Magnesium-Induced Self-Association of Calf Brain Tubulin. II. Thermodynamics†

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ABSTRACT: The thermodynamic parameters of the magnesium ion induced self-association of calf brain tubulin in pH 7.0, 0.01 *M* phosphate buffer containing 10^{-4} *M* GTP, were determined from sedimentation velocity experiments. This reaction proceeds by an isodesmic mechanism terminated by the highly favored formation of a closed ring shaped polymer of degree of association 26 ± 4. Analysis of the variation of the apparent dimerization constant in the isodesmic mechanism shows that this self-association is characterized by positive enthalpy, entropy, heat capacity, and molar volume changes, as well as the binding of one additional magnesium ion, which is probably not involved as a bridge between the protein molecules. The addition of the last monomeric subunit has a free energy which is about three times that of dimer formation. Under the conditions of these experiments, tubulin binds 48 ± 5 magnesium ions with a free energy of −2.8 kcal/mol.

In the preceding paper (Frigon and Timasheff, 1975), it was shown by velocity sedimentation studies that the stoichiometry of the magnesium-induced self-association of calf brain tubulin at pH 7.0 in PG buffer[1] is satisfactorily described by the formation of a progressive series of aggregates, which is terminated by a polymer with most probable degree of association $n = 26$, all species being in rapid equilibrium with the 110,000 molecular weight monomer.[2] The intrinsic association constants of the consecutive steps in the association, k_i, were found to be equal up to $n = 25$. The formation of the highest aggregate with $n = 26$, which is best described by a closed ring structure, is highly favored, indicating that $k_{26} > k_i$. In this paper the thermodynamics of this reaction will be described.

Materials and Methods

Calf brain tubulin was purified, and the experimental samples were prepared as described in the preceding paper (Frigon and Timasheff, 1975). All ultracentrifuge experiments were performed as before and the data were analyzed by the methods described in the preceding paper.

Magnesium Binding. The binding of magnesium ions to tubulin was examined in (PG)[1] buffer at 25.00 ± 0.05° by two different methods: (a) titration of tubulin solutions with $MgCl_2$ while measuring free Mg^{2+} ion activity with a divalent cation-specific ion-exchange electrode; (b) measuring free Mg^{2+} ion concentration by the method of Smith (1955) in solutions from which the protein had been sedimentated out by high-speed centrifugation.

The average number of Mg^{2+} ions bound to tubulin, $\bar{\nu}$, was calculated, as the difference between observed free Mg^{2+} concentration and the total Mg^{2+} added. The data were expressed as $\bar{\nu}$ vs. $-\ln a_{Mg^{2+}}$; $a_{Mg^{2+}}$ is the $MgCl_2$ ion pair activity (Robinson and Stokes, 1959).

Results

Free Energy of the Self-Association. Using the model deduced for the self-association of tubulin and described in the preceding paper (Frigon and Timasheff, 1975), a semiempirical approach to the measurement of the free energy of interaction between tubulin molecules was developed. At low protein concentrations, the self-association can be treated almost exclusively as a linear indefinite association in which the initial dimerization constant of the reaction defines the standard free energy of interaction between each *n*-mer and monomer of the equilibrium mixture (Van Holde and Rosetti, 1967; Adams and Lewis, 1968). Therefore, the thermodynamic properties of the progressive self-association have been examined exclusively in terms of an apparent dimerization constant, K_2^{app}. This parameter was calculated from the weight-average sedimentation velocity of the boundary. At higher protein concentrations, at which the end-product polymer appears in significant amounts, the last step of the polymerization was treated in independent manner, as a monomer–*n*-mer equilibrium.

In this way, the self-association of calf brain tubulin in the presence of magnesium ions has been successfully analyzed in terms of a progressive isodesmic association up to degree of polymerization, $n = 25$, with identical association constants $k_2 = k_3 = \cdots = k_i = \cdots = k_{24} = k_{25}$. The addition of the last monomer to form the 26-mer proceeds with an association constant $k_{26} > k_i$, the reaction being terminated by this step. In the preceding paper, the sedimentation velocity data were characterized by a combination of the proper hydrodynamic parameters, the dimerization constant, K_2, and an overall polymerization constant K_n (for the reaction $26A \rightleftharpoons A_{26}$). These thermodynamic parameters of tubulin polymer formation are presented in

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[1] Abbreviation used is: PG, buffer consisting of 0.01 *M* sodium phosphate at pH 7.0, and containing 1×10^{-4} *M* GTP.

[2] It should be emphasized, at the risk of introducing minor confusion, that in the analysis of the data, the tubulin dimer, molecular weight 110,000, will be referred to as the "monomer" of the self-association reaction, unless indicated otherwise.

Table I: Free Energy of Tubulin 42S Polymer Formation at 20°.

