Promotion of Microtubule Assembly by Oligocations: Cooperativity between Charged Groups

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ABSTRACT: The rate and, to a lesser degree, the extent of microtubule assembly from rat brain tubulin is enhanced by oligocations such as polyamines, melittin, polybasic drugs, oligolysines, and oligoarginines. The effect is cooperative for degrees of polymerization up to seven for oligolysines and up to five for oligoarginines and is interpreted as an interaction with up to seven closely spaced anionic charges. Microtubules so formed appear to be normal by electron microscopy, and by salt, colchicine, and cold sensitivities. Lysyl residues in excess of seven (or five for arginine) in larger oligomers interact nearly noncooperatively. Separation of lysyl charges by intercalation of alanyl residues reduced assembly promoting potency for hexalysines. The cooperative portion of the response is most likely associated with the highly acidic extreme C termini of tubulin because their removal with limited subtilisin treatment markedly reduces oligolysine potency. However, some cooperative interactions with oligocations can also occur with more widely spaced anionic charges elsewhere in tubulin. The potential role of oligocations in the intracellular regulation of microtubule assembly is discussed.

Tubulin is a heterodimer composed of two similar acidic monomers, α and β tubulin, of ∼50 kDa each. The α monomer has 16 and the β monomer has 22 excess anionic charges in pig brain tubulin. Most mammalian tubulins have similar excess carboxylates. The anion excess has a highly asymmetric distribution—of the 20 C terminal amino acids in each monomer, α tubulin has 10 and β tubulin 11 Glu or Asp residues without intervening basic amino acids. Post-translational γ glutamylation of either C terminus can accentuate the charge asymmetry by up to six additional Glu residues each (*1*−*3*); a recent report shows that α-4 tubulin may have as many as 11 added Glu residues (*4*). This anion excess is an important factor that regulates the equilibrium between dimer and microtubules. A portion of these C terminal anions can be removed by limited proteolysis with subtilisin. This cleaves α tubulin after residue 438 and β tubulin after residue 433, removing seven coded acidic residues in α tubulin of a total of 16 and 8 acidic residues in rat brain β tubulin of a total of 22. In this product, called tubulin S,[1] polymerization is markedly favored as measured by a lowered critical concentration for polymerization (*5*−*7*). The same result can be achieved by methylation or amidation of the carboxylates involved, although this reaction is not limited to the C termini (*8*). In addition, it has been known for a long time that basic proteins or polymers promote tubulin assembly to microtubules or other forms (*9*−*13*), and many subsequent studies have confirmed this (e.g., refs *14*−*18*). These findings led to the deduction, and later demonstration, that microtubule associated proteins (MAPs), such as MAP_2 or tau, owed a part of their assembly-promoting properties to the repeat basic patches along their primary sequence (*7, 18, 19*).

Recently we showed that monocations can also promote tubulin polymerization, albeit at high concentration. Charge alone was sufficient (*20*), but the additional ability for hydrogen bonding to the anions as, for example, in guanidine hydrochloride, markedly enhanced the potency of the cation, whereas methyl substitution for the hydrogens diminished or abolished this enhancement (*21*). The potency of the cations yielded a selectivity series characteristic for poly-glutamic acid (*22*), suggesting that the glu-rich C termini of α and β tubulin were involved in the cation effect. The close spacing of these residues in the C termini raised the possibility that there might be multiple charge interactions with the C termini if oligocations were used. In the present study we have used diamines, polyamines, oligolysines, and oligoarginines to examine this question.

MATERIALS AND METHODS

Materials. Polyamines and some polylysine preparations were from Sigma, oligolysine and oligoarginine hydrochlorides (to DP = 5) were from Research Plus Inc. Larger oligolysine, oligoarginine, and alanine/lysine peptides were prepared by Mr. George Poy, NIDDK, with an Applied Biosystems 430A Peptide Synthesizer. They were purified by HPLC using a MonoS cation exchange column. 4-Trifluoromethyl-7VKKR-coumarone was from AnaSpec Inc., hexadimethrine HBr from Aldrich, netropsin from Serva, hirudonine from ICN, and the bis-guanidino alkanes were the generous gift of Dr. Jack Folk, NIDR. Melittin 15−26 was a gift from Schering AG.

