Interaction of Tubulin and Microtubule Proteins with Vanadate Oligomers†

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ABSTRACT: Microtubule assembly is known to be regulated by the phosphorylation of microtubule-associated proteins (MAPs), and is thus sensitive to phosphatase inhibitors. We have investigated the direct interaction between phosphatase inhibitors (vanadate, sodium fluoride, and okadaic acid) and microtubule proteins. Vanadate self-assembles into oligomers, primarily dimer, tetramer, and decamer in 0.1 M Pipes, pH 6.9. Oligomer concentrations and their direct binding to tubulin and MAPs were determined by ^{51}V NMR. The assembly of microtubule protein (MTP) is strongly inhibited by decavanadate binding to MAPs and only weakly inhibited by tetravanadate binding to MAPs. Decavanadate will inhibit both MAP2 and tau-induced assembly. Decavanadate binds to MAP2 at 26 sites [$K_a \geq (1.0–1.3) \times 10^5$ M^{-1}]. The mechanism appears to involve competitive binding to MAPs, presumably at or near the microtubule binding domains, and reduced affinity for microtubules. The assembly of MAP-free, phosphocellulose-purified tubulin (PC-tubulin) is only weakly inhibited by decavanadate, although decavanadate binds to tubulin at four independent sites ($K_a \geq 1.0 \times 10^5$ M^{-1}). Monomeric vanadate, a strong phosphatase inhibitor, does not interact with tubulin or MAPs, and thus does not bind to the exchangeable nucleotide binding site on tubulin. Sodium fluoride stimulates both PC-tubulin and MTP assembly by a nonspecific effect, probably involving water structure formation. Wyman analysis suggests an absence of direct or specific binding to tubulin (d ln K/d ln [NaF] = 0.214). NaCl is nearly as effective in promoting assembly of PC-tubulin, but inhibits MTP assembly. Okadaic acid has no effect on the assembly of MTP or PC-tubulin under standard assembly conditions.

The posttranslational phosphorylation of both tubulin (Edde et al., 1989) and microtubule-associated proteins (MAPs)[1] (Goedert et al., 1992) is believed to be important in the regulation of assembly of microtubules. The phosphorylation/dephosphorylation cycle of MAPs is especially important in the conversion of interphase cells and stable microtubules to mitotic cells and dynamic microtubules (Glickman et al., 1992). Since phosphatase inhibitors are frequently used in the investigation of cell cycle processes, we began a systematic study of typical phosphatase inhibitors (vanadate, sodium fluoride, and okadaic acid) and their direct effect on microtubule assembly. Orthovanadate is an inhibitor of tyrosine phosphatases (Gordon, 1991; Pot & Dixon, 1992). Monomeric vanadate is believed to mimic phosphate, and act as a competitive inhibitor or a transition-state analog. The dramatic ability of vanadate to exchange its inner coordination sphere with other ligands suggests a strong preference for ligand donors in the active site. Experiments with vanadate are complicated by the fact that vanadate tends to form oligomers, and the species composition of a solution is pH, temperature, buffer, and vanadate concentration dependent (Pettersson et al., 1983, 1985; Chasteen, 1990; Correia et al., 1994). Microinjection, cell culture, or cell-free extract experiments are often done at pH values and vanadate concentrations where oligomers may be present (Sawin & Mitchison, 1991), unless care is taken to remove them (Goodno, 1982; Gordon, 1991). Sodium fluoride is a phosphatase inhibitor (Khandelwal, 1977; Brautigan & Shriner, 1988) that may act at the phosphorylation site as an analog of P_i in the presence or absence of aluminum or beryllium (Lange et al., 1986; Combeau & Carlier, 1988; Carlier et al., 1988, 1989; Murphy & Coll, 1992). In the presence of EDTA, it is specific for serine and threonine phosphatases (Brautigan & Shriner, 1988). Okadaic acid is an inhibitor of serine and threonine phosphatases (Hardie et al., 1991; Sim, 1991; Holmes & Boland, 1993), and will inhibit phosphatase 2A at a concentration of 10 nM (Cohen, 1991).

The polymerization and NMR experiments described here, using purified pig brain tubulin (PC-tubulin) and microtubule protein (MTP), indicate that both NaF and vanadate oligomers affect tubulin critical concentrations. NaF lowers the critical concentration of PC-tubulin and MTP due primarily to formation of water structure. The vanadate decamer binds with high affinity to tubulin, but only weakly inhibits the assembly of PC-tubulin. On the other hand, vanadate decamer and tetramer bind to MAPs, but only decamer strongly inhibits MTP assembly and increases the critical concentrations by a mechanism involving release of MAPs from microtubules. Other vanadate species, including monomeric vanadate, appear to have little or no effect on microtubule assembly. Okadaic acid does not directly interact with tubulin or MAPs.

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[1] Abbreviations: c_c, critical concentration; DMSO, dimethyl sulfoxide; DTE, dithioerythritol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; MAP, microtubule-associated protein; MTP, microtubule protein; PCA, perchloroacetic acid; PAGE, polyacrylamide gel electrophoresis; PC-tubulin, phosphocellulose-purified tubulin; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; whole MAPs, fraction of MTP that binds to a PC column and is eluted with 0.75 M NaCl.

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EXPERIMENTAL PROCEDURES

Reagents. Deionized (Nanopure) water was used in all experiments. $MgSO_4$, EGTA, GDP (type I), GTP (type II-S), glutaraldehyde, glycerol, Pipes, sodium fluoride, sodium orthovanadate, Sephacryl-400, and BSA were all purchased from Sigma Chemical Co. Tris, 2-mercaptoethanol, and polyacrylamide were from Fisher Biotech. Okadaic acid was purchased from Boehringer Mannheim. Genistein was from Calbiochem, and SDS (95%) was purchased from Mallinckrodt. Sephadex G-50 was from Pharmacia. The poly(vinyl formal) solution in ethylene dioxide and the uranyl acetate for electron microscopic experiments were from Polysciences, Inc.

Tubulin and Microtubule Protein Purification. Microtubule protein (MTP) from pig brain was purified by two cycles of warm/cold polymerization/depolymerization. MAP-free tubulin (PC-tubulin) was obtained by the same procedure followed by phosphocellulose chromatography to separate tubulin from MAPs (Williams & Lee, 1982; Correia et al., 1987). Tubulin concentrations were determined spectrophotometrically (Detrich & Williams, 1978) (ξ_{278} = 1.2 L/g-cm) or by the method of Bradford (1976) calibrated with tubulin. MTP concentrations were determined by the method of Bradford (1976). Proteins were equilibrated into the appropriate buffer by column centrifugation (Penefsky, 1977).

MAP Purification. Whole MAPs were eluted from the PC column with 0.75 M NaCl, 0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, and 2 mM DTE, pH 6.9. A 30% cut enriched in MAP2 was obtained by precipitation with 30% $(NH_4)_2SO_4$. A 45% cut enriched in tau was obtained by precipitating the supernatant with 45% $(NH_4)_2SO_4$. Further purification of MAP2 was based upon the method of Kowalski and Williams (1993). The 30% pellet was resuspended in 0.1 M Mes, 0.75 M NaCl, and 10 mM DTE, pH 6.8, made 4–5 mg/mL in BSA, and boiled for 9 min. The precipitate was spun out at 34 000 rpm for 30 min at 4 °C in a type 35 rotor, and the supernatant was precipitated with 45% $(NH_4)_2SO_4$. The pellet was resuspended in 0.1 M Mes, 0.35 M NaCl, and 2 mM DTE, pH 6.8, clarified at 14 000 rpm in an Eppendorf centrifuge, Model 5415C, and chromatographed on a Sephacryl-400 column (0.9 × 90 cm), equilibrated in 0.1 M Mes, 0.75 M NaCl, and 2 mM DTE at 4 °C, to obtain pure MAP2. The leading fractions were assayed by SDS–PAGE for purity from MAP2 breakdown products. Pure fractions were pooled, and the concentration of MAP2 was estimated with the extinction coefficient of Hernandez et al. (1986), ϵ_{275} = 0.33 L g^{-1} cm^{-1}. Further purification of tau was performed by resuspending the 45% pellet in 0.1 M Mes, pH 6.8. The solution was made 2.5% PCA and incubated on ice for 15 min. The soluble tau fractions were precipitated with 45% $(NH_4)_2SO_4$; the pellet was resuspended in 0.1 M Mes, pH 6.8, and purified over a hydroxylapatite column with a 5–500 mM KPO_4 gradient (Cleveland et al., 1977). Fractions were assayed by SDS–PAGE for purity, and the concentration of pooled tau fractions was estimated with the extinction coefficient of Butner and Kirschner (1991), ϵ_{278} = 0.29 L g^{-1} cm^{-1}.