$C_{Mg^{2+}}$ (M)	k_2', fitted (M^{-1})	ΔG_2° (kcal/mol)	k_p', fitted (M^{-25})	ΔG_p° (kcal/mol)	γ	ΔG_γ° (kcal/mol)	ΔG_R
0.008	1.23×10^4	−5.5	2.27×10^{109}	−147	1.3×10^7	−9.5 ± 3	−2.5 ± 3
0.016	1.83×10^4	−5.7	1.76×10^{115}	−155	4.8×10^8	−11.6 ± 3	−4.4 ± 3

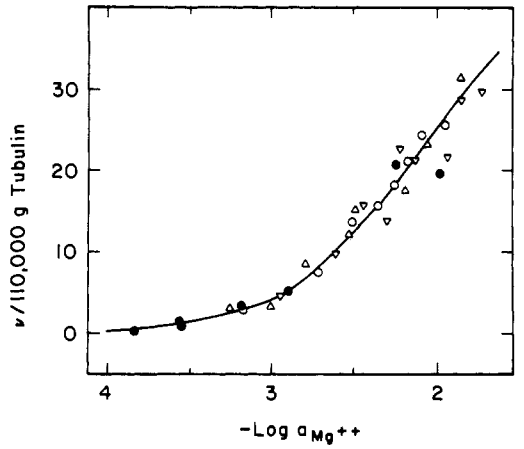

FIGURE 1: Binding isotherm for the interaction of Mg^{2+} with tubulin. The line through the data was calculated by using parameters obtained from the Scatchard plot. (○, ▽, △) Points obtained from separate experiments by continuous titration; (●) points obtained from individual solutions after sedimentation of the protein.

Table I. The equilibrium constants, k_i', are reported in units of $(l./mol)^{i-1}$. These are related to K_i, in units of $(ml/mg)^{i-1}$, by

$$k_i' = \frac{K_i M_1^{i-1}}{i} \quad (1)$$

where M_1 is the monomer molecular weight (110,000). It should be noted that the dimer formation constant, k_2', is equal to k, the intrinsic association constant for the incorporation of additional monomeric subunits into the growing polymer chain. The isodesmic mechanism is valid for the addition of all monomeric subunits, including the step $A_{25} + A \rightleftharpoons A_{26}$, as long as ring closure does not occur. The free energy of the formation of the closed ring polymer from tubulin dimers, ΔG_p°, can be expressed as

$$\Delta G_p^\circ = -RT \ln (k_2^{n-1}\gamma) \quad (2)$$

where n is 26 and $-RT \ln \gamma$ is the additional free energy of converting an open-ended n-mer to a closed ring polymer in which all of the n subunits are in an identical local environment; i.e., it is the free energy of formation of the extra bond needed to close the ring, without transfer of a new particle from solution to the polymer. It is equal to

$$\Delta G_\gamma = \Delta G_b + \Delta G_R \quad (3)$$

where ΔG_b is the free energy of forming the bond and ΔG_R is the additional free energy associated with the overall effect of ring closure on the polymer.

The free energy of forming the last bond, ΔG_b, can be related to the free energy of dimer formation, ΔG_2, by taking into account the statistical factor associated with the loss of freedom of ways in which the contact may be made, and the fact that the closing of the last bond does not entail the loss of entropy associated with the decrease in the number of particles in solution each time that an additional monomer molecule is added to the chain. During chain extension, each step can occur at either end of the chain and either site on the added monomer, giving a statistical factor of 4. The entropic term is equal within a close approximation (Aune, K. C., and Timasheff, S. N., unpublished) to $RT \ln (x_n/x_{n-1}x_1)$, where x_i is the mole fraction of species i. Then, at unit molality of reacting components

$$\Delta G_\gamma = \Delta G_2 - RT \ln \frac{x_n}{x_{n-1}x_1} + RT \ln 4 + \Delta G_R \quad (4)$$

Setting $n = 26$, and introducing the values of ΔG_p° and ΔG_2° from Table I into eq 2 results in $\Delta G_\gamma^\circ = -10.6 \pm 3$ kcal/mol. Then, by eq 4, $\Delta G_R = -3.5 \pm 3$ kcal/mol; i.e., ring closure may be accompanied by a gain of an additional small increment of favorable free energy. Such additional free energy need not be regarded as indicative of the difference between the last step and the chain-building steps, either in the mechanism of bond formation or the number of water molecules released. It can be fully accounted for in terms of additional entropic considerations. A free energy change of −3.5 kcal/mol corresponds to an entropy increase of 12 eu. The ring-closing step does not entail the loss of translational entropy involved in the addition of each monomeric subunit to the growing chain, which for a tubulin dimer can be estimated as 60 entropy units (Glasstone, 1946). This should be compensated, however, by the loss of the configurational entropy of the open structure (Steinberg and Scheraga, 1963). While the net effect of nonspecific entropy changes is derived from the difference between rather uncertain large numbers, these considerations support the conclusion that the nature of the intermolecular contacts formed in the last bond are most probably identical with those of the other 25 intersubunit bonds.