Rat brain tubulin was prepared from microtubule protein (*23*) by sequential polymerization in 1.6 M Mes, pH 6.9, and 1.0 M sodium glutamate (*24*): solid, neutralized Mes

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; DMSO, dimethyl sulfoxide; DP, degree of polymerization; tubulin S, tubulin treated by limited subtilisin proteolysis to remove the extreme C termini from both monomers.

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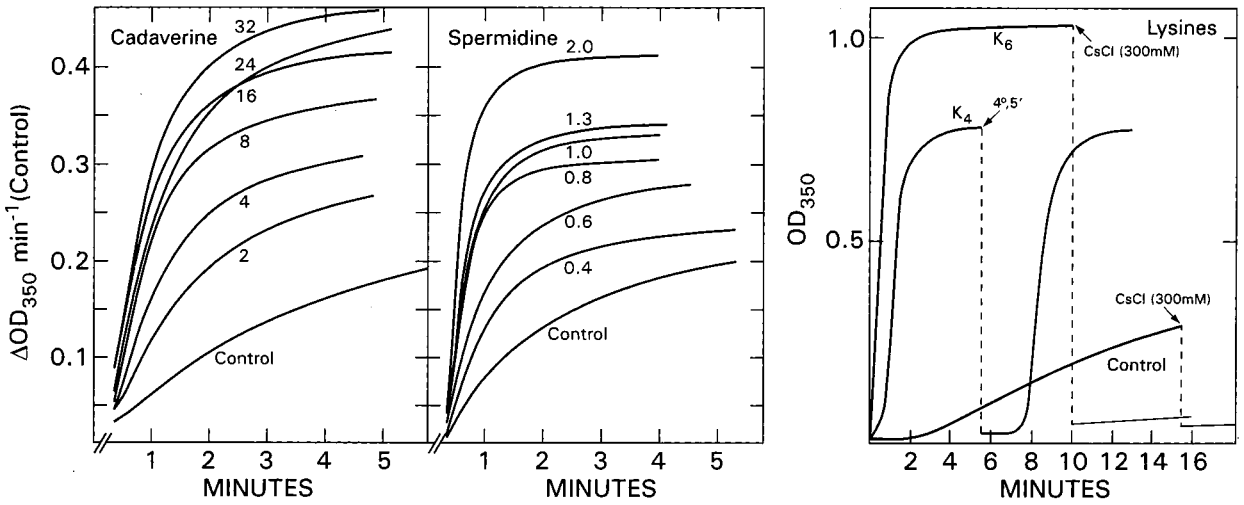

FIGURE 1: Polymerization progress curves of rat brain tubulin treated with oligocations. Pure tubulin (12 μM) in Mes assembly buffer with 0.8 mM GTP at 25.5 °C. Left and center panels with 10 μM taxol. Right panel with 15% DMSO. Left panel: cadaverine in mM; center panel: spermidine in mM; right panel: hexalysine (K_6) 200 μM and tetralysine (K_4) 600 μM; at the arrows 300 mM CsCl was added or cooling on ice was instituted for 5 min.

was added to microtubule protein containing 1 mM dithiothreitol and 1 mM GTP (2 M, 530 mg/mL). Final concentrations were adjusted to 1.6 M Mes, and 1 mM each for GTP and dithiothreitol. The mixture was incubated for 1 h at 37 °C and centrifuged in a Beckman Ti45 rotor at 30 000 rpm, at 33 °C for 45 min. Pellets were resuspended in ~1/3 the starting volume with cold 1.0 M sodium glutamate, pH 7.0, sonicated, left on ice for 2 h, and centrifuged at 4 °C for 30 min in a Ti70.1 rotor at 35 000 rpm. The supernatant solution was brought to 0.1 mM dithiothreitol and 1 mM GTP, incubated at 37 °C for 45 min, and centrifuged in a Ti70.1 rotor for 30 min at 35 000 rpm at 33 °C. Pellets were resuspended in Mes assembly buffer (1 mM $MgCl_2$, EGTA, in 0.1 M Mes, pH 6.9), sonicated, incubated on ice for 45 min, and centrifuged in a Ti70.1 rotor at 4 °C for 30 min at 35 000 rpm. The supernatant protein concentration was adjusted to 25 mg/mL and drop-frozen and stored in liquid nitrogen. Taxol, a gift from Dr. Matthew Suffness, National Cancer Institute, was dissolved in DMSO; the final DMSO concentration was 1.0%.

