Tubulins are evolutionarily conserved proteins that reversibly polymerize and direct intracellular traffic. Of the tubulin family members, only αβ-tubulin forms stable dimers. We investigated the monomer-dimer equilibrium of rat brain αβ-tubulin using analytical ultracentrifugation and fluorescence anisotropy, observing tubulin in virtually fully monomeric and dimeric states. Monomeric tubulin was stable for a few hours and exchanged into preformed dimers, demonstrating reversibility of dimer dissociation. Global analysis combining sedimentation velocity and fluorescence anisotropy yielded $K_d = 84 \text{ (54–123) nM}$. Dimer dissociation kinetics were measured by analyzing the shape of the sedimentation boundary and by the relaxation of fluorescence anisotropy following rapid dilution of labeled tubulin, yielding $k_{\text{off}}$ in the range $10^{-3}$–$10^{-2}$ s$^{-1}$. Thus, tubulin dimers reversibly dissociate with moderately fast kinetics. Monomer- monomer association is much less sensitive than dimer-dimer association to solution changes (GTP/GDP, urea, and trimethylamine oxide).

Microtubules are a major component of the eukaryotic cytoskeleton, playing an essential role in cell motility, intracellular transport, and cell division. Microtubules are cylindrical polymers formed by assembly of αβ-tubulin heterodimers (hereafter “tubulin dimers”). The microtubule cytoskeleton is subject to fine regulation of its architecture and dynamics. Factors regulating dynamics are classified into stabilizers/promoters such as some microtubule-associated proteins or destabilizers such as stathmin or katanin (1–3). Microtubule function can be regulated via post-translational modifications of the tubulin subunits, such as acetylation, C-terminal deetyrosination, polyglutamylation, and polyglycylation (4). Gene expression is also involved in regulation of the microtubule cytoskeleton, as multiple genes for the α- and β-subunits have been found in eukaryotes (5). For instance, dimer formation is regulated in vivo by sorting of β-tubulin isoforms that exhibit distinct affinities for α-tubulins (6). The maintenance of cytoplasmic levels of tubulin dimers is dependent on the balance between subunit degradation and assisted folding of tubulin subunits, followed by dimer formation. Steps in this pathway involve interactions with chaperonin containing TCP-1 (also termed TRiC) and with tubulin folding and tubulin-binding cofactors A to C, respectively (7–9). Mutations in tubulin that are responsible for several developmental diseases (often called “tubulinopathies”), map to the secondary structural elements implicated in the intradimer contacts, some of them conferring resistance to anticancer drugs (10, 11). All these regulatory mechanisms are thought to produce heterogeneity in pools of cytoplasmic tubulin thereby increasing microtubule diversity (12). Thus, regulation of tubulin function occurs both at the level of dimer formation as well as in microtubule polymerization.

The α- and β-tubulins are members of the tubulin/FtsZ superfamly of proteins (13–15), whose eukaryotic and prokaryotic members share the ability to bind guanine nucleotides and participate in the formation of linear polymers. Among these proteins, only α- and β-tubulins form dimers that are stable in isolation. Others may form a dimer as a step in the polymerization reaction, such as BtubA/B (16), or may polymerize directly from the monomer state to a polymeric state, such as FtsZ (17), but these transient dimeric species are not populated in dilute solutions. In the latter case, the intersubunit contact surfaces are all alike, whereas in the former the surfaces that form dimers are slightly different from those that allow dimers to associate to form protofilaments. In the case of α- and β-tubulins, the monomer-monomer (intradimer) contact surfaces are significantly more extensive compared with the dimer-dimer (interdimer) longitudinal contact surfaces (2, 18), so that αβ-tubulin heterodimers are stable in non-polymerizing conditions.

In the presence of GTP and at 37 °C, the tubulin dimers cooperatively polymerize into microtubules with a critical concentration that can vary greatly depending on solution conditions (19, 20). Investigations have also shown that the tubulin dimer itself is in reversible equilibrium with its α- and β-subunits, in a reaction not coupled to polymerization into microtubules. The reported values for the dissociation constant of the dimer, $K_{\text{d}}$, vary by more than 2 orders magnitude in the range 0.002–2 μM (21–29). However, one study inferred a significantly lower value ($K_{\text{d}} = 10^{-11}$ M), reporting a slow rate of tubulin dimer...

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dissociation \( k_{\text{off}} \sim 10^{-5} \text{ s}^{-1} \) (29). Most of these studies used
brain tubulin obtained from different mammals, meaning that the
protein preparations contained multiple tubulin isoforms as
well as different post-translational modifications (5, 30). A
study of tubulin dissociation (as well as polymerization) using
ubin from other sources and/or with different post-transla-
tional modifications would be useful in understanding the role
of post-translational modifications and multiple isoforms in
tubulin function and more specifically in the stabilization of
the dimer. These considerations led us to revisit the question of
mammalian brain tubulin dimer dissociation, exploiting im-
proved biophysical techniques that are now available.

In this study, we used sedimentation velocity (SV)\(^3\) and fluo-
rescence anisotropy (FA) to characterize the thermodynamics and
kinetics of the tubulin dimer dissociation. Sedimentation
velocity-analytical ultracentrifugation (SV-AUC) is a versatile
and rigorous methodology for the study of protein-protein
interactions (31). Recent advances in theoretical and computa-
tional tools for solving the sedimentation-diffusion-reaction
process occurring in the analytical ultracentrifuge, described by
the Lamm equation (LEq) (32), make the SV-AUC technique
capable of characterizing the thermodynamics and kinetics of
protein-protein interactions free in solution (33). Application
of SV to high affinity interactions is greatly facilitated by new
instrumental and analytical techniques that have extended the
detection limits of SV into the picomolar range for the
detection and analysis of fluorescently labeled molecules (34,
35). Furthermore, we applied new global multimethod anal-
ysis techniques (36) to combine the results of SV with com-
plementary FA data. We have combined these methods to
study tubulin at an unprecedentedly low concentration and
over an unprecedentedly wide range of concentrations, dem-
onstrating reversible submicromolar dimer dissociation with
moderately fast kinetics and revealing a stable tubulin
monomer state.