Critical Concentrations. Polymerization of MTP or PC-tubulin was monitored using a Gilford Response II UV–VIS scanning spectrophotometer equipped with a cooling Peltier cell holder. MTP solutions (450 μL) at 1.75–3.5 mg/mL in the absence or presence of phosphatase inhibitors in 100 mM Pipes, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$, and 1 mM GTP were degassed for 30 min on ice, and base-line data at 4 °C were obtained at 350 nm. PC-tubulin solutions were prepared in the same way with the addition of 2 M glycerol to the buffer. The temperature was increased to 37 °C, and solutions were monitored at 350 nm until plateau values were obtained. Solutions were cooled to 0 °C to verify cold sensitivity and record a second base line. The change in optical density between the base-line and peak values was plotted against MTP or PC-tubulin concentrations, and the critical concentrations were obtained from the *x*-axis intercept. Data were combined from two or more separate experiments to determine each critical concentration. All PC-tubulin critical concentrations were verified by polymerization and pelleting experiments as follows: PC-tubulin was polymerized, and microtubule samples (500 μL) were spun at 60 000 rpm and 37 °C for 3 min in a TL100 Beckman tabletop centrifuge. The critical concentration was determined from the protein concentration of the supernatant by the method of Bradford (1976). The MTP critical concentrations could not be verified in this way because of variable amounts of MAPs in the MTP supernatants, i.e., variable response to the Bradford assay (see Results below).

A stock 100 mM vanadate solution in 100 mM Pipes, pH 6.9, was prepared, stored at 4 °C, and used as a starting solution for all vanadate experiments. The effect of the changing composition of vanadate solutions over time on critical concentrations was monitored as follows: A 1 mM vanadate solution was prepared from the 100 mM stock in 100 mM Pipes, pH 6.9, 2 mM EGTA, and 1 mM $MgSO_4$ and stored at 4 °C. In experiments with MTP, protein was equilibrated by column centrifugation (Penefsky, 1977) with 1 mM vanadate after 3, 24, 48, 92, 192, or 216 h of incubation. A series of MTP concentrations were then prepared with a final vanadate concentration in all solutions of 750 μM. For a zero time point (fresh vanadate), MTP solutions were made 750 μM in vanadate by direct dilution from a 100 mM stock. However, due to the high number of binding sites for vanadate decamer on MAPs (n = 26) and tubulin (n = 4), these solutions were buffered and contained 750 μM total, but not free, vanadate (see Figure 2). PC-tubulin polymerizations were carried out at 750 μM vanadate by directly diluting tubulin with 100 mM vanadate stock or by column centrifugation into 1 mM stock vanadate allowed to incubate 18–24 h at 4 °C.

The effects of NaF or okadaic acid on critical concentrations were monitored by adding appropriate amounts of stock phosphatase inhibitor solutions prior to polymerization. Okadaic acid stock was prepared in DMSO at 500 or 50 μM. Stocks were used immediately or stored at −20 °C. MTP or PC-tubulin solutions were made 0.05, 0.1, 1, 2.5, or 5.5 μM okadaic acid and critical concentrations determined as above. Control solutions were made 0.2% DMSO, comparable to the amount added to MTP or PC-tubulin with the okadaic acid. NaF (500 mM) and sodium chloride (500 mM) solutions were made fresh in 100 mM Pipes, 2 mM EGTA, and 1 mM $MgSO_4$ for each polymerization experiment. PC-tubulin experiments were carried out in 0, 25, 50, 75, or 100 mM NaF. In addition, in order to evaluate the effect of ionic strength on PC-tubulin polymerization, NaCl was added to 0, 10, or 30 mM NaF to a final ionic strength of 50 mM, and critical concentrations were determined as above. MTP critical concentration experiments were carried out in the presence of 50 mM NaF or 50 mM NaCl. In other experiments, MTP was equilibrated into 1 mM vanadate (24-h solution), and fresh sodium fluoride was added for final concentrations of 750 μM vanadate and 50 mM NaF.

A tyrosine kinase inhibitor, genistein, was used in combination with vanadate (1 mM stock solution used at 24 h) to determine if changes in the degree of phosphorylation due to the presence of active enzymes in MTP contribute to the increased critical concentration found under these conditions (see Results below). Genistein stock (1 mM) in DMSO was prepared and stored at −20 °C or used immediately at a final concentration of 5 μM in polymerization experiments in the presence of 750 μM vanadate.

MAP Binding Assays. In order to determine if phosphatase inhibitors affect MAP binding to tubulin, polymerization and pelleting experiments were carried out. MTP samples (500 μL, 3 mg/mL) were prepared as above for critical concentration experiments, in the absence or presence of phosphatase inhibitors: 750 μM vanadate (fresh dilution from 100 mM stock or 1 mM stock at 24 h), 2.5 μM okadaic acid, or 50 mM NaF. NaCl (50 mM) was used as a control. Samples were warmed 15 min at 37 °C, and then microtubules were pelleted at 100 000 rpm, 10 min, and 37 °C in a TL100 Beckman tabletop centrifuge. Supernatants were diluted in 300 μL of SDS sample buffer, and pellets were resuspended with 300 μL of SDS sample buffer. Supernatants (45 μL) and pellets (6 μL) were loaded onto 8% polyacrylamide gels. Slab gels were stacked at pH 6.8 and resolved at pH 8.8 (Laemmli, 1970). Following electrophoresis, gels were stained with Coomassie brilliant blue R-250.

In other experiments, 15 μM PC-tubulin was polymerized in the presence of purified tau (1.5 μM), in 100 mM Pipes, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$, and 1 mM GTP in the presence or absence of 750 μM vanadate (fresh dilution from the 100 mM stock or 1 mM stock at 24 h). Absorbance was monitored at 350 nm, as in the critical concentration experiments above, and at plateau, 300-μL samples were pelleted as above (100 000 rpm, 10 min at 37 °C). Microtubule pellets were resuspended in 200 μL of SDS sample buffer, and supernatants were diluted in 200 μL of sample buffer. Supernatants and pellets were analyed by SDS–PAGE. Gels were transfered to PVDF filters (Towbin et al., 1979) and reacted with tau–1 monoclonal antibodies (Binder et al., 1985). A second reaction was carried out with goat anti-mouse alkaline phosphatase-conjugated antibodies and developed with BCIP and NBT.

Nucleotide Binding Assay. Since phosphatase inhibitors may affect tubulin critical concentrations by enhancing or interfering with nucleotide binding, GXP/tubulin determinations were made as described by Croom et al. (1985). PC-tubulin samples (500 μL, 2 mg/mL) in 100 mM Pipes, pH 6.9, 2 mM EGTA, and 1 mM $MgSO_4$ were incubated with 1 mM GDP or GTP in the presence or absence of 750 μM vanadate (using 100 mM stock solution or a 1 mM solution 24 h after preparing) for 30 min on ice. Samples were centrifuged through Sephadex G-50 columns (Penefsky, 1977), equilibrated with 100 mM Pipes, pH 6.9, 2 mM EGTA, and 1 mM $MgSO_4$, and then diluted to about 1 mL. The tubulin concentration was determined by UV spectroscopy at 278 nm. Samples were made 3.5% PCA, vortexed, and left on ice 2 h. A blank was prepared in the same way without tubulin. Samples were centrifuged at 4 °C and 15 000 rpm for 30 min and optical densities of the supernatants determined at 256, 280, and 320 nm (Bock et al., 1956). The concentration of GXP was determined from the extinction coefficient, $\epsilon_{256} = 1.24 \times 10^4\ M^{-1}\ cm^{-1}$. The data at 320 nm were subtracted as the base line, and the ratio 280/256 nm for guanine nucleotide was verified to be 0.65.