Effect of Magnesium on the Self-Association. Binding. The results of the Mg^{2+} ion binding measurements at pH 7.0, 25°, are presented in Figure 1, where the observed number of Mg^{2+} ions bound per 110,000 g of tubulin, $\bar{\nu}$, has been plotted as a function of the negative logarithm of Mg^{2+} activity, $a_{Mg^{2+}}$. Analysis of the data according to the Scatchard equation

$$\frac{\bar{\nu}}{a_{Mg^{2+}}} = k'(n - \bar{\nu}) \quad (5)$$

resulted in a straight line fit through the points indicating that magnesium ions are bound to tubulin at n independent binding sites with identical binding constants, k' (Klotz, 1953). Although the binding of Mg^{2+} to tubulin involves interaction between charged species in solution, no electrostatic corrections appear to be necessary, and the apparent contant, k', is a good measure of the intrinsic binding constant (Klotz, 1953; Tanford, 1963). The values obtained were $n = 48 \pm 5$ and $k' = 106 \pm 5$ l./mol, giving a standard free energy of binding of Mg^{2+} to tubulin at pH 7.0, 25°, of −2.8 kcal/mol.

Using these parameters, the binding isotherm was calculated with the expression

Table II: Dependence of the Dimerization of Tubulin on Magnesium Ion Concentration.

$C_{Mg^{2+}}$ (M)	$a_{Mg^{2+}}$[a]	Fitted K_2^{app} (ml/mg)	k_2^{app}[b] (l./mol)	ΔG° (kcal/mol)
0.005	0.0061	0.140 ± 0.001	0.75×10^4	−5.2
0.008	0.0092	0.224 ± 0.001	1.23×10^4	−5.5
0.010	0.0112	0.276 ± 0.001	1.52×10^4	−5.6
0.016	0.0164	0.472 ± 0.001	2.60×10^4	−5.9

[a] Mean ionic activity of $MgCl_2$. [b] Taking 110,000 for the molecular weight of the monomer.

$$\bar{\nu} = \frac{nk'a_{Mg^{2+}}}{1 + k'a_{Mg^{2+}}} \quad (6)$$

It is shown as the solid line in Figure 1. The good agreement obtained supports the conclusion that a large number of magnesium ions are weakly bound to tubulin. There is no evidence for a small number of very tightly bound magnesium ions, although these would be very difficult to detect in the presence of the large nonspecific binding.

Self-Association. The effect of Mg^{2+} concentration on the self-association of tubulin was calculated from the data of Figure 4b of the preceding paper. The values of the deduced dimerization constants are given in Table II, and the $\bar{s}_{20,w}$ vs. concentration curves calculated from these parameters are compared with the experimental points in Figure 2. It is seen that the agreement is good.

Assuming that the observed equilibrium contant is purely a dimerization constant with no secondary reactions, such as conformational changes, the dependence of K_2^{app} on the solvent variables at constant temperature and pressure can be expressed as (Aune et al., 1971)

$$\mathrm{d}\ln K_2^{app} = \left(\frac{\partial \ln K_2^{app}}{\partial \ln a_{H^+}}\right)_{a_{H_2O},a_x} \mathrm{d}\ln a_{H^+} + \left(\frac{\partial \ln K_2^{app}}{\partial \ln a_{H_2O}}\right)_{a_{H^+},a_x} \mathrm{d}\ln a_{H_2O} + \left(\frac{\partial \ln K_2^{app}}{\partial \ln a_x}\right)_{a_{H^+}a_{H_2O}} \mathrm{d}\ln a_x \quad (7)$$

where a_{H^+} is the hydrogen ion activity, a_{H_2O} is the activity of water, and a_x is the activity of other solute species. If the activities of buffer components are invariant, x = Mg^{2+} (as $MgCl_2$). Then, at constant pH, eq 7 may be rewritten as (Wyman, 1964; Tanford, 1969)

$$\frac{\mathrm{d}\ln K_2^{app}}{\mathrm{d}\ln a_{Mg^{2+}}} = \Delta\bar{\nu}_{Mg^{2+}} - \frac{n_{Mg^{2+}}}{n_{H_2O}}\Delta\bar{\nu}_{H_2O} \equiv \Delta\bar{\nu}_p \quad (8)$$

where $n_{Mg^{2+}}/n_{H_2O}$ is the ratio of the number of moles of Mg^{2+} to the number of moles of water present in the system.