Experimental Procedures

Tubulin Preparation and Fluorescent Labeling—Rat brain
tubulin was purified from total microtubule protein as
described previously (37). Concentrated tubulin (\(\sim 250 \mu M\))
was stored at \(-80^\circ\text{C}\) and kept on ice during sample prepara-
tion. The storage buffer was PM (0.1 M Pipes-KOH, pH 7, 1 mM
MgCl\(_2\)) and was used as the working buffer unless otherwise
specified. For fluorescent labeling, 1 mg of tubulin was polyme-
erized at 30 °C in a total volume of 200 \(\mu L\) in PM buffer + 1 mM
GTP, with addition of 1 mM trimethylamine oxide (TMAO) to
promote polymerization (38). After 15 min of equilibration, the
mixture was adjusted to pH 8 by adding a 20% volume of 1 M
sodium borate, pH 8.7 (except for pyrene maleimide where the
pH was kept neutral). The NHS esters dyes (amine-reactive)
or pyrene maleimide (thiol-reactive) were dissolved in DMSO and
added to a 10-fold molar excess relative to tubulin concentra-
tion and incubated for \(\sim 1\) h at 30 °C. The mixture was centri-
fuged at 100,000 \(\times g\) for 15 min to harvest the polymerized
protein. The microtubules were dissolved in cold PM buffer
and centrifuged again to remove insoluble aggregates. The poly-
merization cycle was repeated once, and the final soluble tubu-
lin was passed through a gel filtration micro Bio-Spin P6 col-
umn (Bio-Rad) previously equilibrated in cold PM buffer. The
protein concentration and labeling ratio were determined spec-
trophotometrically using the following extinction coefficients:
\[ \text{tubulin} = 112.155 \ (\text{M cm})^{-1} \] at 276 nm (39); DyLight-488 NHS ester = 70,000 \(\text{(M cm)}^{-1}\) at 493 nm; Dylight-550 NHS ester = 150,000 \(\text{(M cm)}^{-1}\) at 562 nm; and pyrene maleimide = 30,000 \(\text{(M cm)}^{-1}\) at 345 nm. Labeling ratios were 40 and 20% for DyLight-
488 (tubulin-488) and Dylight-550 (tubulin-550), respectively,
and 80% for pyrene maleimide (tubulin-345). In all cases, 50–60% of the starting protein material was recovered. An inde-
pendent estimation of protein concentration was made
employing the Bradford assay with BSA as the calibration
standard.

Sedimentation Velocity Analytical Ultracentrifugation—Sed-
imentation velocity experiments were conducted in Optima
XL-A analytical ultracentrifuges (Beckman Coulter, Indianap-
olis, IN) equipped with absorbance optics or with a fluores-
cence detection system with a 10-milliwatt laser emitting at 488
nm (AVIV Biomedical, Lakewood, NJ). SV experiments were
carried out at 50,000 rpm in an 8-hole An-50 Ti rotor at 20 °C,
using 400 \(\mu L\) of samples in 12-mm double-sector charcoal-filled
Epon centerpieces, using standard procedures as described in
more detail elsewhere (40). To accommodate a large number of
samples in absorbance experiments, data were acquired in the
intensity mode (41). Tubulin mixtures were prepared by dilut-
ing the stock solution to reach the desired protein concentra-
tion, allowing for \(\sim 2\) h of temperature and chemical equilibra-
tion prior to sedimentation. Unlabeled tubulin was detected
using absorbance at 230 nm for concentrations below 2 \(\mu M\) or at
280 nm at concentrations above 2 \(\mu M\). Labeled tubulin was
detected with fluorescence optics setting the focal depth to 4
\(\mu M\). The density and viscosity of PM buffer at 20 °C were mea-
sured, respectively, using a densitometer model DMA 5000 M
and a microviscometer model AMVn (both from Anton Paar
Inc., Ashland, VA), resulting in density (\(\rho\)) = 1.0138 g/ml and
viscosity (\(\eta\)) = 1.0881 cP; the partial specific volume (\(\nu_{\text{bar}}\)) of
tubulin was set to 0.736 ml/g (42). The density and viscosity of
PM buffer + 0.3 \(M\) urea were (\(\rho\)) = 1.01867 g/ml and viscosity
(\(\eta\)) = 1.1003 cP, and those of PM + 0.3 \(M\) TMAO were (\(\rho\)) =
1.0143 g/ml and viscosity (\(\eta\)) = 1.1604 cP. These values were used
to calculate the \(s_{20, w}\) of tubulin samples.

Analysis of Sedimentation Velocity Data Using the c(s) Distribu-
tion Model—SV data analyses were carried out in SEDFIT
software (version 14.4), using the built-in continuous sedimen-
tation coefficient distribution model \(c(s)\) (43). For absorbance-
detected SV data, the distribution was discretized with a grid of
100 to 300 \(s\) values between 0 and 20 to 25 \(S\) (dependent on the
presence of traces of rapidly sedimenting material, with
an additional small discrete component to account for buffer sig-
nal at 230 nm. For diffusion deconvolution, a scaling law for
compact particles was used with the average frictional ratio
allowed to refine in the fits, along with the meniscus position.
For pseudo-absorbance analysis, we accounted for the time-

\(^3\)The abbreviations used are: SV, sedimentation velocity; FA, fluorescence
anisotropy; AUC, analytical ultracentrifugation; TMAO, trimethylamine
oxide; r.m.s.d., root-mean-square deviation; GMMA, global multimethod
analysis; cP, centipoise; LEq, Lamm equation.

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and radius-invariant noise. Maximum-entropy regularization of the \( c(s) \) model was set to a confidence level of 0.68. In the case of fluorescence data, files were sorted using SEDFIT tools, mainly to remove randomly occurring defective scans, and then initially analyzed with the standard \( c(s) \) model. For high intensity samples (with high signal-to-noise ratio), the built-in fluorescence tools were summoned, as described before (44), to account for imperfections in the alignment of the fluorescence optics, temporal drifts in the signal, and excitation beam shadowing at the bottom of the cell.

The best-fit \( c(s) \) distribution model was judged based on minimization of the root-mean-square deviation of the fit (r.m.s.d.), and on the shape of the residuals plot, both reported by SEDFIT software. The ratio of the r.m.s.d. to the total loading signal (r.m.s.d./total loading signal) was expressed as a percentage to indicate the level of statistical noise in the detection of the SV data (percentage noise ratio). For instance, a 100% noise ratio indicates that the sedation boundary is of the same amplitude as the noise of data acquisition, whereas for 1% noise ratio (or lower), the sedation boundaries are clearly seen in the SV data. The values of \( s_{20,w} \) were obtained by integration of \( c(s) \) distributions that fit the data well and therefore accurately describe the mass balance (31). A consistent integration range from 2 to 8 S was chosen to account for the monomer and dimer species. The 68% confidence intervals associated with \( s_{20,w} \) were determined by \( F \)-statistics considering variations in the meniscus position over the quality of the best-fit \( c(s) \) distribution in SEDFIT. This was achieved by searching the range of values of the meniscus position that worsen the \( \chi^2 \) of the fit by a critical factor set to a confidence level of 68% and by assuming that the ratio of the reduced \( \chi^2 \) of two fits is distributed according to a Fisher distribution (\( F \)-statistics). For isotherm analysis, the parameters describing tubulin dimer dissociation equilibrium were determined in SEDPHAT (version 10.58), by nonlinear regression. The confidence intervals of the best-fit parameters were obtained by searching the range of values of \( s_{20,w} \) or \( K_d \) that caused a relative increase in the reduced \( \chi^2 \) of the fit, by a critical factor set to a confidence level of 68%, and by assuming that the ratio of the \( \chi^2 \) of two fits is distributed like a Fisher distribution (\( F \)-statistics). The high resolution plots of SV data were obtained with the software GUSSI 1.0.8 (45).