Electron Microscopy and Microtubule Length Distribution. Electron microscopic examination of negatively stained specimens of all solutions described above was carried out in order to verify that normal microtubules were present. Microtubule length distributions were determined in the presence or absence of vanadate or okadaic acid. PC-tubulin solutions were prepared as above for critical concentration experiments in the absence or presence of vanadate (final concentration 750 μM) at two time points: immediately after dilution from the 100 mM stock or at 24 h after preparing the 1 mM stock. In addition, PC-tubulin solutions with 2.5 μM okadaic acid were prepared as above. Tubulin samples were warmed at 37 °C for 10 min, diluted 1:1 with fresh 2% glutaraldehyde, and left at room temperature for 5 min. Copper grids (75 mesh; Polysciences, Inc.) were prepared with 0.25% (w/v) poly(vinyl formal) solution in ethylene dioxide and carbon-coated. The procedure for specimen preparation was described by Vonck and van Bruggen (1990). Grids were laid on top of 10-μL drops of tubulin/glutaraldehyde solutions for 1 min and then washed with 2 drops of water and negatively stained for 1 min with 1% uranyl acetate. Grids were examined and photographed using a Zeiss EM 10 electron microscope. Microtubule lengths were measured on photographs using a digitizing tablet and SigmaScan software. Only microtubules with both ends clearly visible were measured. A minimum of 170–200 microtubules were measured in each of 2 separate experiments for each condition, and data were combined to obtain distributions and averages.

NMR Experiments. One-dimensional ^{51}V NMR spectra were recorded at 65.82 MHz on a Bruker 250-MHz NMR spectrometer using a 10-mm tunable probe. Samples were run with a deuterium lock; all measurements were obtained at ambient temperature. The parameters used were the following: pulse angle, 90°; sweep width, 50 000 Hz; acquisition time, 0.082 s, with no relaxation delay between pulses. An exponential line-broadening function of 0–20 Hz was applied prior to Fourier-transform to the frequency domain with use of a 8K data set zero-filled to 16K. Relative peak areas were obtained by integration of the 1D ^{51}V spectra, on a Sunstation using NMR1 software developed by New Methods Research, Inc., East Syracuse, NY. Concentrations of the various vanadate species in each sample were calculated using the known total vanadium concentrations and corrected to molar units using the size of the vanadium *n*-mer. For the tubulin, MTP, and MAP binding experiments, solutions were mixed 2 parts protein (2 mL) with 1 part 3 or 6 mM vanadate (1 mL) to give a final concentration of 1 or 2 mM total vanadate/0.1 M Pipes, pH 6.9. The 3–6 mM vanadate was made by dilution of a 10 mM VO_4 stock in 0.1 M Pipes, pH 6.9. If used immediately, the solution contains monomer, dimer, tetramer, and decamer forms of vanadate (see Figure 3). If allowed to equilibrate overnight at room temperature (or 8 days at 4 °C), only monomer, dimer, and tetramer forms are present (see Figure 3). The relatively slow kinetics of vanadate reequilibration at neutral pH (Pettersson et al., 1983, 1985) allow detection of the species which bind to a protein using ^{51}V NMR without significant reequilibration of the unbound species during the data collection time. Vanadium species bound to proteins exhibit line broadening. This is due to lengthening of the rotational correlation time and/or a reduction in the symmetry of the electric field due to a protein donor atom in the coordination sphere of the vanadium (Drago, 1992).

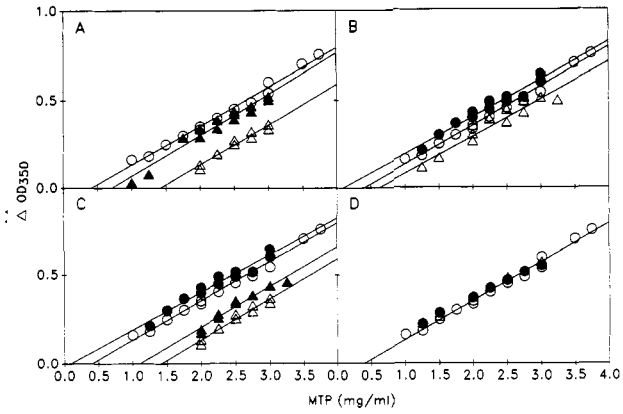

FIGURE 1: Critical concentrations of MTP in the absence or presence of phosphatase inhibitors. The x-axis intercepts of all plots indicate the critical concentrations. In all panels, (○) represent MTP polymerized in the absence of phosphatase inhibitors. The lines are linear least-squares fits. (A) Polymerization in the presence of 750 μM vanadate. MTP was equilibrated into a 1 mM stock that had been aged 24 h (△), or 8 days (▲) at 4 °C. (B) Polymerization in the presence of 50 mM NaF (●) or 50 mM NaCl (△). (C) Polymerization in the presence of 50 mM NaF (●); 750 μM vanadate from a 1 mM solution aged 1 day (△); 50 mM NaF and 750 μM vanadate from a 1 mM solution aged 1 day (▲). (D) Polymerization in the presence of 0.2% DMSO (△) or 2.5 μM okadaic acid in DMSO (●). The line in panel D is a linear fit of only the MTP data.

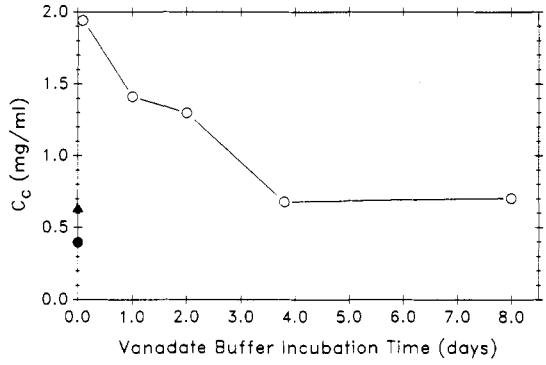

FIGURE 2: Critical concentration of MTP in 750 μM vanadate vs time of incubation of 1 mM vanadate at 4 °C. The filled circle (●) corresponds to no vanadate. The filled triangle (▲) corresponds to direct addition of vanadate from a 100 mM stock (see Results). All other samples (○) were equilibrated into 1 mM vanadate by column chromatography and diluted to 750 μM and a range of appropriate MTP concentrations to determine c_c. The decrease in inhibition of polymerization corresponds to a decrease in the amount of decavanadate present as determined by NMR (see Figure 3).