A plot of the data of Table II according to eq 8 gave a straight line with a slope, $\Delta\bar{\nu}_p = 1.15 \pm 0.15$. Thus, if dimerization does not entail a change in the number of water molecules interacting with tubulin, the formation of each tubulin–tubulin contact is accompanied by the binding of one additional magnesium ion. On the other hand, if it is assumed that $\Delta\bar{\nu}_{Mg^{2+}} = 0$, i.e., that the entire observed effect is due to the release of water from the protein, then, at the experimental $MgCl_2$ concentrations ($\sim 10^{-2}$ M), eq 8 would require the leaving of ca. 5×10^3 molecules of water in the formation of each tubulin–tubulin contact. This is totally unreasonable. The leaving of 100–200 molecules of water, which is a reasonable value for the formation of, for example, hydrophobic contacts, would make a contribution of at most 0.05 to $\Delta\bar{\nu}_p$. It seems, therefore, most likely that the dimerization reaction is accompanied by the binding of one additional Mg^{2+} ion. This binding is, however, not necessarily an integral part of the association mechanism. It could simply be the consequence of the change in the electrostatic free energy of the protein which must accompany the dimerization reaction, or of the formation of a new liganding site when two tubulin monomers come into contact.

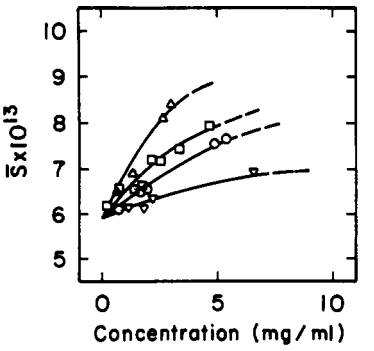

FIGURE 2: Effect of Mg^{2+} on the weight-average sedimentation coefficient of tubulin as a function of protein concentration, in PG buffer, pH 7.0, 20°. The points are the experimentally determined $\bar{s}_{20,w}$, and the lines are least-squares fits to the data. The Mg^{2+} ion concentrations are: (△) 0.016; (□) 0.010; (○) 0.008; (▽) 0.005 M.

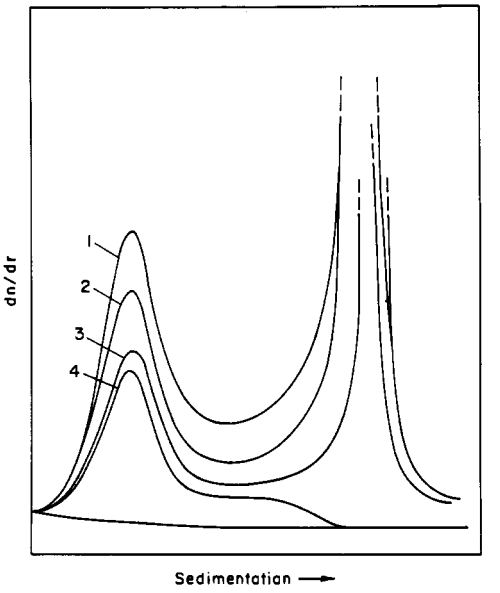

FIGURE 3: Sedimentation velocity profiles of tubulin in PG, pH 7.0, 20°, 0.016 M $MgCl_2$, as a function of temperature. The speed was 48,000 rpm. The sedimentation times, temperatures, and protein concentrations are given as follows: (1) 33 min, 5.5° (the curve for 10° nearly indistinguishable), 20 mg/ml; (2) 21 min, 20°, 20 mg/ml; (3) 17 min, 30°, 19 mg/ml; (4) 13 min, 37°, 5 mg/ml.

Effect of Temperature. The sedimentation velocity behavior of tubulin, in pH 7.0 PG buffer containing 0.016 M $MgCl_2$, was examined as a function of temperature between 5.5 and 37°. The observed sedimentation patterns are shown in Figure 3. The area under the slow peak in the reaction boundary decreases with an increase in temperature indicating an enhancement of self-association at higher temperatures. At each temperature, the protein concentration dependence of the sedimentation patterns was that of a Gilbert system. The dependence of the sedimentation velocities of the peaks on protein concentration, measured at dif-

Table III: Temperature Dependence of the Dimerization of Tubulin in the Presence of 0.016 *M* $MgCl_2$.[a]

T (°K)	Fitted K_2^{app} (ml/mg)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)	$\Delta S_u°$ (eu)
303.15	0.355 ± 0.001	−5.92	4.6	35	43
293.15	0.282 ± 0.001	−5.59	3.2	30	38
283.15	0.248 ± 0.005	−5.32	1.8	25	33
278.65	0.233 ± 0.001	−5.21	1.1	23	31

[a] ΔC_p = 135 ± 45 cal/deg.

ferent temperatures, gave $s_{20,w}$ vs. C curves similar to those of Figure 4 of the preceding paper.

From these results, the dimerization constant, K_2, was calculated by using the weight-average sedimentation coefficients of patterns at concentrations below the onset of bimodality. The values of K_2 (in ml/mg) are presented in the second column of Table III.