**Fluorescence Anisotropy Measurements**—Steady-state fluorescence anisotropy was recorded in an ISS PC1 spectrofluorimeter (ISS, Champaign, IL). For \( L \)-form anisotropy measurements, the left-side detector was set in photon counting configuration with a calculated \( G \)-factor between 1.03 and 1.05. T-format anisotropy measurements allowed a 2-fold increase in data collection rate. In this configuration, the left-side detector was used as for \( L \)-format measurements, and the right-side detector was used in photon counting mode with the monochromator set to zero order position. The calculated \( G \)-factor in these conditions was between 7.5 and 8.5. For samples containing tubulin-488, the excitation wavelength was 480 nm (8-nm bandpass), and the fluorescence emission was collected through a 530-nm long-pass filter to block scattered light. For tubulin-345, the excitation was set at 345 nm (8-nm bandpass), and the emission was collected employing a 400-nm long-pass filter. In the dilution experiments, the samples were prepared at the desired protein concentration, by dilution, and equilibrated at the working temperature previous to each measurement. In dilution experiments where different solvent conditions were examined, the dilution buffer was replaced by the desired solution. Titration experiments consisted of the addition of increasing concentrations of unlabeled tubulin into a “tracer monomer solution” of labeled tubulin dissolved in PM buffer. The tracer solution was obtained by serially diluting the labeled protein stock to 5–10 nm until the signal was just sufficient for anisotropy measurements by the instrument. The tracer solutions were centrifuged at 15,000 \( \times g \) at 4 °C during 15 min before each experiment. Measurement of dilution-induced anisotropy relaxation kinetics consisted of taking an aliquot (3 \( \mu l \)) of the temperature-equilibrated starting solution of tubulin, directly diluting and mixing it in PM buffer (600 \( \mu l \)) in the cuvette in the sample holder of the T-format instrument, and recording anisotropy as a function of time. All measurements were made in quartz cuvettes at 20 °C. The best-fit parameters describing labeled tubulin dimer dissociation were calculated in SEDPHAT using the same procedure described for isotherm analysis of SV data.

**Global Multimethod Analysis of Weight Average Sedimentation Coefficient and of Fluorescence Anisotropy Isotherms**—To arrive at a consistent best-fit estimate of the dimerization equilibrium constant, all binding isotherms were analyzed in SEDPHAT by global multimethod analysis (GMMA) (36) using the built-in monomer dimer self-association model. In this simple model, it is assumed that tubulin \( \alpha \)- and \( \beta \)-monomers are hydrodynamically indistinguishable from each other, and therefore, the system is treated as the association of identical subunits according to Reaction 1 and Equation 1,

\[
K_a = \frac{2M}{D}
\]

**REACTION 1**

\[
K_s = \frac{k_{on}}{k_{off}} = \frac{[D]}{[M]^2}
\]

(Eq. 1)

where \( K_a \) is the equilibrium association constant, and \( k_{on} \) and \( k_{off} \) are the association and dissociation rate constants of the dimer, respectively. At a given total concentration, mass action law provides the monomer and dimer concentrations, and the measured isotherms take the form shown in Equation 2,

\[
S_w = \frac{S_M e_M c_M + S_D e_D K_s c_M^2}{e_M c_M + e_D K_s c_M}
\]

(Eq. 2)

where \( c_M \) and \( c_D \) are the concentration of monomer and dimer; \( K_s \) is the equilibrium association constant; \( S_M \) and \( S_D \) denote \( s \) values; and \( e_M \) and \( e_D \) are the extinction coefficients of each species, respectively. For anisotropy isotherms, the same equation applies after replacing species’ sedimentation coefficients with anisotropy values and extinction coefficients with species’ fluorescence signal amplitudes. Neither fluorescence anisotropy nor SV data provided any indication of dimerization-de-
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...dependent changes in absorbance or emission quantum yields, such that $\varepsilon_D = 2\varepsilon_M$.

Data sets from different titration and dilution isotherms with different fluorophores were globally fit with the dimer dissociation constant as a global parameter for all data sets. For anisotropy data, species anisotropy values were treated as global parameters, while allowing for separate anisotropy values for tubulin labeled with different fluorophores. For SV data, the monomer s value was fixed to the monomer peak observed by c(s) analysis under the most dilute condition (see under “Results”), and the dimer s value was treated as a global parameter to be refined. Weighting of data sets was carried out solely on the basis of statistical confidence intervals in each data point. In the GMMA best fit, the 68% confidence intervals of the best-fit parameters were determined using $F$-statistics as described above.

Lamm Equation Modeling of SV Data for Interacting Systems—Sedimentation velocity data were globally fit in SEDPHAT with the built-in model for the kinetic monomer-dimer self-association equilibrium (33). The data processing, software configuration, initial parameters, and fitting procedure were handled similarly as explained (46). The global fit comprised data at six nominal concentrations from 50 nM to 2 $\mu$M, constrained by partial boundary modeling (47) over a range of 1.18–1.32, the corresponding molecular mass estimated in the range 96–105 kDa, consistent with the dimer species. At lower tubulin concentrations (0.2 and 0.03 $\mu$M), $K_d$ was obtained with the following single

$$K_d = \frac{(r_D - r_M) \times \exp(-k_{off}) + r_M}{r_M}$$

where $r_M$ and $r_D$ are the limiting anisotropies of the monomer and dimer species, respectively, and $k_{off}$ is the first-order dissociation rate constant. The 68% confidence intervals were calculated using the built-in software tools.

Pelleting Assay for Determination of Critical Concentration—The critical concentration ($C_C$) for polymerization of tubulin was measured as the concentration of non-polymerized tubulin at steady state of polymerization (19). Briefly, tubulin was polymerized at a concentration of 10 $\mu$M in PM buffer (0.1 M Pipes, pH 7, 1 mM MgCl$_2$) supplemented with 10% v/v dimethyl sulfoxide (DMSO), in a 60-$\mu$l volume at 30 °C for 30 min. Polymerization reactions were set up in two halves. One-half of the final sample volume (or 30 $\mu$l) contained tubulin dissolved in PM buffer at 2 $\times$ the desired concentration (20 $\mu$M). The other 30 $\mu$l contained PM buffer + 20% v/v DMSO, and the ligands GTP/GDP, or the co-solvents urea or TMAO, or NaCl, at 2 $\times$ the desired concentration. The reaction was begun by mixing the two 30-$\mu$l samples and incubating at 30 °C. The samples were centrifuged at 20,000 $\times$ g at 30 °C for 30 min, and the top 50% volume was removed to measure the concentration of soluble protein by the Bradford assay (similar results were obtained by centrifuging at 100,000 $\times$ g during 10 min). This is the concentration of dimeric (non-polymerized) tubulin in steady-state equilibrium with microtubule polymer, which is taken as the critical concentration ($C_C$). In these conditions, the polymerization of tubulin in the presence of 0.5 mM GTP resulted in a $C_C = 4.7 \pm 0.3$ $\mu$M. The critical concentrations of labeled tubulins were found to be similar to that of unlabeled tubulin indicating that the labels did not damage the polymerization capabilities of these tubulin preparations.