RESULTS

Polymerization of MTP in the Presence of Vanadate. Figure 1A shows the results of MTP polymerization in the presence of 750 μM vanadate. We observed an unusual dependence upon the age of the 1 mM vanadate solution. (By age, we mean the time a 1 mM vanadate solution in 0.1 M Pipes, pH 6.9, is incubated at 4 °C after dilution from a more concentrated stock, typically 100 mM, and prior to being used to equilibrate tubulin and initiate a polymerization study.) For example, when a 1 mM vanadate solution was aged 24 h at 4 °C, the MTP critical concentration increased by about 1.0 mg/mL, while polymerizations using a 1 mM vanadate solution aged 8 days showed a critical concentration near that of the control (Δc_c = 0.3 mg/mL; Figure 1A). Thus, time course experiments were carried out to determine the effect of aging vanadate solutions on the critical concentration of MTP (Figure 2). As can be seen, initially the critical concentration of MTP is significantly increased by the presence

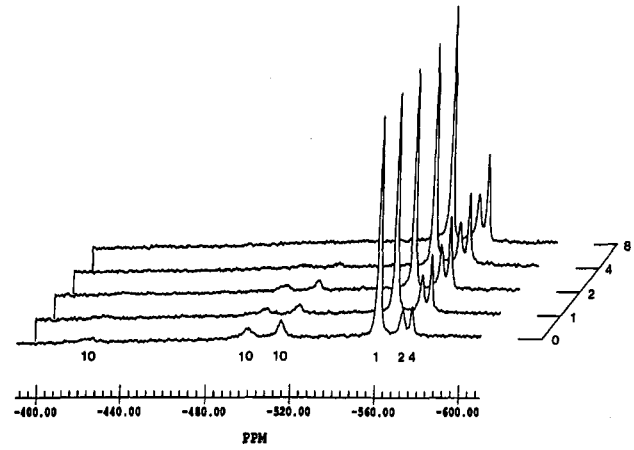

FIGURE 3: ^{51}V NMR of 3 mM vanadate, 0.1 M Pipes, pH 6.9, incubated at 4 °C for 0, 1, 2, 4, and 8 days. Note the loss of area under the decamer (labeled 10) peaks, corresponding to $V_{10}O_{28}^{6-}$, and the increase in the area of peaks corresponding to monomer (1), dimer (2), and tetramer (4), corresponding to $H_2VO_4^{1-}$, $H_2V_2O_7^{2-}$, and $V_4O_{12}^{4-}$, respectively. The half-time of reequilibration is estimated to be 3.3 days. At 25 °C, the half-time for decamer reequilibration is 9 h.

of 750 μM vanadate (Δc_c = 1.5 mg/mL), but incubation of the vanadate for 8 days returns the c_c to a value close to the control. The vanadate solutions are initially yellow, due to the presence of decavanadate, but gradually become clear. Therefore, we investigated the kinetics of vanadate species composition changes by ^{51}V NMR (Figure 3). NMR spectra of vanadate solutions taken over 8 days are shown in Figure 3, indicating the predominant vanadate species. It can be seen that the height of the decamer peaks declines, while the tetramer, dimer, and monomer peaks gradually increases over 8 days. The loss of inhibition of polymerization appears to be due to the disappearance of decamer. The half-time for reequilibration of decamer to tetramer, dimer, and monomer at 4 °C is estimated to be 3.3 days.[2] Electron microscopy revealed no difference in morphology of MTP microtubules in the presence or absence of vanadate (fresh or aged solutions). The addition of a tyrosine kinase inhibitor, 5 μM genistein, did not alter this vanadate effect (data not shown), suggesting it is not directly related to phosphorylation (Gordon, 1991). Thus, these data suggest that decavanadate strongly inhibits the polymerization of MTP, while tetrameric, dimeric, and/or monomeric vanadate only weakly affect polymerization. This could be due to direct interaction with tubulin or MAPs or both.

Polymerization of PC-Tubulin in the Presence of Vanadate. Small effects on critical concentrations were observed when vanadate was present in PC-tubulin solutions (Figure 4A). When vanadate was added directly from the 100 mM stock (fresh vanadate), a small increase in critical concentration was found (Δc_c = 0.2 mg/mL; Figure 4A). However, when the 1 mM vanadate stock was used 24 h after preparation, this effect was not observed, and the c_c values of PC-tubulin and PC-tubulin with vanadate were identical. These differences were verified by pelleting microtubules and determining the concentration of the supernatants. The weak effect of vanadate observed in Figure 4A was further tested by determining length distributions. The microtubules are slightly longer in the

[2] The experiment shown was done in the absence of EGTA, a chelator of monomeric vanadate (Gordon, 1991), and a common additive to microtubule polymerization buffers. The EGTA–vanadate complex has a broad ^{51}V NMR signal centered at −525 ppm. The inclusion of EGTA does not affect the kinetics of decamer reequilibration.

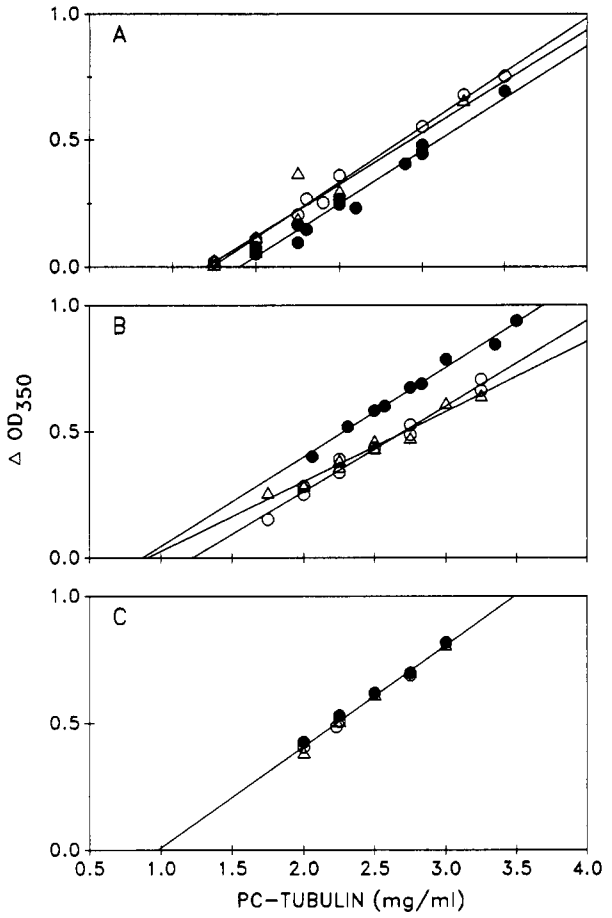

FIGURE 4: Critical concentrations of PC-tubulin in the absence or presence of phosphatase inhibitors. In all panels, PC-tubulin in the absence of phosphatase inhibitors is indicated by open circles (○). The lines are linear least-squares fits to the appropriate data set, except where noted. Critical concentrations were determined as in Figure 1. (A) 750 μM vanadate added directly from a fresh 100 mM vanadate solution (●) or 750 μM vanadate from a 1 mM vanadate stock allowed to aged 24 h prior to equilibration of the protein by column centrifugation (△). (B) 50 mM NaF (●) or 50 mM NaCl (△). (C) 2.5 μM okadaic acid (●) or 0.2% DMSO (△). The line in panel C is a joint linear fit to all the data shown. Note that the critical concentrations for native PC-tubulin vary for different tubulin preparations, and therefore the control critical concentrations may be different in each panel.

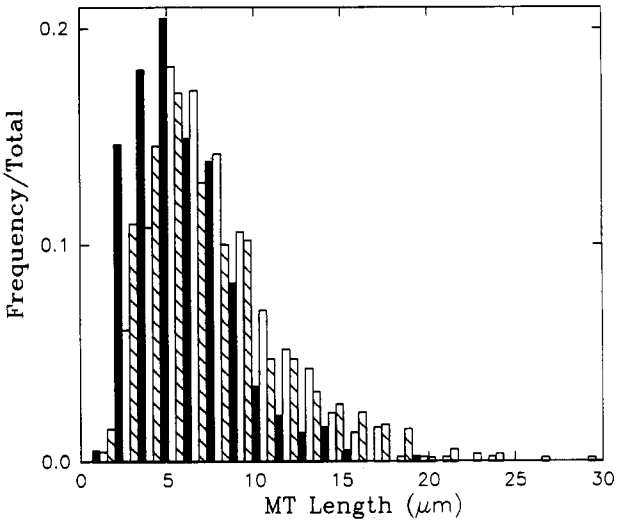

FIGURE 5: Length distribution of PC-tubulin microtubules in the presence or absence of vanadate. By *t*-test, there is a small effect of vanadate on PC-tubulin microtubule length distributions. The average lengths with fresh vanadate (white bars, 7.02 ± 3.5 μm) or vanadate incubated 24 h (hatched bars, 6.99 ± 4.53 μm) are slightly longer than with no vanadate black bars, 5.52 ± 2.87 μm).