The van't Hoff plot for this system was found to have a pronounced curvature; therefore, the data were fit to the equation

$$\ln K_2^{app} = a + b(1/T) + c \ln T \qquad (9)$$

which is a truncated form of the integrated van't Hoff equation (Glasstone, 1947). The values of the free energy, $\Delta G°$, the enthalpy, $\Delta H°$, the entropy, $\Delta S°$, and the heat capacity, ΔC_p, changes in the dimerization reaction are given by

$$\Delta G° = -RT \ln K_2^{app}$$
$$\Delta H° = R(cT - b)$$
$$\Delta S° = (\Delta H° - \Delta G°)/T$$
$$\Delta C_p = Rc \qquad (10)$$

The resulting values of the thermodynamic parameters are presented in Table III, where $\Delta S_u°$ is the unitary entropy change (Gurney, 1953; Kauzmann, 1959). As can be seen, the formation of a tubulin dimer from two 110,000 molecular weight "monomers" in the presence of magnesium ions is characterized by a positive enthalpy change, a positive entropy change, and a small positive change in heat capacity.

Effect of Pressure. If self-association is accompanied by a change in molar volume, and if the end product is large, the formation of polymer becomes a sensitive function of pressure. In an ultracentrifuge cell, the equilibrium constant, $K(P)$, of polymer formation at any pressure P_x, corresponding to position x in the cell, is related to the equilibrium constant under reference conditions (usually atmospheric pressure), $K(0)$, by (Harrington and Kegeles, 1973)

$$\ln K(P) = \ln K(0) - \frac{1}{RT}\int_{x_0}^{x} \Delta V \left(\frac{\partial P}{\partial x}\right)_T dx \qquad (11)$$

where ΔV is the molar volume change of the reaction for the formation of 1 mol of polymer, P is the pressure, and x_0 is the position of the meniscus in a centrifuge cell.

In the case of a self-associating system, a positive molar volume change results in polymer dissociation which increases progressively from the meniscus to the bottom of the centrifuge cell. This leads to negative gradients of polymer toward the bottom of the cell and convective disturbances within the solution are expected in the plateau region below the boundary (Kegeles, 1970, Harrington and Kegeles, 1973).

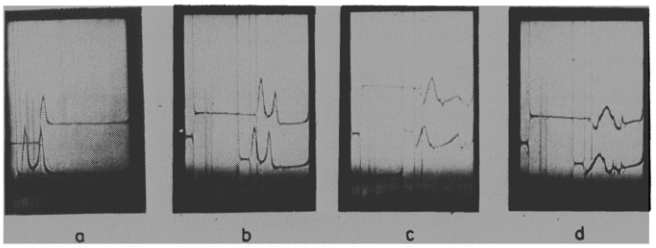

FIGURE 4: Effect of changing rotor speed on the sedimentation of tubulin. Frame a shows the sample of protein at 5.6 mg/ml (standard cell) and 1.1 mg/ml (wedge cell) after 5 min of sedimentation at 48,000 rpm. Aliquots of the same stock solution, diluted to 4.6 mg/ml into both cells, are shown in the successive photos, ending at the right. The cumulative sedimentation times after reaching a speed of 40,000 rpm are given with the speed at which the record was made: (b) 6 min (40,000); (c) 13.5 min (after acceleration to 60,000); (d) 19 min (after deceleration to 40,000). The solutions were in PG at pH 7 and 20° with 0.016 *M* Mg^{2+} and 0.02 *M* NaCl. About 1 mm of oil was layered over the standard cell sample and about 6 mm of oil over the wedge cell sample. The bar angle was 60°.

Such pressure effects on an association reaction can usually be detected by changes in the qualitative nature of the sedimentation velocity profiles induced either by varying rotor speed or by increasing the pressure at the meniscus of the solution by overlaying a mineral oil (Josephs and Harrington, 1967, 1968). Furthermore, a strong pressure effect results in a positive concentration gradient throughout the cell with the result that the schlieren pattern does not return to the base line both in the region between the peaks and in that centrifugal to the rapidly sedimenting peak. In fact, it can be expected to slope upward toward the bottom of the cell in both of these regions (Josephs and Harrington, 1968).

In order to test whether the self-association of tubulin is affected by pressure, experiments were carried out in which the hydrostatic pressure over the protein solution was varied by layering varying amounts of oil over it. The results, shown in the first two frames of Figure 4, indicate that the effect is weak. In frame a, the bottom pattern shows the biomodality characteristic of 42S polymer formation. It should be noted, that the schlieren pattern essentially returns to the base line centrifugally to the rapid peak, even though, at the experimental speed, 48,000 rpm, the pressure at the bottom of the cell is 200 atm. Layering of 0.1 and 0.6 cm of oil over identical protein solutions, shown as the lower and upper patterns of frame b, did not produce any major changes in the appearance of the pattern. In the presence of the thicker layer of oil (at higher pressure), however, the area distribution under the peaks shows relatively more slowly sedimenting material than in the solution at lower pressure. Rapid acceleration to 60,000 rpm (frame c) resulted in severe convection in the cell, as did subsequent rapid deceleration to 40,000 rpm (frame d). These effects may be attributed to increasing pressure with increasing speed, and vice versa, with the concomitant changes in the equilibrium distribution of species in the self-associating system, although such convection may be induced by the rapid acceleration and deceleration processes themselves.