Results

Tubulin Dimer Dissociation Examined by Sedimentation Velocity Experiments—We carried out SV experiments over an ~10,000-fold range in protein concentration, bracketing the literature range for tubulin dimer dissociation constant ($K_d = 0.002$–2 $\mu$M) (21–29). Multiple SV experiments using different detection systems were required to cover this concentration range. Initially, we characterized unlabeled tubulin using absorbance detection. Typical experimental sedimentation boundary profiles are shown in Fig. 1, A–C. The absorbance radial scans show clear sedimentation boundaries in the concentration range of 0.2–2 $\mu$M, allowing precise calculation of the c(s) distribution model (solid lines in Figs. 1 and 2A). At concentrations below 0.2 $\mu$M, the fitting precision was reduced due to the noise of data acquisition, as shown in Fig. 1 by the r.m.s.d. and the increase in the percentage noise ratio shown in parentheses. Fig. 2A depicts the family of representative c(s) distributions obtained from these experiments. For example, at a concentration of 2 $\mu$M tubulin, we observed a predominant peak with maximum at ~5.8 S. With the best-fit average frictional ratio in the range 1.18–1.32, the corresponding molecular mass estimate is in the range 96–105 kDa, consistent with the dimer species, which accounts for ~93% of the total integrated signal. In the same c(s) distribution at 2 $\mu$M, and reproducibly in others, a second very small peak with maximum at ~8 S was observed, representing ~2% of the signal, likely corresponding to a tetramer species. Upon a 4-fold decrease in tubulin concentration (at 0.5 $\mu$M), the 5.8 S peak remained at the same position but with a smaller amplitude, whereas a new peak with maximum at ~3 S was resolved, corresponding to the monomer species. At lower tubulin concentrations (0.2 and 0.03 $\mu$M in Fig. 2A), the dimer peak maximum showed a small shift from 5.8 to ~5.5 S and decreasing amplitude. Similarly, the monomer peak maximum moved from 3.4 to ~3.0 S. These shifts in...
position of the peaks as a function of protein concentration are an indication of the kinetics of the underlying dissociation reaction (see Lamm equation modeling below). At 0.03 μM, the population of the monomer species surpassed that of the dimer species. These observations indicate that the \( K_d \) value for tubulin dissociation is likely between 0.03 and 0.2 μM.

Full dissociation of tubulin dimers could be observed using fluorescence-detected SV, which allows characterization of proteins down to picomolar concentrations (35). Tubulins labeled with DyLight 488 NHS ester (tubulin-488) and with DyLight 550 NHS ester (tubulin-550) were analyzed using the same dilution experimental design as with unlabeled tubulin. Three representative SV profiles, with the corresponding best-fits using the \( c(s) \) distribution model, are shown in Fig. 1, D–F. We were able to calculate the \( s_{20, w} \) from SV data showing up to 50% noise ratio, spanning a 2000-fold range in protein concentration, from 0.5 to 2000 nm. The family of \( c(s) \) distributions obtained from these experiments is shown in Fig. 2, B and C. The features previously described for the \( c(s) \) distributions of the absorbance-detected data were also observed in the fluorescence-detected data. At \( \sim 1 \) μM labeled tubulin, we observed two distinct peaks in the range 1–8 S, corresponding to the dimer and monomer species, with maxima at 5.9 and 3.5 S, respectively. After dilution of labeled tubulin to 0.1–0.5 μM, the

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**FIGURE 1.** Sedimentation velocity profiles of tubulin dilution series detected with absorbance optics (A–C) and with fluorescence optics (D–F). The concentrations of unlabeled tubulin in A–C were 2, 0.5, and 0.2 μM, respectively. The concentrations of tubulin-488 in D–F were 120, 12, and 1.2 nM, respectively. In all panels the dots are the radial scans, and the solid lines are the best-fits using the continuous sedimentation coefficient distribution \( c(s) \) in SEDFIT, as described under “Experimental Procedures.” For simplicity, only every 3rd data point recorded and every 3rd scan are shown. Earlier radial scans are shown to the left. The quality of the best-fit \( c(s) \) distribution is shown by the r.m.s.d. and also by the percentage noise ratio (r.m.s.d./total loading signal). The lower plots are the residuals of the fits. AU, absorbance units.
dimer peak displayed a small shift to lower $s$ values, from 5.9 to $\sim5.5$ S, which was accompanied by a small decrease in amplitude. The monomer peak increased upon dilution but remained at virtually the same $s$ value independent of the protein concentration. At 10–50 nM labeled tubulin, the amplitude of the monomer peak increased upon dilution but remained $s$-value independent of the protein concentration. At 1–5 nM, the monomer peak (maximum at $s_{20,w}=3.5$ S) accounted for 90% of the total signal. A comparison of the shape and center of peaks representing the monomer and dimer species in $c(s)$ distributions obtained with absorbance and fluorescence detection showed these were quantitatively similar (Fig. 3A). We concluded that unlabeled and labeled tubulin showed a similar hydrodynamic behavior.

Because we were able to study tubulin at low concentrations that yielded almost all monomers, we analyzed the hydrodynamics of dissociated $\alpha$- and $\beta$-subunits to learn about hypothetical conformational transitions between isolated monomers and monomers incorporated into dimers. This was done by comparing the observed $s_{20,w}$ of the monomer and dimer species ($s_{20,w \text{-}}$monomer = 3.2 ± 0.3 S and $s_{20,w \text{-}}$dimer = 5.9 ± 0.2 S), with the theoretical values calculated from the crystal structure (1JFF) using Hydropro (48), which produced values $s_{\text{monomer}} (\alpha,\beta) = 3.5$ S and $s_{\text{dimer}} = 6.1$ S. From these numbers we may infer that the solution shape of the tubulin dimer, and of the $\alpha$- and $\beta$-subunits, is in accordance with the shapes determined from the crystal structure. In addition, we find no evidence of monomer unfolding sufficient to change the observed $s_w$ values.

**Isotherm Analysis of Tubulin Sedimentation Velocity Data**—
The weight average sedimentation coefficients $s_{20,w}$ were calculated by integration of the best-fit $c(s)$ distributions shown in Fig. 2, $A$–$C$, which rigorously correspond to integration of sedimentation boundaries in the second moment transport method, irrespective of any reaction kinetics (31). Fig. 2D shows the resulting $s_w$ isotherms for dilution experiments obtained with unlabeled tubulin and with fluorescently labeled tubulin. A close inspection of the unlabeled tubulin isotherm (black circles in Fig. 2D), shows good precision in calculated $s_{20,w}$ values in the range from 5 to 0.1 μM, as indicated by small error bars. At lower protein concentrations (<0.1 μM), the precision was significantly reduced due to the lower overall signal. This limitation impeded the correct description of $s_{20,w}$ for the monomer species, based on these data alone. The lowest concentration we could characterize using absorbance detection was 20 nM with ~70% monomer population and $s_{20,w} = 3.9 ± 0.6$ S. In the isotherms of labeled tubulin, which ranged from 2 μM to 0.5 nM (red triangles and green squares in Fig. 2D), an increased precision in $s_{20,w}$ was observed as indicated by the smaller error bars at nanomolar protein concentrations. For instance, at 1 nM tubulin-488 we observed ≥95% monomer species population with a calculated $s_{20,w} = 3.3 ± 0.4$ S. Therefore, the fluorescence detection allowed characterization of tubulin in a virtually pure monomeric state.