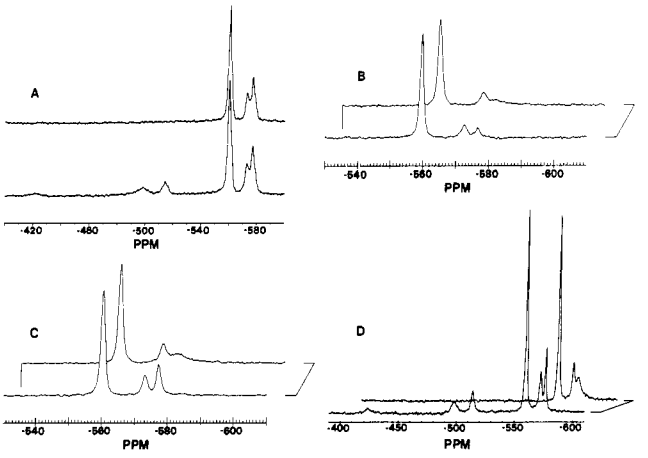

FIGURE 6: NMR spectra of vanadate in the absence and presence of (A) 3.0 mg/mL PC-tubulin, (B) 8.7 mg/mL MTP, (C) 2.1 mg/mL whole MAPs, and (D) 2.1 mg/mL whole MAPs. In (A) and (D), decavanadate is present; in (B) and (C), the vanadate was equilibrated to remove decamer.

presence of fresh 750 μM vanadate or 750 μM vanadate aged 24 h compared to vanadate-free solutions (Figure 5). Thus, vanadate does affect nucleation in glycerol buffer, although the effect on c_c is small (see Figure 5). There is no difference in microtubule morphology in the presence of vanadate. Microtubule polymerization is dependent upon GTP binding to tubulin, and thus vanadate could affect nucleotide binding. For example, Combeau and Carlier (1988) demonstrated vanadate stabilizes actin filaments by interacting with the γ-phosphate binding site. Nucleotide binding experiments showed that there was no detectable difference in the amount of GTP bound to PC-tubulin in the presence or absence of vanadate (fresh or 24 h after dilution to 1 mM; data not shown), indicating that inhibition of GTP binding does not account for the increase in c_c observed in the presence of fresh vanadate.

^{51}V NMR Binding Experiments. To further investigate the interaction of vanadate with tubulin and MAPs, direct binding experiments were conducted by monitoring changes in the ^{51}V NMR signals due to the various vanadate species in the presence of PC-tubulin, MTP, and purified MAP2 (Figure 6). The NMR spectra of 1 mM vanadate stock solutions show decamer, tetramer, monomer, and dimer peaks. PC-tubulin strongly interacts with decavanadate as evidenced by the loss of decavanadate signals at −424, −500, and −516 ppm (Figure 6A).[3] This suggests that the vanadate decamer binds to tubulin and results in an increase in the PC-tubulin critical concentration. MTP interacts with tetramer, as evidenced by the loss of tetravanadate signal at −576 ppm (Figure 6B), and thus, based upon the results with PC-tubulin (Figure 6A), the MAP fraction interacts with tetrameric vanadate. To test this hypothesis, whole MAPs, eluted from a PC column, were interacted with vanadate that had been aged to remove decamer (Figure 6C) or used fresh, and thus contained decamer (Figure 6D). Whole MAPs clearly interact with both tetrameric vanadate and decameric vanadate. Whole MAPs from porcine brain are composed of primarily MAP2 and tau. To investigate the interaction of vanadate

[3] Elsewhere we have shown that tetravanadate binds to tubulin weakly (K_d = 0.16 mM) and can be photoactivated to cleave tubulin at a number of sites on both α-tubulin and β-tubulin (Correia et al., 1994). Decavanadate is not a photoactive cleaver of tubulin.

with these MAPs, polymerizations were conducted with PC-tubulin in the presence of purified MAP2 or purified tau; 750 μM vanadate inhibited both MAP2 and tau-induced assembly (data not shown). Thus, the inhibition of MTP assembly is due to an effect on all MAPs present, and not just an effect on the predominant MAP present in these preparations, MAP2.

To further investigate this interaction, quantitative binding experiments were conducted with PC-tubulin and with purified MAP2. A fixed amount of vanadate was titrated with either PC-tubulin or purified MAP2 and examined by ^{51}V NMR (Figure 7). Decamer concentrations decreased with added protein, but there was no evidence of tetramer binding at these concentrations of MAP2, suggesting weaker affinity. The decamer data can be described by a simple binding isotherm where $K_{app} = nK_a$, the number of independent, noninteracting sites times the association constant (Figure 7C). The maximum r values (moles of V_{10} bound per mole of macromolecule) observed were 3.5 for tubulin and 20 for MAP2. A Scatchard plot of the MAP2 data gave an n of 25.8 and a $K_a = 1.3 \times 10^5$ M^{-1}. (A Scatchard plot of the tubulin data was inconclusive due to extensive scatter.) The data were thus transformed to site concentrations assuming 26 sites on MAP2 and 4 sites on PC-tubulin. The tubulin data were fit to a quadratic, i.e., a single site binding curve (Loontiens et al., 1990), and the results were plotted as [V_{10}]$_{free}$ vs log sites] (Figure 7C, insert). The resultant $K_a = 1.0 \times 10^5$ M^{-1}. A simulated curve for the MAP2 data using the same K_a, 1.1 $\times 10^5$ M^{-1}, and the initial [V_{10}], 27 μM, is consistent with the trend of the data and the estimated value from a Scatchard plot. Thus, we conclude (1) V_{10} binds with the same affinity to PC-tubulin and to MAP2, and (2) there are approximately 4 sites on tubulin and 26 sites on MAP2 for V_{10} binding.[4] These results suggest that the identical binding affinities may correspond to similar binding sites, possibly single side chains or a cluster of side chains on the protein surface. The extended structure of MAP2 accounts for the large number of sites relative to tubulin. Presumably only a few of these sites are in the microtubule binding tandem repeats. Thus, when vanadate is added to MTP, 4 sites on tubulin and 26 sites on MAP2 compete for V_{10} binding and partially buffer the inhibitory effect. This is why direct addition of 750 μM vanadate from a 100 mM stock does not cause significant inhibition of MTP assembly [see Experimental Procedures and Figure 2 (▲)]. However, when MTP is equilibrated by chromatography, the free concentration of the initial V_{10} solution is maintained at 30–40 μM, and the sites in the tandem repeats are nearly saturated, thus leading to inhibition. As the vanadate species reequilibrate, the inhibition decreases with decreasing decamer concentration. We cannot exclude, and would in fact predict, binding to a single repeat causing a reduction in MAP2 affinity for microtubules. This is similar to what is observed for tau binding (Butner & Kirschner, 1991). We also cannot exclude a cooperative interaction between V_{10} sites in nonrepeat regions, or cooperative effects caused by MAP–MAP interactions (Wallis et al., 1993).