In order to obtain a more quantitative picture of the dependence of tubulin self-association on pressure, experiments were carried out in which two aliquots of a protein solution were centrifuged simultaneously, with the overlaying of 0.6 cm of oil over one sample. At 48,000 rpm, this

corresponds to applying a pressure of 84 atm at the meniscus. The increase in pressure resulted in a redistribution of area under the boundary profiles toward slower moving material, indicating that the self-association of tubulin is accompanied by a positive molar volume change. Although an exact calculation of ΔV is not possible without knowledge of the effect of pressure on each equilibrium parameter, a reasonable estimate could be made, using the observation that, under all conditions examined, the self-association of tubulin displays a pseudo-Gilbert character (Cann, 1970). Therefore, if a change in pressure does not induce changes in the mechanism or stoichiometry of the reaction, identical sedimentation patterns should correspond to identical association constants, and a direct comparison of patterns should be sufficient to obtain a good estimate of the latter. In this way, in experiments carried out in 0.016 M $MgCl_2$ and 0.02 M NaCl at 20°, it was possible to estimate intrinsic dimerization constants of 1.54×10^4 l./mol (no oil) and 1.27×10^4 l./mol (0.6 cm of oil). The magnitude of the change in molar volume which accompanies self-association was calculated from these values of k_i. The uncertainty in these values is actually not critical, since the change in partial specific volume, $\Delta\bar{v}$, which accompanies polymer formation, is calculated from the difference between the logarithms of the equilibrium constants:

$$\Delta\bar{v} = -\frac{RT}{M_P}\frac{[\ln K(P) - \ln K(0)]}{P_x} \qquad (12)$$

where M_P is the molecular weight of the polymer, and P_x is the pressure at position x in the cell. For the formation of tubulin dimer, M_P = 220,000, these equilibrium constants led to a value of $\Delta\bar{v}$ of approximately 2.5×10^{-4} ml/g. For the formation of 1 mol of a 2.86×10^6 molecular weight polymer (n = 26), this would amount to a total volume change of ca. 700 ml. At 48,000 rpm, $\Delta\bar{v} = 2.5 \times 10^{-4}$ ml/g results in a pressure effect at 0.5 cm centrifugally to the meniscus of [log $k_{26}'(0)$ − log $k_{26}'(P)$] = 1.1. Since at 20°, in the presence of 0.016 M $MgCl_2$, in PG buffer, k_{26}' is 1.76×10^{115} (l./mol)^{25}, an error by one power of ten introduced by the pressure effect falls essentially within the limits of uncertainty of the analysis used and does not introduce an uncertainty of more than 1% in the free energy of polymer formation. Because of the uncertainties in this analysis, however, the value of $\Delta\bar{v} = 2.5 \times 10^{-4}$ ml/g should be regarded as approximate. Doubling this value would not affect seriously the present analysis. In the case of myosin polymerization, Josephs and Harrington (1967) found that a similar change in $\bar{v}$ did have a strong effect on the sedimentation patterns. This can be attributed to the difference in molecular weight of the polymer in the two systems, that in myosin being ten times greater than that of the tubulin polymer. As shown by eq 12, the effect of pressure on the equilibrium constant of a self-associating system augments by ten orders of magnitude for each order of magnitude of increase of the molecular weight of the polymer. It appears, therefore, that the self-association product of tubulin is just not sufficiently large to be very seriously affected by an increase in pressure at the conditions used. On the other hand, such changes in $\bar{v}$ are sufficient (Timasheff et al., 1975) to account for the reported depolymerization of microtubules under pressure (Inoue, 1952; Tilney et al., 1966).

Discussion

The magnesium-induced self-association of calf brain tubulin at pH 7.0 can be described reasonably well by the simple model of an isodesmic association to a degree of polymerization, n = 26, followed by a ring-closing step with additional favorable free energy. The individual steps in the self-association proceed with an identical association constant, k_i, equal within a close approximation to the dimerization constant, k_2. The final unimolecular ring closing step equilibrium constant, γ, is related to k_2 by

$$\gamma = 14.1 k_2 k_R \qquad (13)$$

Since k_R has a value of ~400, the addition of the last monomeric subunit with the closing of the ring has a free energy which is almost three times that of the addition of a subunit to the growing chain. As a result, the formation of the final aggregate in closed ring form is highly favored thermodynamically and the polymerization reaction is driven to the end step.