Although similar $s_{20,w}$ values for dimer species were observed in the isotherms of unlabeled tubulin, tubulin-488, and tubulin-550, only the data from the isotherms of labeled tubulin could be employed to estimate an accurate value for $s_{20,w}$ of the...
Tubulin Dimer Reversible Dissociation

Monomer Tracer Sedimentation Velocity Experiments—A signature behavior of a reversibly dissociating system (here tubulin dimers), is the ability of the free monomer to associate into dimers in response to an increment in protein concentration. This experiment requires that the monomer be stable for the time of the experiment (~3 h) and be capable of responding to added protein. We tested these predictions, using the sedimentation velocity experiment, by preparing a tracer monomer solution (by diluting tubulin-550 to 5 nM) and adding either nothing or unlabeled tubulin to a final concentration of 1 μM after ~1 h of incubation at room temperature. The resulting best-fit c(s) distributions of these experiments are shown in Fig. 3B. It is clear from the shape of the distributions that 5 nM tubulin-550 was nearly all monomer and stable for the time of the experiment. It is also clear that addition of free unlabeled tubulin shifted the equilibrium of the tubulin-550 to a majority of dimer. These conclusions are supported by the integrated s_{20, w} which was indistinguishable from that of pure labeled tubulin at 1 μM. Additionally, the shift in the apparent s_{20, w} for the monomer upon addition of unlabeled tubulin is indicative of a reversible equilibrium (see below). Taken together, these experiments demonstrate the reversibility of tubulin dissociation under our experimental conditions.

Tubulin Dimer Dissociation Examined by Fluorescence Anisotropy Experiments—Having shown the reversible dissociation of labeled tubulin dimers by SV, we used steady-state FA to independently measure the tubulin dimer K_d values. The FA experiment provides a reproducible and inexpensive method to characterize tubulin dimer dissociation. In a first approach, we performed dilution of tubulin labeled with pyrene maleimide (tubulin-345) and observed a concentration-dependent decrease in fluorescence anisotropy (Fig. 4A). The decrease in FA occurred in the range of concentrations in which we observed dissociation of the tubulin dimer using SV experiments. However, the lowest concentration of tubulin-345 we could measure by FA was between 5 and 10 nM, which is ~10-fold higher than the lowest concentration measured by fluorescence-detected SV (0.5 nM). Therefore, in the FA experiment a maximum ~90% dimer dissociation may be detected. Excimer formation was previously reported with tubulin labeled using pyrene maleimide (49), and it would complicate interpretation of our data. However, no evidence of excimer fluorescence was observed in the emission spectra of tubulin-345 at any concentration (data not shown). The isotherm of tubulin-345 dilution was fit using the monomer-dimer self-association model, and the best-fit parameters are shown in Table 1 (under heading Fluorescence anisotropy). The resulting K_d = 125 (59–262) nM showed no statistical difference from the K_d determined using SV (p = 0.9).

Monomer Tracer Fluorescence Anisotropy Experiments—We employed the monomer tracer experimental design and fluorescence anisotropy to test the ability of labeled tubulin monomers to exchange into preformed dimers. In these experiments a low concentration of fluoscently labeled tubulin was

monomer species. In addition, the fair overlap of the three isotherms indicated a similar dissociation behavior of the three samples. Therefore, based on the complementary information provided by sedimentation velocity experiments made with unlabeled and labeled tubulin samples, we analyzed the combined isotherms using the monomer-dimer self-association model. The solid line in Fig. 2D is the best-fit of this global analysis, and the resulting parameters are shown in Table 1 (under the column Sedimentation velocity). It is seen in the isotherm of the combined data that all measured s_{20, w} values are distributed within 1 S.D. of the best-fit over the full range of tubulin concentrations (dotted lines in Fig. 2D), consistent with a similar dissociation behavior for unlabeled and both labeled tubulins. Thus, from analysis of combined SV experiments, we may conclude that rat brain tubulin dissociates with K_{d-SV} = 64 (45–91) nM and that s_{20, w-monomer} = 3.2 (3.0–3.5) S and s_{20, w-dimer} = 5.9 (5.8–6.2) S.

FIGURE 3. Similar hydrodynamic behavior between unlabeled and labeled tubulin is shown by superposition of the corresponding best-fit c(s) distributions (A). In these experiments, unlabeled tubulin (s_{20, w} = 4.9 ± 0.1 S) was detected using absorbance optics (dashed line), and tubulin-550 (s_{20, w} = 4.8 ± 0.02 S) was detected using fluorescence optics (solid line). The reversibility of tubulin dimer dissociation is demonstrated by the monomer tracer sedimentation velocity experiment (B). Tubulin-550 was diluted to 5 nM (solid line), where >90% dissociation was observed according to the c(s) distribution analysis, with an observed s_{20, w} = 3.3 ± 0.4 S. A parallel sample of 5 nM labeled tubulin was titrated with 1 μM unlabeled tubulin (dashed line). It is seen that the addition of unlabeled tubulin shifted the equilibrium to the majority dimer of the fluorescent monomer tracer, with an observed s_{20, w} = 5.5 ± 0.4 S, which is identical to that of pure unlabeled tubulin at 1 μM. The fit quality of the best-fit c(s) distributions is shown as the percentage noise ratio.
TABLE 1
Global parameters determined for tubulin dimer reversible dissociation

| Parameter | Sedimentation velocity | LEq model | Fluorescence anisotropy | GMMA SV + FA |
|-----------|------------------------|-----------|-------------------------|--------------|
| Monomer  | 3.2 (3.0–3.5)          | 3.2 (Fix) | 0.087 (0.084–0.089)     | ND           |
| Dimer     | 5.9 (5.8–6.2)          | 5.9 (5.8–6.2) | 0.167 (0.164–0.171)     | ND           |
| Kd, nm    | 64 (45–91)             | 47 (42–69) | 125 (59–262)            | 84 (54–123)  |
| koff, s⁻¹ | ND                     | 7.5 × 10⁻³ (0.1–11) | 3.1 × 10⁻³ (2.7–3.6)   | ND           |

a Values are the best-fits, and the 68% confidence intervals, determined using F-statistics, are shown in parentheses. Monomer and dimer parameters are koff, w for SV experiments, and limiting anisotropies for FA experiments.

b The analysis was made over the data shown in Fig. 2D.

The global LEq fit was made over the SV data shown in Fig. 6. In this analysis the koff, w of the monomer was fixed.

c The analysis was made over data in Fig. 4A. The best-fit value of koff, w was obtained from data in Fig. 4B.

d GMMA was employed to calculate the value of Kd value across six isotherms combining SV and FA data (for details see “Experimental Procedures” and Fig. 6).

e ND indicates not determined.

Tubulin Dimer Reversible Dissociation

FIGURE 4. Tubulin dimer dissociation determined by fluorescence anisotropy. A, tubulin-345 was serially diluted and incubated to equilibrium, and the fluorescence anisotropy was recorded (solid circles). The error bars are the standard deviations of 20 iterations. The solid line is the best-fit isotherm to the monomer-dimer self-association model. B, T-format fluorescence anisotropy relaxation kinetics (dots) recorded after 200-fold dilution of 2 μM tubulin-345. The solid line is the best fit using a single component model as explained under “Experimental Procedures.” The inset in B show the relaxation kinetics after 50-fold dilution of tubulin-345. The resulting best-fit parameters are detailed in Table 1.

titrated with unlabeled tubulin. This was done using both tubulin-488 and tubulin-345. An increase in anisotropy was observed indicating exchange of free labeled monomers into unlabeled dimers (see below for a view of the actual experimental data). A detailed comparison of the best-fit parameters for each FA experiment is presented in Table 2 (under the heading Fluorescence anisotropy). The estimates of Kd for the monomer tracer experiments using fluorescence anisotropy (or titrations) yielded close to 2 orders of magnitude confidence intervals, which were broader than the dilution using tubulin-345, or the SV estimates discussed above. We conclude from these analyses that the fluorescence anisotropy assay is able to detect tubulin dissociation and monomer exchange, with Kd values consistent with SV results, but with broader confidence intervals on the estimate.