Displacement of MAPs from Microtubules by Vanadate. To verify the mechanism of decavanadate inhibition of MTP assembly, microtubules were pelleted and then the supernatants and pellets run on an SDS gel to demonstrate release of MAPs. Figure 8 presents the results of MTP alone compared with 750 μM vanadate enriched with decavanadate (aged 3 h) or tetravanadate (aged 8 days). Decavanadate clearly causes a release of MAPs (here MAP2 is most clearly visible at the top of the lane), while tetravanadate only causes

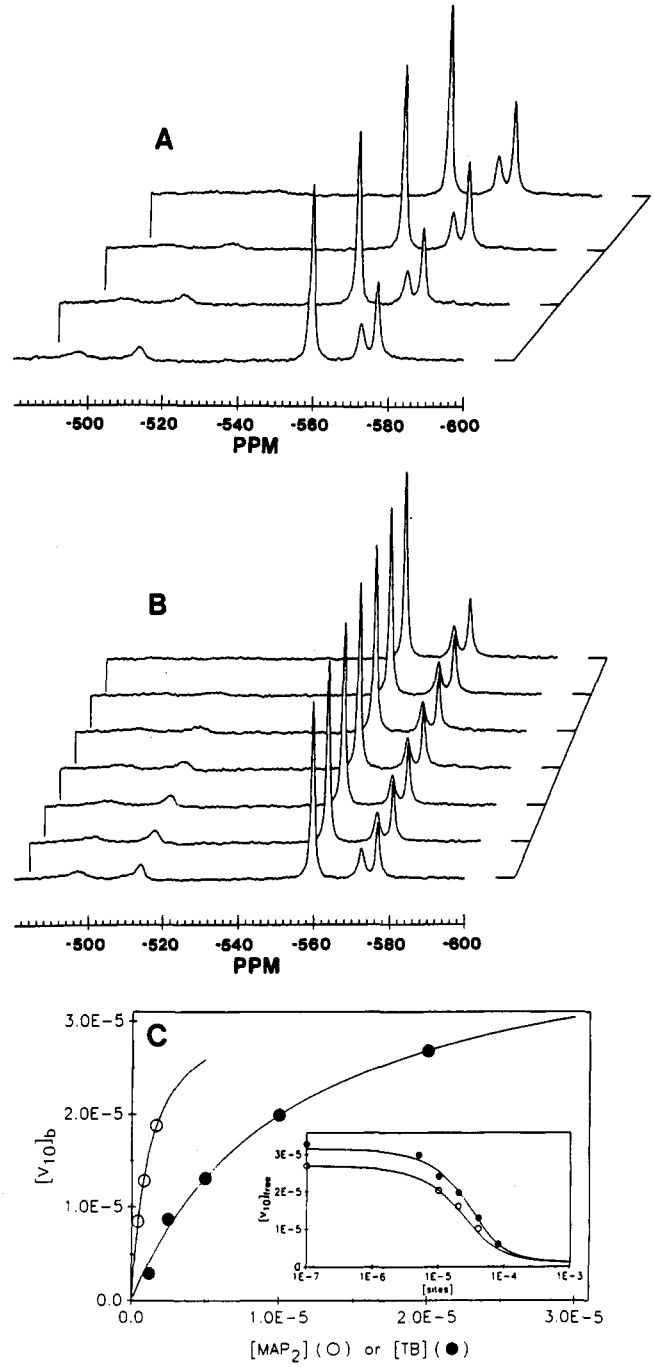

FIGURE 7: Binding of tubulin or MAP2 to decamer by ^{51}V NMR. Note the small decamer peak at –424 ppm is not shown. (A) Titration of 2 mM vanadate with purified MAP2 at 0, 0.42, 0.84, and 1.68 μM (front to back). (B) Titration of 2 mM vanadate with PC-tubulin at 0, 1.25, 2.5, 5.0, 10.0, 20.0, and 30.0 μM (front to back). (C) Bound decamer plotted vs total [MAP2] or total [TB] and fit to a simple binding curve to show the trend of the data. Insert: The data are transformed to [sites] by multiplying the [MAP2] by 26 and the [TB] by 4. The tubulin data are fit to a polynomial form of a binding curve, involving K and adjustable parameters for the initial and final [V_{10}], and displayed as [V_{10}]$_{free}$ vs log sites]. The resultant K_a, 1.0 $\times 10^5$ M^{-1}, is used to simulate an expected binding curve for MAP2. The initial [V_{10}] for each experiment is plotted at 1×10^{-7} to indicate the isotherm limit.

[4] The estimated affinity, 1.0×10^5 M^{-1}, may be a lower limit. This is because the resultant K_d (9.1 μM) is 3–4-fold below the experimental [V_{10}], and thus the curve is shifted by stoichiometric binding; i.e., the midpoint of the titration is at half the initial [V_{10}] (Loontiens et al., 1992; Chaires, 1990). The experiments cannot be done at V_{10} concentrations below the K_d, however, because the V_{10} NMR signal is too small to accurately integrate.

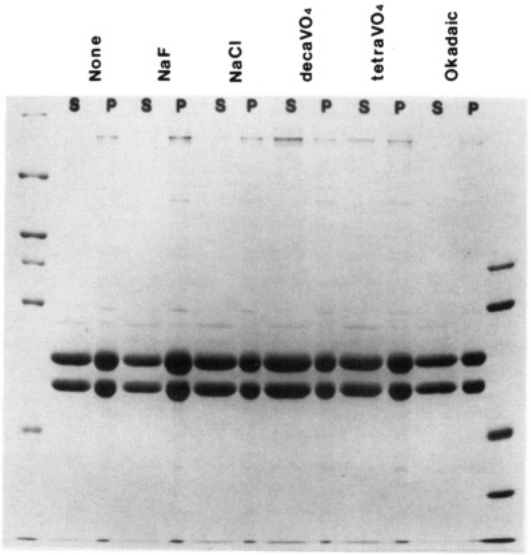

FIGURE 8: SDS–PAGE of MAP binding in the absence or presence of phosphatase inhibitors. MTP was polymerized and pelleted as described under Experimental Procedures. Forty microliters of supernatants or 6 μL of pellets in SDS sample buffer was loaded onto an 8% slab polyacrylamide gel. The gel was stained with Coomassie brilliant blue R-250. Lane 1, high molecular weight markers: myosin, 200 000; β-galactosidase, 116 250; phosphorylase B, 97 400; bovine serum albumin, 66 200; ovalbumin, 45 000. Lanes 2, 4, 6, 8, 10, and 12 are supernatants from MTP only, or MTP with added 50 mM NaF, or 50 mM NaCl, or solutions enriched for decavanadate or tetravanadate (750 μM total), or 2.5 μM okadaic acid, respectively; and lanes 3, 5, 7, 9, 11, and 13 are pellets from the same samples. Lane 14, low molecular weight markers: phosphorylase B, 97 400; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500.

a small release of MAPs, consistent with the small increase observed in c_c. A similar experiment was carried out with purified tau (3 μM) and PC-tubulin (30 μM). Microtubules were pelleted, and Western blot analysis with tau-1 monoclonal antibody was used to determine the effect of fresh or aged vanadate solutions on tau binding to tubulin. The results showed a similar inhibition of tau binding by decavanadate (data not shown). Thus, we conclude that decavanadate binds directly to MAPs and inhibits their binding to microtubules.

Polymerization of MTP in the Presence of Fluoride or Okadaic Acid. The effect of 50 mM NaF on MTP is shown in Figure 1B, where the critical concentration decreases to near zero. With 50 mM NaCl, MTP polymerization is slightly inhibited (Figure 1B). When 50 mM NaF was added to MTP solutions with 750 μM vanadate (aged 24 h), the critical concentration was found to be between the values expected with either additive (Figure 1C). This suggests NaF can partially reverse the influence of decavanadate, possibly by directly stimulating tubulin assembly. No change of critical concentration of MTP was observed when 2.5 μM okadaic acid was added to solutions (Figure 1D).

In order to investigate the effect of these phosphatase inhibitors on MAP binding to microtubules, MTP was polymerized in the presence of 50 mM NaF or NaCl, or 2.5 μM okadaic acid, and then the microtubules were pelleted. SDS–PAGE was used to analyze the MAP composition of the supernatants and pellets (Figure 8). When 50 mM NaCl is in solution, MAPs are weakly displaced, suggesting an inhibition of MAP binding to tubulin, and consistent with the slight inhibition observed (Figure 1B). NaF or okadaic acid does not cause a release of MAPs.