Rat brain tubulin S was prepared from rat brain tubulin (3−5 mg/mL) in diluted Mes assembly buffer with 1 mM GTP by addition of subtilisin (Carslberg) in a weight ratio of 1:100 and incubated at 30 °C for 45 min. The reaction was stopped with 0.1% phenylmethyl sulfonyl fluoride in DMSO, placed on ice for 20 min, and centrifuged at 18000*g* at 4 °C for 20 min in a Beckman Ti70.1 rotor. The supernatant showed >98% β_s and >95% α_s. Under these conditions subtilisin did not cleave tubulin at other sites. Protein was assayed by the bicinchoninic acid method and tubulin S was drop-frozen and stored in liquid nitrogen.

Polymerization Methods. Both taxol- and DMSO-driven polymerization was employed using turbidity development at 350 nm in 250 μL cells (1 cm light path) at 25.5 ± 0.3 °C in Mes assembly buffer, pH 6.9, in thermostated cell holders of a Cary 219 instrument. In most studies polymerization was carried out in 10% DMSO or 10 μM taxol, and 0.8 mM GTP, and the reaction was started by addition of tubulin to prewarmed (27 °C) reagents. Except as indicated, ionic strength was not varied in these experiments. Rates of polymerization were measured starting at 20−30 s after addition of tubulin to the prewarmed mixture of reagents. Progress curves were continued until a clear falling-off of the rate was established (4−15 min). In some experiments, polymerization was followed to plateau turbidity (up to ~30 min), followed by cooling on ice for 5−10 min in order to measure the extent of cold depolymerization. Stored tubulin S preparations were cleared by 5 min centrifugation in an Airfuge running at 30 psi before use. The critical concentrations of this material (measured by turbidity development) varied from 0.5 to 0.9 μM.

Electron Microscopy. Tubulin (21 μM) was incubated with hexalysine or hexaarginine as above for 30 min, centrifuged at room temperature for 10 min in an Airfuge at 30 psi, decanted, and exposed to a glutaraldehyde−tannic acid fixing solution for 30 min at room temperature and then at 4 °C overnight. Markham rotations were carried out by Mr. Jan Endlich of JFE Enterprises, Brookville, MD, to whom we are most grateful. For negative staining, tubulin samples were preincubated with pentalysine or pentaarginine for 30 min at 37 °C, applied to carbon/Formvar grids for 10−15 s, and stained with 1% filtered uranyl acetate.

RESULTS

Polyamines. Rat brain tubulin polymerized at 25.5 °C in 10 μM taxol and 0.8 mM GTP (in Mes assembly buffer, pH 6.9) showed an increase in both the rate and, to a lesser degree, the extent of OD_{350} generation with various diamines as exemplified by cadaverine (1,5-diamino pentane) in Figure 1, left panel. Rates (ΔOD_{350}/min) increased up to a maximum concentration of 32 mM, followed by decreased polymerization at higher concentrations (data not shown). For ease of comparison of potent and less potent enhancers of polymerization, we used as criterion the concentration of oligoamine required to attain a 3-fold increase in maximal rate as shown in Table 1. While the highest concentrations may contribute to the ionic strength of the medium, this effect is not significant at concentrations required to triple the maximal rate. Moreover, with trivalent amines and oligolysines (see below), no significant change in the ionic strength occurs. The maximum rate increase was 6−7-fold, whereas the extent of OD generation never exceeded 2−3-fold in

Table 1: The Effect of Diamines, Polyamines, and Certain Guanidinium Derivatives on the Rate of Polymerization of Rat Brain Tubulin[a]

compound	concentration for triple rate: whole molecule (mM)	concentration for triple rate: per N or guanidino (mM)
diamines (HCl)		
1,2-ethylenediamine	2.5	5.0
tetramethyl ethylenediamine	10	20
1,3-diamino propane	5	10
putrescine	4	8
tetramethyl putrescine	8−10	16−20
cadaverine	2.1	4.2
1,6-diamino hexane	4.5	9
polyamines (HCl)		
spermidine	0.6	1.8
spermine	0.1	0.4
hexadimethrine (Polybrene)(21mer)	0.5 (μM)	0.02
oligoguanidines (HCl)		
1,3-diguanidino propane	1	2
1,4-diguanidino butane	2	4
1,5-diguanidino pentane	1.7	3.4
netropsin	1.1	2.2
hirudonine (diguanidino-spermidine)	0.24	0.72