Thermodynamics of Tubulin Dimer Dissociation, Global Multimethod Analysis—The dissociation of the tubulin dimer has been preferentially studied using analytical ultracentrifugation but also using fluorescence anisotropy (21–28). These two biophysical methods rely on different molecular properties and provide independent information for determination of equilibrium constants. This is the basis of GMMA where the thermodynamic parameters of the underlying chemical reactions are simultaneously calculated across multiple data sets, from replicate experiments as well as from different experimental methods (50). Here, we have applied GMMA to the combined data from sedimentation velocity (three SV isotherms) and fluorescence anisotropy experiments (three FA isotherms). Given the equivalence of the binding isotherms produced by SV and FA methods, in the fitting routine the global parameter shared across the combined data is the equilibrium constant. The local parameters are the sedimentation coefficients (shared among SV isotherms) and the limiting anisotropies (shared among isotherms of tubulin-345) of the monomer and dimer species. The resulting parameters are shown in Table 1. Also, for a better look of the experimental data employed for these analyses, the GMMA fit projected over each data set is shown in Fig. 5. Thus, the global estimate of Kd,GMMA = 84 nM (54–123) considers the contribution of two independent methods, of two different experimental designs (dilution and titration), and the utilization of various tubulin samples (including unlabeled tubulin and tubulin labeled with three different dyes).

Kinetics of Tubulin Dissociation Determined Using Lamm Equation Modeling of Sedimentation Velocity Data—In the SV experiment, a mixture of interacting proteins will display a sedimentation boundary characteristically different from a mixture of non-interacting proteins. Thus, for a monomer-dimer system, slow dissociation kinetics will produce two peaks centered at the s values of the monomer and dimer species, with changing amplitudes as a function of concentration. In contrast, fast dissociation kinetics will produce a single broad peak reflecting an effective particle with a time-average state with increasing s values and amplitudes, as a function of increasing protein concentration (40). This is the basis for modeling of SV data using Lamm equation solutions coupled to reaction fluxes (LEq), which provides thermodynamic as well as kinetic information for the underlying chemical equilibrium. In the case of
tubulin dissociation, a first diagnostic of the kinetic regime was obtained by inspection of the best-fit \( c(s) \) distributions presented in Fig. 2. We observed monomer and dimer peaks that exhibited changes in relative populations dependent on protein concentration, as well as small shifts in the center of the peaks. These small shifts suggest that reversible dissociation of the tubulin dimer occurs on a slow time scale but one comparable with the characteristic time of the sedimentation experiment.

To further this analysis, a 6-sample dilution series of unlabeled tubulin (diluted from 2 down to 0.05 \( \mu \)M) was globally fit using the LEq model for monomer-dimer self-association (as detailed under “Experimental Procedures”). The sedimentation boundaries with the global fit to the LEq model are shown in Fig. 6. The LEq model resulted in an excellent fit to the absorbance radial scans of the partial boundary data (2–8 S), as evidenced by the residuals of the plots and the statistical analyses performed on the data (Table 1). Initially, we performed a global fit by floating all adjustable parameters (data not shown). The \( s_{20,w} \) of the monomer species could not be determined with accuracy, and the best fit value of \( K_d \) was 50% lower than expected from isotherm analysis. We next fixed the \( s_{20,w} \) of the monomer to the value estimated using isotherm analysis (consistent with the monomer \( c(s) \) peak of the fluorescence detected SV data in the most dilute sample) and continued the optimization routine. As seen in Table 1, this allowed calculation of \( s_{20,w,dimer} \) and \( K_d \) with the same confidence as with the isotherm analysis indicating the model successfully described the experimental data. The best fit value of the \( k_{off} \) was 7.5 \( \times \) \( 10^{-3} \) \( s^{-1} \), with a 2-order of magnitude statistical precision (1 \( \times \) \( 10^{-3} \)–1 \( \times \) \( 10^{-2} \) \( s^{-1} \)). This suggests that the kinetic regime of

### Table 2: Summary of parameters calculated for various experiments of tubulin dimer dissociation

Each isotherm was modeled individually (locally), i.e. not a global analysis.

| Parameter | Tubulin dilution | Tubulin-488 dilution | Tubulin-550 dilution | Tubulin-488 titration | Tubulin-345 titration | Tubulin-345 dilution |
|-----------|------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| SFV       | SV               | FA                   | SFV                 | FA                   | SFV                 | FA                   |
| Monomer, \( s_{20,w} \) or anisotropy | 3.0 (1.5–3.1) | 3.2 (2.9–3.4) | 3.2 (3.0–3.2) | 0.243 (0.238–0.247) | 0.094 (0.091–0.097) | 0.087 (0.084–0.089) |
| Dimer, \( s_{20,w} \) or anisotropy | 5.9 (5.8–6.2) | 6.0 (5.8–6.4) | 5.7 (5.6–5.8) | 0.280 (0.271–0.299) | 0.153 (0.145–0.162) | 0.167 (0.164–0.171) |
| \( K_d \), nM | 39 (15–74) | 40 (23–69) | 65 (51–83) | 347 (71–1650) | 265 (57–1250) | 125 (59–262) |
| \( k_{off} \), s\(^{-1} \) | | | | | | |

*Values are the best-fits with 68% confidence intervals in parentheses.
The effect of solvent variables in the tubulin dimer dissociation constant and the critical concentration for polymerization

| Condition       | Dissociation constant (nM) | Critical concentration (μM) |
|-----------------|-----------------------------|-----------------------------|
| PM buffer       | 96 (84–107)                 | 1.0                         |
| + GDP 0.5 mM    | 76 (71–81)                  | 0.8                         |
| + GTP 0.5 mM    | 76 (69–83)                  | 0.8                         |
| + Urea 0.3 M    | 115 (101–131)               | 1.2                         |
| + TMAC 0.3 M    | 93 (84–103)                 | 1.0                         |
| + NaCl 0.5 M    | 51 (48–54)                  | 0.5                         |

The values of $K_d$ were determined using the fluorescence anisotropy assay. The experiment in PM buffer was the control condition. Values reported are the best-fit with 68% confidence intervals in parentheses. The anisotropy of the monomer species was fixed for these analyses.

Tubulin dimer reversible dissociation

Tubulin dimer reversible dissociation

FIGURE 6. Tubulin dimer dissociation sedimentation velocity data analyzed by global analysis using Lamm equation modeling for interacting systems. A 6-sample dilution series was detected using absorbance at 230 nm. The protein concentrations in A–F were as follows: 2; 1; 0.5; 0.2; 0.1, and 0.05 μm, respectively. The raw data were fit in SEDPHAT with the built-in monomer-dimer self-association equilibrium model, using the partial boundary modeling approach, between 2 and 8 S. The resulting best-fit parameters including $s_{20,w}$ of tubulin dimer, $K_{off}$, and $K_{on}$ values are shown in Table 1. In all panels the dots are the absorbance readings; the solid lines represent the best-fits of the model; and the lower plots are the residuals of the fits. For simplicity, only every 3rd data point recorded and every 3rd scan are shown. Earlier radial scans are shown to the left. The r.m.s.d. of the fits are shown above each panel, and also in parentheses as the percentage noise ratio. G, best-fit c(s) distributions obtained from the SV profiles shown in A–F. The shift of the monomer and dimer peaks is an indication of reversibility and of moderate to fast kinetics of dissociation.