Polymerization of PC-Tubulin in the Presence of Fluoride or Okadaic Acid. When 25, 50, 75, or 100 mM NaF was added to PC-tubulin solutions, the critical concentration decreased with increasing amounts of NaF. These differences in critical concentrations were independently verified by pelleting experiments. K_p, the propagation constant for subunit addition to the growing end of the microtubule, may be defined as $K_p = 1/c_c$ (Lee & Timasheff, 1977). A Wyman plot of ln K_p vs ln [NaF] gave a value of d ln K/d ln [NaF] = 0.214 (Wyman, 1964; Lee & Timasheff, 1977). The positive slope indicates that ions are preferentially bound to the polymer form relative to the tubulin subunits. The value 0.214 means that one ion is bound per four to five subunits assembled. "Binding" is used in the thermodynamic sense, meaning not necessarily bound to a specific site, although specific site binding is not excluded. Titrations with NaCl and NaF mixed together, to give a total concentration of 50 mM, lowered the critical concentration to the same extent as observed with NaF alone. This observation indicates a nonspecific ionic strength effect (Figure 4B). [Note, however, that the slope of the ΔOD_{350} vs [TB] curves is very different for NaCl than for NaF or the control experiment (Figure 4B; see Discussion).] These results demonstrate that the lower critical concentration is not due to direct interaction of F^- with tubulin, since the chloride salt is as effective as the fluoride salt, and therefore that the effect is not a specific ion effect, but rather a general ionic strength effect on polymerization. This could arise from screening of charge repulsion between tubulin subunits, probably due to the interaction of Na^+ with the acidic carboxyl region of tubulin subunits. This is consistent with the partial reversal of NaF on the decavanadate inhibition effect described above. Finally, when okadaic acid was added, there was no effect on the critical concentration (Figure 4C). This result was verified by microtubule pelleting experiments. Mean microtubule lengths in the presence of 2.5 μM odadaic acid were the same as found for PC-tubulin alone, 6.02 ± 2.25 μm vs 5.52 ± 2.87 μm (see Figure 5).

DISCUSSION

It is generally assumed that phosphatase inhibitors only affect dephosphorylation events [see Gordon (1991) for a discussion of this point]. The experiments reported here were undertaken to test for the direct interaction of typical phosphatase inhibitors on microtubule protein. NaF stimulates MTP assembly while NaCl inhibits MTP assembly. This suggests that the formation of water structure by the more kosmotrope ion F^- stabilizes polymer formation and compensates for an ionic strength effect on MAP binding (Collins & Washabaugh, 1985). This assignment would be consistent with the anion dominance of the Hofmeister effects on water structure. With PC-tubulin, both NaF and NaCl are stimulatory, in part because of the different polymerization conditions. In 2 M glycerol, polymerization is driven by preferential interactions with glycerol (Lee & Timasheff, 1977), and the anion effect is relatively unimportant. Thus, increasing ionic strength will stimulate assembly through an effect of Na^+, unless MAP binding is essential for microtubule stabilization, and then ionic strength will inhibit polymerization by reducing MAP binding. Note that NaCl also causes a dramatic change in the slope of the ΔOD_{350} vs [TB] plot for PC-tubulin. The slope is 0.278 for NaCl vs 0.374 ± 0.043 for all the other PC-tubulin data presented. This is not due to a significant change in microtubule lengths or morphology, as verified by EM. This effect could be due to disruption or enhancement of macroscopic microtubule organization (bundling or aligned macroscopic domains) by NaCl. Current studies in our laboratory are concerned with the influence of

microtubule ordering on turbidity and the wavelength dependence of light scattering.

Our results clearly show that monomeric vanadate and okadaic acid have no direct effect on the polymerization of microtubule protein or PC-tubulin. However, vanadate oligomers do affect polymerization. Decavanadate weakly inhibits PC-tubulin polymerization and strongly inhibits MAP binding to microtubules. Tetravanadate also binds to MAPs but only weakly inhibits MAP binding to microtubules and MAP-stimulated microtubule assembly. Thus, care must be taken in preparing vanadate solutions for biological studies involving microtubules. Vanadate oligomers form in a pH, temperature, buffer, and vanadate concentration dependent manner (Pettersson et al., 1983, 1985; Chasteen, 1990; Correia et al., 1994). The titration of vanadate with H^+ generates oligomers, especially decamers, that slowly reequilibrate in neutral solutions (Goodno, 1982; Pettersson et al., 1983, 1985). As we report here, overnight incubation at room temperature or extensive incubation in the cold will reequilibriate decameric vanadate to smaller oligomeric forms. The final fraction of oligomer is, of course, vanadate concentration dependent. Boiling basic vanadate solutions (pH 10) is reported to convert all oligomers to monomeric vanadate (Goodno, 1982; Gordon, 1991), although basic solutions are not convenient for microinjection or cell culture studies, and direct addition of vanadate to buffered solutions near neutral pH can cause a significant shift in pH (Gordon, 1991). Heating of concentrated vanadate solutions (100 mM) near neutral pH (7.8) is reported to remove colored decavanadate (Combeau & Carlier, 1988), although this will not necessarily dissociate smaller oligomeric forms, i.e., tetramer.

The locations of the multiple sites of decavanadate binding to MAPs or tubulin are not known. Vanadate is reported to interact with numerous amino acids. Being a so-called "hard acid", vanadate has an affinity for "hard bases", i.e., oxides, alkoxides, etc. (Ho, 1975). It readily binds to alcohol groups to form esters (Gresser & Tracey, 1985), and it is known to interact with serine and tyrosine groups of protected amino acids and peptides and possibly the ϵ-amino group of lysine (Crans et al., 1989, 1992; Tracey & Gresser, 1986; Jaswal & Tracey, 1991; Wittenkeller et al., 1991). Binding to thiolate ($R-S^-$) cysteine or thioether (R–S–R) methioine, which are soft bases, would be less likely (Crans & Simone, 1991; Huheey, 1983). Numerous workers have shown the tendency of tetravanadate to bind to proteins with high affinity (Cremo et al., 1989, 1990; Ringel et al., 1990). These binding sites probably represent clusters of positive charge on the protein surface. The binding site for tetrameric vanadate is believed to involve basic residues, two lysines and an arginine for Cu,Zn-superoxide dismutase (Wittenkeller et al., 1991), and a cluster of basic residues for myosin (Ringel et al., 1990). By analogy, MAP2 has 28 sites where 2–4 basic amino acids are contiguously clustered. If exposed on the surface, these could represent decamer binding sites. None of these basic clusters are in the tandem microtubule binding domains. However, one cluster is in the first spacer region (QVQIVTKKID; Lewis et al., 1988), and decavanadate binding to this region could disrupt interactions with tubulin. A similar cluster of positive charge occurring in the first linker region of human tau (KVQIINKKLD; Jakes et al., 1991) has recently been implicated in microtubule binding (Goode & Feinstein, 1994). (The porcine tau sequence is not known.) The structure of decavanadate (Figure 9) suggests a large complementary region of positive charge on any protein surface may be sufficient for binding. A potential method of determining the

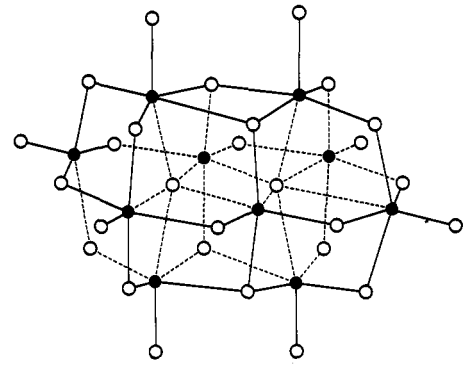

FIGURE 9: Perspective view of the structure of decavanadate ($V_{10}O_{28}^{6-}$) as described by Swallow et al. (1966) and Debaerdemaeker et al. (1982). The atoms shown are vanadium (●) and oxygen (○). The unit cell is approximately 16.8 × 10.2 × 10.9 Å.

binding sites for vanadate decamer is photoactivated cleavage of MAPs. However, recent experiments suggest that tetravanadate and not decavanadate is a photoactive cleaver of tubulin (Correia et al., 1994). If this also applies to MAP2 and tau, then photocleavage experiments would not reveal the sites of decavanadate binding unless decamer and tetramer bound to the same sites. Our titration experiments suggest that decavanadate binds with a higher affinity to MAP2 than tetravanadate, but this is not necessarily consistent with different interaction sites.

