that the rate of polymerization of 10 μM tubulin is half-maximal with 0.25 μM GTP (MacNeal et al., 1977). The 0.25 μM value was incorrectly taken as an estimate of the dissociation constant for GTP; again there is no reason to expect the kinetic dependence on the nucleotide concentration to parallel the concentration dependence for nucleotide binding.

(f) From measurements of fluorescence quenching, a dissociation constant for GTP has been reported to be equal to 8×10^{-7} μM (Karr & Purich, 1978). However, it was not demonstrated that the maximal fluorescence quenching was associated with the binding of 1 mol of nucleotide to tubulin; this was arbitrarily assumed to be the case. If the maximal fluorescence quenching is associated with saturation of a minor component with nucleotide, then the reported dissociation constant is not related to the properties of the tubulin E site.

Appendix

Derivation of the Equation for Calculating the Amount of Nucleotide Remaining Associated with the Protein, upon Movement through an Increment of an Exclusion Column

The equilibrium equation

$$\frac{(T)(G)}{(T\text{–}G)} = K_D$$

where T = free tubulin, T–G = bound GDP, G = GDP unbound, T + T–G = T_T = total tubulin, K_D = GDP dissociation constant, and G + T–G = G_T = total GDP can be rewritten for our experimental conditions as

$$K_D = \left(\frac{\text{nmol of T}}{2\text{ mL}}\right)\left(\frac{\text{nmol of G}}{4.66\text{ mL}}\right)\Big/\left(\frac{\text{nmol of T–G}}{2\text{ mL}}\right) = \left(\frac{\text{nmol of } T_T - \text{nmol of T–G}}{2\text{ mL}}\right) \times \left(\frac{\text{nmol of } G_T - \text{nmol of T–G}}{4.66\text{ mL}}\right)\Big/\left(\frac{\text{nmol of T–G}}{2\text{ mL}}\right)$$

where (a) K_D will be expressed in micromolarity and (b) it is explicitly taken into account that the protein occupies only the void volume, but unbound GDP occupies the sum of void volume and inner-bead volume. The value of 4.66 mL for this sum is calculated from data (Fisher, 1969) for water regain and bed volume per gram dry weight of resin.

This equation becomes

$$(4.66\text{ mL})(K_D)(\text{nmol of T–G}) = (\text{nmol of } T_T - \text{nmol of T–G})(\text{nmol of } G_T - \text{nmol of T–G})$$

or

$$0 = (\text{nmol of T–G})^2 - (\text{nmol of T–G})[(\text{nmol of } T_T) + (\text{nmol of } G_T) + (4.66\text{ mL})(K_D)] + (\text{nmol of } T_T)(\text{nmol of } G_T)$$

Solving for bound GDP (nmol of T–G) by the quadratic formula

$$\text{nmol of T–G} = 1/2[\text{nmol of } G_T + \text{nmol of } T_T + (4.66\text{ mL})K_D - [[\text{nmol of } G_T + \text{nmol of } T_T + (4.66\text{ mL})K_D]^2 - 4(\text{nmol of } T_T)(\text{nmol of } G_T)]^{1/2}] \quad (1)$$

The quantities nmol of T–G and nmol of T_T refer to the number of nanomoles in 2.0 mL of void volume; nmol of G_T refers to the sum of nmol of T–G in 2.0 mL of void volume plus nmol of G in 4.66 mL of void volume and associated inner-bead volume.

We now calculate the amount of total nucleotide (G_T) remaining in the 4.66 mL (corresponding to 2 mL of void volume plus 2.66 mL of related inner-bead volume) of solution associated with the bulk of the protein moving down the column during chromatography. The amount remaining will depend on the extent to which the protein has penetrated the column. Although the sample can be considered to undergo an infinite number of equilibrations with the column inner-bead volume during passage through the column, for the purpose of this calculation we attribute 636 0.1-mL void-volume increments and equilibrations during passage; the results so obtained are in good agreement with results obtained with many more (1272) increments of 0.05 mL.

The amount of total nucleotide in 4.66 mL remaining at the nth 0.1-mL void-volume increment, $(\text{nmol of } G_T)_n$, is equal to the amount of total nucleotide remaining at the $(n-1)$th 0.1-mL increment, $(\text{nmol of } G_T)_{n-1}$, minus the amount of unbound nucleotide lost to the inner-bead volume; since the inner-bead volume corresponding to a 0.1-mL void-volume increment is equal to 0.133 mL [= 0.1 mL[(4.66 – 2.00)/2.00]], at each increment there is lost 2.85% (= 0.133/4.66) of the unbound nucleotide. Therefore

$$(\text{nmol of } G_T)_n = (\text{nmol of } G_T)_{n-1} - 0.0285(\text{nmol of G})_{n-1}$$