Tubulin dissociation is intermediate between fast and slow kinetics (33).

Kinetics of Tubulin Dissociation Measured by Relaxation of Fluorescence Anisotropy—We used the relaxation of fluorescence anisotropy upon dilution of tubulin-345 to measure the apparent dissociation rate, $k_{off}$. Starting from an equilibrated solution of 2 μm tubulin-345, we performed dilutions directly into a cuvette containing PM buffer while recording T-format fluorescence anisotropy. Typical traces of these experiments are shown in Fig. 4B, where a 200-fold dilution of tubulin-345 (final concentration ~10 nM) resulted in an overall change in anisotropy $\Delta r = 0.05$, a value 20 times greater than the standard deviation of any single measurement (S.D. ~0.003). The characteristic single component decay observed in this trace is $k_{off} = 3.1 \pm 0.5 \times 10^{-3} \text{s}^{-1}$. We also observed a faster relaxation in fluorescence anisotropy for some dilution experiments. For instance, when a 50-fold dilution was tested (final concentration ~40 nM), the overall change in fluorescence anisotropy was lower with $\Delta r = 0.02$, which is eight times above the S.D. (inset in Fig. 4B). In this experiment, a single component was observed with $k_{off} = 1.2 \pm 0.1 \times 10^{-2} \text{s}^{-1}$, which is 4-fold faster than found in the previous experiment. In a series of experiments where a clear fluorescence anisotropy relaxation was observed, the measured $k_{off}$ values were contained in the interval between these two measurements. Thus, we can establish a range between $10^{-3}$ and $10^{-2} \text{s}^{-1}$ for the apparent dissociation rate constant of tubulin-345. These values are in good agreement with $k_{off}$ calculated using LEq modeling for unlabeled tubulin.

Solution Variables and Dimer Dissociation—Having analyzed the equilibrium and kinetic behavior of tubulin dimer dissociation under our standard conditions, we wished to examine the effect of changing the solution. Many studies have reported the sensitivity of tubulin polymerization (or dimer-dimer association) to changes in solution composition by changes in the critical concentration for polymerization, and some previous reports on dimer dissociation (or monomer-monomer association) examined this as well by measuring effects on dimer $K_d$. Here, we examined four changes to the solution as follows: addition of ligands 1) GTP or GDP at 0.5 mM; addition of co-solvents 2) urea; or 3) trimethylamine N-oxide (TMMAO), each at 0.3 mM; and addition of 4) NaCl at 0.5 mM. We used the fluorescence anisotropy experiment to measure the dimer $K_d$ value and a microtubule pelleting assay to measure the $C_{cr}$ (Table 3). Our results show that monomer-monomer association differs in sensitivity to solution composition com-
pared with dimer-dimer association. Addition of 0.5 mM GTP or GDP had a mild effect on the dimer \(K_d\) compared with our standard conditions with no added nucleotide \((K_d = 96 \pm 12\) nM). Because the buffer contains 1 mM MgCl₂, tubulin should be saturated with nucleotide under both conditions \((51)\). Urea, which strongly inhibits polymerization at 0.5 m (52), had little or no effect on dimer \(K_d\) when added to 0.3 M. TMAO, which strongly promotes polymerization (38), failed to perturb \(K_d\) beyond the confidence intervals found under standard conditions when added to 0.3 M. In contrast, addition of 0.5 M NaCl induced 2-fold decrease in \(K_d\) indicating that monomer-monomer association is favored at high ionic strengths. We also measured the sedimentation coefficients \(s_{20, w}\) of tubulin samples in the presence of 0.3 M urea or 0.3 M TMAO in sedimentation velocity experiments \(\text{(yellow diamonds in Fig. 2D)}\), and the samples showed no differences from the control condition in PM buffer thereby confirming the findings of fluorescence anisotropy experiments.

To test the sensitivity of dimer-dimer association to changes in solvent conditions, we measured the critical concentration \(C_C\) of tubulin polymerization in conditions similar to those in Ref. 53. The measured values of \(C_C\) are shown in Table 3. It is seen that the critical concentration was modified under conditions that did not alter the \(K_d\) values \(\text{(monomer-monomer association)}\). The control condition in the presence of 0.5 mM GTP resulted in \(C_C = 4.7 \pm 0.3\) \(\mu M\). GDP and urea inhibited tubulin polymerization by increasing the \(C_C\) at least 2-fold, whereas TMAO favored polymerization by inducing a 3-fold decrease in \(C_C\). In contrast, NaCl did not alter the value of \(C_C\) by more than 50%. We may conclude from this series of experiments that monomer-monomer association is less sensitive to allosteric ligands \(\text{(GTP/GDP)}\), or a structure destabilizer (urea) or stabilizer \(\text{(TMAO)}\), or increased ionic strength, than is dimer-dimer association \(\text{(polymerization)}\).

**Discussion**

The reversible polymerization of αβ-tubulin has inspired numerous and continuing biochemical and structural analyses. The same is not true for the process of reversible monomer-dimer equilibrium. A number of studies on the subject were published more than a decade ago \((21–29)\). These studies used different quantitative methods to measure the tubulin dimer dissociation, and reported \(K_d\) values in the range 0.002–2 \(\mu M\), under diverse experimental conditions and using different technologies. The values reported in these studies are listed in Fig. 7 in a graphical depiction using box plots and also in tabulated form. Because most of previous studies did not report error estimates for the reported values of \(K_d\), we estimate those in Fig. 7 to allow comparison to the explicit statistical confidence intervals \(\text{(CI)}\) that we present for the current data. It is seen that the values of \(K_d\) determined using either sedimentation equilibrium-AUC or FA are specifically in the range 2–840 nM. However, these estimations depended largely on extrapolation because the tubulin concentrations used were in the range 0.1–10 \(\mu M\), which are well above the corresponding \(K_d\) values. Exceptionally, Caplow and Fee \((29)\) used surface plasmon resonance with biotinylated tubulin dimers and reported a slow dissociation kinetics \(\text{(}k_{\text{off}} \approx 10^{-5} \text{ s}^{-1})\) from which a \(K_d = 10^{-11} \text{ M}\) was calculated, concluding that the tubulin dimer dissociation is an energetically unfavorable process. This last value is in obvious conflict with all previous determinations, probably due to the different experimental design involving biotinylated tubulin dimers attached to a streptavidin-coated surface \((29)\). Despite this, the failure to produce native recombinant tubulin expressed in bacteria and this last report are often cited to argue that the tubulin dimer does not dissociate, for example in studies of the \textit{in vivo} folding pathway of tubulin, which has been shown to be dependent on cytoplasmic chaperonin-containing TCP-1 and folding cofactor tubulin-binding cofactors A to C \((7–9)\).