[a] All experiments were carried out in Mes assembly buffer containing 0.8 mM GTP and 10 μM taxol at 25.5 °C. Values are the concentrations required to triple the control rates as measured by ΔOD_{350}/min.

experiments where plateau values were allowed to accumulate. There is also a marked effect on nucleation of microtubule assembly as judged by decreased latent periods for initiation of turbidity as shown in Figure 1 (right panel). Similar results were obtained with oligolysines (data not shown). There was no great difference as a function of chain length between the diamines tested, but all are 5−10 times more potent (lower concentration required) than the monoamine, methylamine, tested previously (*20*). As in the case of the monoamine and guanidinium chlorides (*21*), methylation reduced potency, as shown by tetramethyl ethylenediamine and tetramethyl putrescine. On the other hand, the addition of one or two extra nitrogens (primary or secondary) as in the polyamines, spermidine (Figure 1, center panel), or spermine, led to increased polymer-promoting potency by a factor of 10−20. Enhancement of polymerization with these polyamines has been previously noted to form normal appearing microtubules (*25*). The same is true for the addition of guanidino groups. Thus, the bis guanidino alkanes of chain length 3−5 are 10−20 times more potent on a molar basis than guanidine-HCl (*21*), and the bisguanidino spermidine, hirudonine, is more potent still. The nonintercalating bisguanidino DNA binding agent, netropsin, promotes increased OD_{350} in the concentration range characteristic for bis guanidino compounds. The 21mer, hexadimethrine or Polybrene, a quarternary amine antiheparin agent, proved extremely potent and could be used over only a very limited concentration range before precipitation occurred; the potency is high for this 21mer, even when expressed on a per N basis. Similar observations have been made for related compounds (*26*). The resulting potency per N is the following: monoamines < diamines < bisguanidino compounds < triamines or amine-guanidino compounds < tetramines < polyamines with more than 4 N. Pentamidine (4,4′diamidino-α, ω-diphenoxypentane), an antitrypanosomal drug, showed only very slight stimulation and did not achieve a tripling of the rate of polymerization (data not shown).

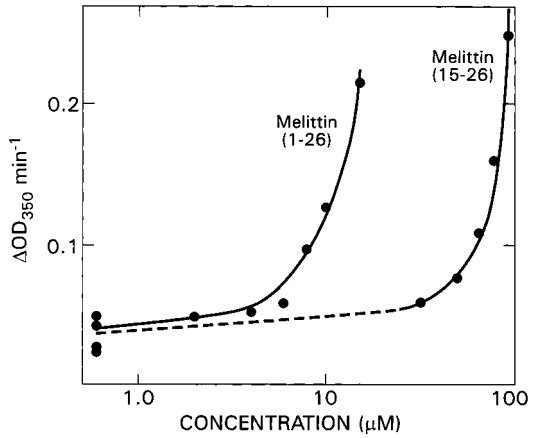

FIGURE 2: Promotion of tubulin polymerization by melittin and melittin fragment 15−26; 8.4 μM rat brain tubulin, 10 μM taxol in Mes assembly buffer at 25.5 °C. GTP was reduced to 0.4 mM because it induced clouding of the mixture. Data were collected as in Figure 1 and maximal rates of polymerization are plotted against peptide concentration.

To see if a larger, natural, oligoamine also increased the polymerization rate, we used the bee venom peptide, melittin, with two α-helices connected by a proline hinge (*27*) (GIGAVLKVLTTGLPALISWIKRKRQQNH2), and +6 charges including the N terminal amino group, four of which are adjacent. It is a potent promoter of assembly, and, as shown in Figure 2, is ∼7-fold more potent than its basic (+4) C terminal portion, melittin 15−26 (again assessed as the concentrations to triple the polymerization rate = 10 and 70 μM, respectively). This suggests that the two charges of the N terminal portion contribute significantly to assembly enhancement. The potency of the (+4) mellitin fragment is not very different from that of tetralysine (see below).