In an analysis of the thermodynamics of the simplest, dimer formation step, it was shown that the reaction proceeds with positive enthalpy, positive entropy, positive molar volume, and small positive heat capacity changes. The changes in the first three thermodynamic parameters are consistent with a mechanism of self-association which involves the release of water molecules. This could be the result of either the formation of hydrophobic contacts or of electrostatic interactions (Timasheff, 1973). The latter does not seem very likely, since the self-association of tubulin does not show any simple dependence on the ionic strength of the medium, but various ions seem to affect it in a complicated manner (Frigon, 1974). The positive heat capacity change, however, is inconsistent with a mechanism involving the release of water molecules. It may be related to a conformational change or to ligand binding. Ventilla et al. (1972) have reported that the circular dichroism spectrum of porcine brain tubulin between 220 and 240 nm changes with a change in temperature. It is not known whether the same is true of the calf brain protein.

In a self-association reaction, if the monomer exists in a state of equilibrium between two conformational states and only one can dimerize, the equilibrium constant between the two states of the protein, A and A*, must be considered if the method of determining the association constant, k_a^{app}, involves measurement of the mass distribution of the species (Timasheff and Townend, 1968), as is the case with sedimentation experiments. The same is true if ligand binding is involved. Taking the case of dimerization, the two consecutive reactions may be written as

$$A + X \overset{k_1}{\rightleftharpoons} A^*X$$

$$2A^*X \overset{k_2}{\rightleftharpoons} A_2{}^*X_2 \qquad (14)$$

where X is a ligand. The apparent dimerization constant is then

$$k_2{}^{app} = \frac{[A_2{}^*X_2]}{[A + A^*X]^2} = \frac{k_2 k_1{}^2[X]^2}{(1 + k_1[X])^2} \qquad (15)$$

and

$$\Delta H_2{}^{app} = \Delta H_2 + \frac{2\Delta H_1}{1 + k_1} \qquad (16)$$

It can be shown that, with $\Delta C_{p,2}$ (the true change in heat capacity of dimer formation) negative, $\Delta C_{p,2}{}^{app}$ will assume a positive value only upon satisfaction of the following condition:

$$\Delta C_{p,1} - \frac{(1+k_1)}{2}\left[\frac{2k_1}{(1+k_1)^2}\frac{\Delta H_1^2}{RT^2} - \Delta C_{p,2}\right] > 0 \quad (17)$$

where $\Delta C_{p,1}$ is the heat capacity change of the first step.

Equations similar to 14–17 can be applied directly to simpler cases, in which either a conformational change or ligand binding is required for self-association, by simply omitting from them the feature which does not apply. When the two reactions are linked, however, k_1 takes on the character of an apparent constant and the relation between intrinsic constants becomes more complicated (Tanford, 1961; Timasheff et al., 1966).

Turning to the effect of magnesium ions, it is not possible to state at present whether the additional ligand, bound on association, is involved in the intermolecular bond. The free energy of binding of magnesium to tubulin, −2.8 kcal/mol, is too weak to generate ligand concentration gradients across the sedimenting reaction boundary (Cann, 1970; Cann and Goad, 1972) and to disturb the Gilbert character of the patterns. On the other hand, application of eq 14 and 15 to tubulin dimerization, with k_1 = 106 l./mol and k_2^{app} values taken from column 4 of Table II, results in a $\Delta G_2°$ value of −6.5 kcal/mol, independent of magnesium ion concentration. This calculation, while strongly implicating magnesium ions in the mechanism of tubulin self-association, nevertheless does not establish their direct participation in interprotein bond formation. It is equally probable that magnesium acts either through an alteration of the charge distribution on the surface of the protein molecules or through an induction, or enhancement, of a conformational change required for self-association. This question is at present under investigation in our laboratory.

The validity of the present analysis requires that the nature of the sedimentation patterns be controlled neither by the redistribution of ligand during the course of sedimentation, nor by the kinetics of the reaction (Cann and Kegeles, 1974). That the first criterion is met has been shown above. The second requirement seems to be satisfied by the rapid reequilibration of the system whenever perturbed.

A further potential complication in the use of sedimentation velocity for the study of self-associating systems with large end products is the pressure dependence of the equilibrium. An approximate analysis of this effect, reported in this paper, has shown that, in the present case, the effect is not sufficiently large to affect seriously the analysis of the data, although a small uncertainty is introduced into the final thermodynamic parameters deduced.

Finally, one last remark is necessary about the reproducibility of the results. During the course of these studies, the quantitative behavior of the protein often varied from one preparation of tubulin to another, although the qualitative character of the observed effects remained unaltered. Within single preparations of the protein, tubulin behaved reproducibly; therefore, each of the reported studies, e.g., effect of magnesium ion concentration or effect of temperature, was carried out on a single preparation of the protein. The results, within each of these studies, are, thus, self-consistent and the reported variations of the equilibrium constants with changes in environment valid, as is the hydrodynamic analysis. The absolute values of the equilibrium constants are, however, subject to an uncertainty. Comparison of dimerization constants deduced from the most divergent preparations of tubulin lead to an estimated uncertainty of ±0.5 kcal/mol in the free energy of dimer formation. While it would be desirable to eliminate this uncertainty, rigorous control of a variety of procedures and operating conditions within the laboratory did not lead to any improvement in reproducibility. Efforts are continuing in this direction; there is no possible control, however, over the source of the protein—namely, over the age, breed, and feeding history of the animals processed at the slaughterhouse.