The results of this study are most applicable to experiments *in vitro*. Vanadate has been used for microinjection and cell culture studies to ascertain the role of phosphatases on various cellular processes [reviewed in Gordon (1991)]. Smith (1983) suggested the mitogenic properties of vanadate were similar to those of colchicine, and proposed a common mechanism of action. Sawin and Mitchison (1991) found vanadate at 500 μM concentrations caused spindle disruption in cell-free extracts, suggesting a possible role for interaction with MAPs or other factors involved with microtubule stabilization. A role for vanadate oligomers may be indicated. Cell culture work is often done at 1–5 mM vanadate [see Gordon (1991)], concentrations that would favor oligomers. However, transport across the cell membrane will probably be limited to monomeric vanadate, although this is not known. Once in the cell, the reducing conditions will eventually convert vanadate to vanadyl (VO_2^+) (Gordon, 1991). Nonetheless, our results with MTP are consistent with previous reports that vanadate has a wide range of effects that are not directly related to phosphatase inhibition (Combeau & Carlier, 1988; Gordon, 1991).

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REFERENCES

Binder, L. I., Frankfurter, A., & Rebhun, L. I. (1985) *J. Cell Biol. 101*, 1371–1378.

Bock, R. M., Ling, N.-S., Morell, S. A., & Lipton, S. H. (1956) *Arch. Biochem. Biophys. 62*, 253–264.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Brautigan, D. L., & Shriner, C. L. (1988) *Methods Enzymol. 159*, 339–346.
Butner, K. A., & Kirschner, M. W. (1991) *J. Cell Biol. 115*, 717–730.
Carlier, M.-F., Didry, D., Melki, R., Chabre, M., & Pantaloni, D. (1988) *Biochemistry 27*, 3555–3559.
Carlier, M.-F., Didry, D., Simon, C., & Pantaloni, D. (1989) *Biochemistry 28*, 1783–1791.
Chaires, J. B. (1990) in *Molecular Basis of Specificity in Nucleic Acid–Drug Interactions* (Pullman, B., & Jortner, J., Eds.) pp 123–136, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Chasteen, N. D. (1990) *Vanadium in Biological Systems*, 225 pp, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Cleveland, D. W., Shu-Ying, H., & Kirschner, M. K. (1977) *J. Mol. Biol. 116*, 207–225.
Cohen, P. (1991) *Methods Enzymol. 201*, 389–398.
Collins, K. D., & Washabaugh, M. W. (1985) *Q. Rev. Biophys. 18*, 323–422.
Combeau, C., & Carlier, M.-F. (1988) *J. Biol. Chem. 263*, 17429–17436.
Correia, J. J., Baty, L. T., & Williams, R. C., Jr. (1987) *J. Biol. Chem. 262*, 17278–17284.
Correia, J. J., Lipscomb, L. D., Dabrowiak, J. C., Isern, N., & Zubieta, J. (1994) *Arch. Biochem. Biophys. 309*, 94–104.
Crans, D. C., & Simone, C. M. (1991) *Biochemistry 30*, 6734–6741.
Crans, D. C., Bunch, R. L., & Theisen, L. A. (1989) *J. Am. Chem. Soc. 111*, 7597–7607.
Crans, D. C., Sudhakar, K., & Zamborelli, T. J. (1992) *Biochemistry 31*, 6812–6821.
Cremo, C. R., Grammer, J. C., & Yount, R. G. (1989) *J. Biol. Chem. 264*, 6608–6611.
Cremo, C. R., Long, G. T., & Grammer, J. C. (1990) *Biochemistry 29*, 7982–7990.
Croom, H. B., Correia, J. J., Baty, L. T., & Williams, R. C., Jr. (1985) *Biochemistry 24*, 768–775.
Debaerdemaeker, T., Arrieta, J. M., & Amigo, J. M. (1982) *Acta Crystallogr. B38*, 2465–2468.
Detrich, W. D., III, & Williams, R. C., Jr. (1978) *Biochemistry 17*, 3900–3907.
Drago, R. S. (1992) *Physical Methods in Chemistry*, Saunders, New York.
Edde, B., Denoulet, P., de Nechaud, B., Koulakoff, A., Berwald-netter, Y., & Gros, F. (1989) *Biol. Cell 65*, 109–117.
Glickman, N. R., Parsons, S. F., & Salmon, E. D. (1992) *J. Cell Biol. 119*, 1271–1276.
Goedert, M., Cohen, E. S., Jakes, R., & Cohen, P. (1992) *FEBS Lett. 312*, 95–99.
Goode, B. L., & Feinstein, S. C. (1994) *J. Cell Biol. 124*, 769–782.
Goodno, C. C. (1982) *Methods Enzymol. 85*, 116–123.
Gordon, J. A. (1991) *Methods Enzymol. 201*, 477–482.
Gresser, M. J., & Tracey, A. S. (1985) *J. Am. Chem. Soc. 107*, 4215–4220.
Hardie, D. G., Haystead, T. A. J., & Sim, A. T. R. (1991) *Methods Enzymol. 201*, 469–476.
Hernandez, M. A., Avila, J., & Andreu, J. M. (1986) *Eur. J. Biochem. 154*, 41–48.
Ho, T.-L. (1975) *Chem. Rev. 75*, 1–20.
Holmes, C. F. B., & Boland, M. P. (1993) *Curr. Opin. Struct. Biol. 3*, 934–943.
Huheey, J. E. (1983) *Inorganic Chemistry*, 3rd ed., Harper and Row, New York.
Jakes, R., Novak, M., Davison, M., & Wischik, C. M. (1991) *EMBO J. 10*, 2725–2729.
Jaswal, J. S., & Tracey, A. S. (1991) *Can. J. Chem. 69*, 1600–1607.
Khandelwal, R. L. (1977) *Biochim. Biophys. Acta 485*, 379–390.
Kowalski, R. J., & Williams, R. C., Jr. (1993) *J. Biol. Chem. 268*, 9847–9855.
Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.
Lange, A. J., Arion, W. J., Burchell, A., & Burchell, B. (1986) *J. Biol. Chem. 261*, 101–107.
Lee, J. C., & Timasheff, S. N. (1977) *Biochemistry 16*, 1754–1764.
Lewis, S. A., Wang, D., & Cowan, N. J. (1988) *Science 242*, 936–939.
Loontiens, F. G., Regenfuss, P., Zechel, A., Dumortier, L., & Clegg, R. M. (1990) *Biochemistry 29*, 9029–9039.
Murphy, A. J., & Coll, R. J. (1992) *J. Biol. Chem. 267*, 5229–5235.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Pettersson, L., Andersson, I., & Hedman, B. (1985) *Chem. Scr. 25*, 309–317.
Pettersson, L., Hedman, B., Andersson, I., & Ingri, N. (1983) *Chem. Scr. 22*, 254–264.
Pot, D. A., & Dixon, J. E. (1992) *Biochim. Biophys. Acta 1136*, 35–43.
Ringel, I., Peyser, Y., & Muhlrad, A. (1990) *Biochemistry 29*, 9091–9096.
Sawin, K. E., & Mitchison, T. J. (1991) *J. Cell Biol. 112*, 941–954.
Sim, A. T. R. (1991) *Mol. Neurobiol. 5*, 229–246.
Smith, J. B. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6162–6166.
Swallow, A. G., Ahmed, F. R., & Barnes, W. H. (1966) *Acta Crystallogr. 21*, 397–405.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Tracey, A. S., & Gresser, M. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 609–613.
Vonck, J., & van Bruggen, E. F. J. (1990) *Biochim. Biophys. Acta 1038*, 74–79.
Wallis, K. T., Azhar, S., Rho, M. B., Lewis, S. A., Cowan, N. J., & Murphy, D. B. (1993) *J. Biol. Chem. 268*, 15158–15167.
Williams, R. C., Jr., & Lee, J. C. (1982) *Methods Enzymol. 85*, 376–408.
Wittenkeller, L., Abraha, A., Ramasamy, R., de Freitas, D. M., Thiesen, L. A., & Crans, D. C. (1991) *J. Am. Chem. Soc. 113*, 7872–7881.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 223–286.