By definition

$$(\text{nmol of } G_T)_{n-1} = (\text{nmol of G})_{n-1} + (\text{nmol of T–G})_{n-1}$$

so that, by substitution

$$(\text{nmol of } G_T)_n = (\text{nmol of } G_T)_{n-1} - 0.0285[(\text{nmol of } G_T)_{n-1} - (\text{nmol of T–G})_{n-1}]$$

$$(\text{nmol of } G_T)_n = 0.9715(\text{nmol of } G_T)_{n-1} + 0.0285(\text{nmol of T–G})_{n-1} \quad (2)$$

This value of $(\text{nmol of } G_T)_n$ so calculated and the value for $(\text{nmol of } T_T)_n$ were corrected for the observed protein dilution (see below) and then substituted into eq 1 to calculate $(\text{nmol of T–G})_n$ for use in the successive calculation of $(\text{nmol of } G_T)_{n+1}$ by eq 2. The correction for protein dilution was carried out as

$$\text{corrected } (\text{nmol of } G_T)_n = (\text{nmol of } G_T)_n(\text{dilution factor})$$

$$\text{corrected } (\text{nmol of } T_T)_n = \text{corrected } (\text{nmol of } T_T)_{n-1}(\text{dilution factor})$$

The dilution factor was determined as follows. Assuming that the protein undergoes dilution in each increment by a constant fraction of the existing concentrations, then

$$\text{final concentration} = (\text{initial concentration})(\text{dilution factor})^{636}$$

$$(\text{dilution factor})^{636} = \frac{\text{final concentration}}{\text{initial concentration}}$$

$$636 \ln(\text{dilution factor}) = \ln\frac{\text{final concentration}}{\text{initial concentration}}$$

$$\text{dilution factor} = \exp\left(\frac{\ln\dfrac{\text{final concentration}}{\text{initial concentration}}}{636}\right)$$

Finally, the amount of GDP associated with the eluted tubulin was taken to be equal to the expression

$$(\text{nmol of T–G})_{636} + \frac{2.00}{4.66}[(\text{nmol of G}_T)_{636} - (\text{nmol of T–G})_{636}]$$

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Nuclear Magnetic Resonance Studies of the Binding of Trimethoprim to Dihydrofolate Reductase†

P. J. Cayley, J. P. Albrand,‡ J. Feeney,* G. C. K. Roberts, E. A. Piper, and A. S. V. Burgen

ABSTRACT: The resonances of the aromatic protons of trimethoprim [2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine] in its complexes with dihydrofolate reductases from *Lactobacillus casei* and *Escherichia coli* cannot be directly observed. Their chemical shifts have been determined by transfer of saturation experiments and by difference spectroscopy using [2′,6′-$^2H_2$]trimethoprim. The complex of 2,4-diamino-5-(3′,4′-dimethoxy-5′-bromobenzyl)pyrimidine with the *L. casei* enzyme has also been examined. At room temperature, the 2′,6′-proton resonance of bound trimethoprim is very broad (line width >30 Hz); with the *E. coli* enzyme, the resonance sharpens with increasing temperature so as to be clearly visible by difference spectroscopy at 45 °C. This line broadening is attributed to an exchange contribution, arising from the slow rate of "flipping" about the C7–C1′ bond of bound trimethoprim. The transfer of saturation measurements were also used to determine the dissociation rate constants of the complexes. In the course of these experiments, a decrease in intensity of the resonance of the 2′,6′-proton resonance of free trimethoprim on irradiation at the resonance of the 6 proton of free trimethoprim was observed, which only occurred in the presence of the enzyme. This is interpreted as a nuclear Overhauser effect between two protons of the *bound* ligand transferred to those of the *free* ligand by the exchange of the ligand between the two states. The chemical shift changes observed on the binding of trimethoprim to dihydrofolate reductase are interpreted in terms of the ring-current shift contributions from the two aromatic rings of trimethoprim and from that of phenylalanine-30. On the basis of this analysis of the chemical shifts, a model for the structure of the enzyme–trimethoprim complex is proposed. This model is consistent with the (indirect) observation of a nuclear Overhauser effect between the 2′,6′ and 6 protons of bound trimethoprim.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. The enzyme is of considerable pharmacological interest as the target for the "anti-folate" drugs. One of these, trimethoprim (I), is an effective antibacterial agent, selectively inhibiting bacterial dihydrofolate reductase by virtue of binding up to 50 000 times more tightly to the bacterial enzyme than it does to the mammalian enzyme (Hitchings & Burchall, 1965).

As part of a wider study aimed at understanding the factors determining inhibitor binding to dihydrofolate reductase, we have been using high-resolution NMR spectroscopy to study

† From the Division of Molecular Pharmacology (P.J.C., J.P.A., J.F., G.C.K.R., and A.S.V.B.) and Computer Science Laboratory (E.A.P.), National Institute for Medical Research, Mill Hill, London NW7 1AA, England. *Received March 8, 1979.*

‡ Present address: Laboratoire de Chimie Organique Physique, Centre d'Etudes Nucleaires de Grenoble, 85XF 38041 Grenoble Cedex, France.

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