Almost all of the previous studies of tubulin dimer dissociation used mammalian brain tubulin. At present, the diversity of the tubulin family has become clear, and consequently the utility of using mammalian brain tubulin to represent all tubulins is open to question. At the same time, the methods available for

### Table of Tubulin Dimer Reversible Dissociation Constants

| Study          | Technique           | \(K_d\) M | # |
|----------------|---------------------|----------|---|
| This work      | SV-AUC + FA (Global)| 8.4±3.5 \(10^{-4}\) | 8 |
| Dietrich, 1978 | SE-AUC              | 8.0 \(10^{-7}\) | 7 |
| Sackett, 1991  | SE-AUC              | 2.0 \(10^{-7}\) | 6 |
| Shaerwin, 1994 | SE-AUC              | 2.0 \(10^{-9}\) | 5 |
| Menendez, 1998 | SE-AUC              | 5.0±2.5 \(10^{-4}\) | 4 |
| Mejillano, 1989| FA                  | 8.4±0.4 \(10^{-7}\) | 3 |
| Panda, 1992    | FA                  | 7.2 \(10^{-7}\) | 2 |
| Caplow, 2002   | SPR                 | 1.0 \(10^{-11}\) | 1 |
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quantitating sub-micromolar dissociation constants free in solution have been greatly improved. For these reasons, we revisited the issue of the $K_d$ for the $\alpha\beta$-tubulin dimer. Furthermore, we plan to examine the $K_d$ values for dimers of a number of different non-neural tubulins to test whether this fundamental property is influenced by the isotypes or the presence of post-translational modifications and to seek differences that may have resulted from evolutionary adaptation.

Mammalian Tubulin Dimer Reversibly Dissociates with Sub-micromolar Affinity—Our study began with the reexamination of mammalian brain tubulin dimer dissociation. Developments in analytical methods, especially in AUC, currently allow examination of samples at nm and even sub-nm concentrations. This allowed us to cover a range of tubulin concentrations from μM, where tubulin should be all dimer based on reported $K_d$ values, to low nm concentrations, where tubulin should be substantially monomeric. Thus, unlike the previous studies, we did not rely on extrapolation to determine the $K_d$ but could examine samples that were both substantially above and below the expected $K_d$. We observed solutions with almost all monomer, and found, perhaps surprisingly, that tubulin monomers were stable at least for a few hours in solution. This stability was manifest in stable hydrodynamic behavior, without evidence of aggregation that might occur due to partial denaturation accompanying dilution (see below). Stability was also demonstrated by function as follows: monomeric tubulin retained the ability to associate with added tubulin to form dimers that showed $K_d$ values comparable with samples produced by dilution from high concentrations of predominantly dimeric tubulin. Quantitatively, the combined results of these experiments, from sedimentation velocity AUC and from steady-state fluorescence anisotropy, yielded a global $K_d$-GMMA $= 84$ nM (54–123). This value is consistent with previous literature (21–28), but it is much better documented in this work. Because the concentration range examined is much more extensive than in previous studies, the statistical confidence intervals are explicit, and this study included for the first time the studies of solutions of essentially pure monomeric tubulin.

Mammalian Tubulin Dimer Dissociates with Moderately Fast Kinetics—We examined the kinetics of dimer dissociation by use of quantitative modeling of SV data based on the Lamm equation and by the relaxation kinetics in fluorescence anisotropy. In principle, if dissociation is slow relative to the time of the centrifugation, monomer and dimer species will cleanly separate and appear as single peaks in the c(s) distribution whose $s_m$ values do not vary with total concentration. If dissociation is fast relative to the centrifugation, a single species with $s$ value intermediate between that of monomer and dimer will be observed (40, 54). An advantage of fluorescence polarization is that it allows direct real time examination of the kinetics of processes under study (55). We were able to use both methods, because the kinetics of rat brain tubulin dimer dissociation were close enough to the required range for Lamm equation modeling of SV data, and the $K_d$ value allowed dissociation to be observed at a concentration with sufficient sample fluorescence intensity to permit analysis by anisotropy. These combined analyses yielded a 1 order of magnitude confidence interval for the apparent dissociation rate: $10^{-2} < k_{off} < 10^{-2}$ s$^{-1}$, indicating moderate kinetics that are neither very fast nor extremely slow. The approximate value of the kinetic association rate constant $k_{on}$ of tubulin dimers was calculated using the global estimate of $K_d$ and the confidence interval determined for the kinetic off-rate, yielding $10^{9} < k_{on} < 10^{10}$ M$^{-1}$ s$^{-1}$. This value is lower than the expected rate for diffusion-controlled association reactions ($k_{on} \sim 10^9$–$10^{10}$ M$^{-1}$ s$^{-1}$) but similar to many other protein-protein association constants (56), indicating that orientation, electrostatics, and/or conformational constraints are important in the association of tubulin monomers (57, 58).

Tubulin Monomer-Monomer Association Is Less Influenced by the Solvent than Dimer-Dimer Association—Because the association of the $\alpha$- and $\beta$-monomers to form the tubulin heterodimer involves protein-protein interaction surfaces that are significantly more extensive from those that form the longitudinal dimer-dimer contact in protofilaments (2, 18, 59, 60), we were interested in comparing the stability of these two interactions. The critical concentration ($C_C$) for polymerization is a measure of the equilibrium binding constant of a dimer to the microtubule, and it can be significantly modulated by solvent changes and additives (19). However, the $C_C$ does not measure directly only the protofilament interaction (referred here as dimer-dimer interaction) but also includes the contribution from lateral interactions between protofilaments and therefore between tubulin dimers. In case of tubulin monomer-dimer equilibrium, less information is available, but different effects of buffer composition have been observed in the $K_d$ values determined using sedimentation equilibrium-AUC and FA (21–28). Here, only mild effects on $K_d$ were observed under various solvent conditions, but in contrast, we observed greater effects on the $C_C$ for polymerization with the same solution changes. These findings suggested a tighter association between $\alpha$- and $\beta$-tubulin in formation of the dimer than the association of dimers to form microtubules.

Concluding Remarks—What is the evolutionary advantage of having a high affinity $\alpha\beta$-tubulin dimer? In some more basal members of the FtsZ/tubulin superfamily, the dimer is less stable that the mammalian tubulin dimer. For instance, the bacterial tubulin BtubA/B is a weak dimer ($K_d = 10 \mu M$), and it has been shown that formation of the dimer is relevant in the context of the polymerization reaction, but the separate subunits are stable and can be easily isolated (16). In case of the more distant prokaryotic homolog FtsZ, the biological significance of the dimer has not been shown beyond that of a role as a kinetic intermediate in polymerization, and the reported dimerization constant is $\sim 10 \mu M$ (61).

We found that although rat brain tubulin dimers are stable in solution at micromolar concentrations, they do undergo reversible concentration-dependent dissociation with $K_d$ values of $\sim 100$ nm. Dimer dissociation at lower concentrations yields monomers that are stable for at least several hours. We found that the interaction between $\alpha$- and $\beta$-tubulin subunits is only mildly influenced by the solvent additives we examined, indicating a tight association between both subunits that may not be easily modulated by changes in solute concentration or composition. Because we were able to measure the tubulin dimer $K_d$ with better accuracy than in previous stud-
ies, we plan to expand these studies to see to what extent dimer dissociation varies in different isoforms of mammalian tubulin, in tubulins from different species, and in the presence of other cosolutes. These points will be the subjects of future studies.

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