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Nonequivalence of the Metal Binding Sites in Vanadyl-Labeled Human Serum Transferrin†

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ABSTRACT: Vanadyl ion, VO(IV), has been used as an electron paramagnetic resonance (EPR) spin label to study the metal-binding properties of human serum transferrin in the presence of bicarbonate. Iron-saturated transferrin does not bind the vanadyl ion. Room temperature titrations of apotransferrin with VO(IV) as monitored by EPR indicate the extent of binding to be pH dependent, with a full 2.0 VO(IV) ions per transferrin molecule bound at pH 7.5 and 9, but only about 1.2 VO(IV) ions bound at pH 6. The EPR spectra of frozen solutions with or without 0.1 *M* $NaClO_4$ at 77 K show that there are two spectroscopically nonequivalent binding sites (A and B) with a slight difference in binding constants. One site (A site) exhibits essentially constant binding capacity in the pH range 6-9, but the other (B site) becomes less available as the pH is reduced below 7. Results with mixed Fe(III)-VO(IV) transferrin complexes suggest that iron shows a slight tendency to bind at the B site over the A site at pH 7.5 and 9.0. Only the B site in both vanadyl and iron transferrins is perturbed by the presence of perchlorate.

Human serum transferrin is an Fe(III) transport protein of about 80,000 molecular weight. It gives up ferric ions to bone marrow and placental tissues in preference to other cells such as liver cells (Morgan, 1972, and references cited therein). The protein is capable of binding two irons per molecule but an anion such as bicarbonate (in biological systems) or oxalate must be present for binding to occur (Aisen et al., 1967; Price and Gibson, 1972a). It has been shown that HCO_3^- is bound stoichiometrically to transferrin in a 1:1 ratio with Fe(III) (Aisen et al., 1969).

Fletcher and Huehns (1967) and Harris and Aisen (1975) have shown that the two sites differ in their ability to donate iron to reticulocytes. Aisen et al. (1973) also report that two different rate constants are needed to describe the exchange of bound CO_3^{2-} during dialysis. Additional evidence for nonequivalence of the sites is the report by Luk (1971) that transferrin binds a full two rare earth ions per protein molecule for the smaller ions Tb^{3+}, Eu^{3+}, Er^{3+}, and Ho^{3+}, but smaller amounts for the ions with larger ionic radii, such as Nd^{3+} and Pr^{3+}.

Circular polarization of luminescence has failed to show any differences in the ligand fields of the two sites of the protein when Tb^{3+} is bound (Gafni and Steinberg, 1974). Solution calorimetry has yielded values of ΔH for addition of the first and second moles of iron to transferrin which are the same within experimental error (Binford and Foster, 1974). EPR experiments and magnetic susceptibility measurements with Fe(III), Co(III), Cu(II), and Mn(III) bound to transferrin in frozen solutions have not demonstrated the presence of more than one type of binding (Aasa and Aisen, 1968; Aisen et al., 1969). However, a careful reexamination of the Fe(III) EPR spectrum suggests that differences do exist between the two metal sites (Aasa, 1972). In the case of Cr(III), EPR spectra indicate the presence of two metal environments. The lines attributed to these species lose spectral identity in a sequential manner when Fe(III) displaces the Cr(III) (Aisen et al., 1969).

Price and Gibson (1972b) have observed that addition of $NaClO_4$ to Fe(III) transferrin produces changes in the EPR spectrum of frozen solutions of the protein suggestive of changes in one site per protein molecule. The relatively sharp peak characteristic of the perchlorate-free species decreases in intensity as $NaClO_4$ is added, and a broader band with a similar *g* value grows in.

Fourier transform ^{13}C nuclear magnetic resonance (NMR) of Fe-transferrin in the presence of $K_2{}^{13}CO_3$ suggests that the anion actually bound may be CO_3^{2-} rather than HCO_3^- (Harris et al., 1974). Other anions reported to bind in place of bicarbonate include oxalate, nitrilotriacetate, and ethylenediaminetetraacetate (Aisen et al., 1967).

The vanadyl(IV) ion, VO^{2+}, has been used to probe metal binding sites in a number of proteins (Chasteen et al., 1973; Fitzgerald and Chasteen, 1974a,b; DeKoch et al., 1974; Francavilla and Chasteen, manuscript in preparation). The success of this probe is due in part to the fact that its EPR spectrum consists of relatively sharp lines which make it possible to distinguish small differences in the spectroscopic *g* and *A* parameters which reflect differences in the metal ion environment. In addition, vanadyl(IV) species exhibit reasonably well-resolved room temperature solution spectra thus avoiding some of the inherent difficulties of

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