Oligolysines. To achieve an increase in polymerization-promoting potency with increasing numbers of nitrogen atoms per ligand without changing charge spacing etc., we examined oligo- and polylysines with a progressive increase in cationic charge. This is particularly critical in tubulin because of the multiple Glu residues present in both α and β C termini. Curves similar to Figure 1 were generated using L-lysines with degrees of polymerization (DP) ranging from 1 to 20. These were then converted to maximal rates as a function of concentration and compared to control rates. As shown in Figure 3, increasing the DP from 1 to 20 led to nearly 4 orders of magnitude reduction in the molar concentrations required for promoting tubulin polymerization. Although lysine was initially thought not to promote polymerization (*28*), it was later found to bind to tubulin, albeit at much higher concentrations (*29*). All rate vs concentration curves were sigmoidal where measurements were possible, but for a number of curves the high concentration range could not be determined because rates were too fast to measure or, in certain cases, precipitation supervened. For the series K_2 to K_{10}, as well as two polylysine preparations used here with average DP of 12 or 20, the molar potency increased with increasing DP.

The lower oligolysine concentration required to increase the rate of assembly is not merely due to the increased total concentration of lysyl residues or positive charges, for when comparisons are made on the basis of monomer concentration, a biphasic response, which is a function of DP, can be

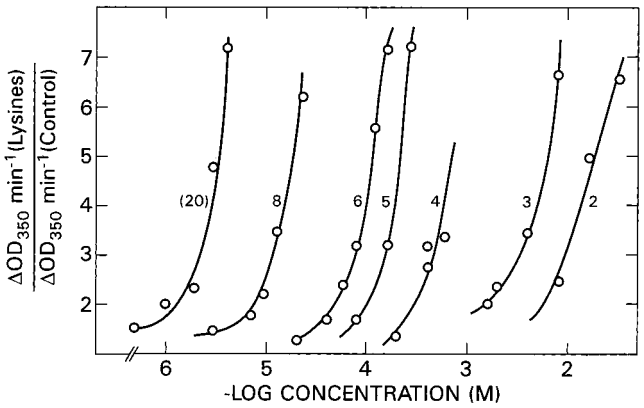

FIGURE 3: Oligolysine-promoted microtubule assembly from 8.4 to 10 μM tubulin as a function of the concentration of oligolysine of different degrees of polymerization, DP; the numbers on the curves indicate the DP, thus 2 = lysyl-lysine, etc.; parentheses indicate average degree of polymerization calculated from molecular mass. Mes assembly buffer with 0.8 mM GTP, 10 μM taxol at 25.5 °C. Because different protein preparations were used in some cases, all values are the ratios of the maximal polymerization rate in the presence of lysine oligomers to the maximal control polymerization rate.

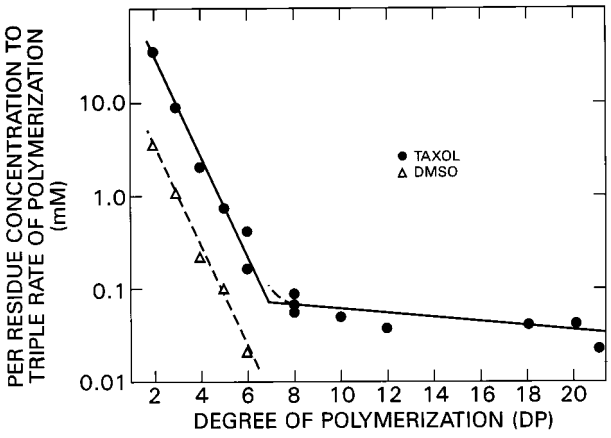

FIGURE 4: The concentrations required to attain a 3-fold increase in the rate of tubulin polymerization calculated on a *per lysyl residue* basis and plotted as a function of the DP for lysines. The per lysyl residue concentration is calculated from data in Figure 3 by multiplying the oligomer concentration by the DP. Tubulin (12 μM) in Mes assembly buffer with 0.8 mM GTP and 10 μM taxol at 25.5 °C.

demonstrated. As shown in Figure 4, when plotting the residue concentration required to triple the rate of polymerization as a function of DP, there is a progressive increase (>2 orders of magnitude) in the *per residue* potency until the DP is seven. With DP > 7, the *per residue* potency approaches a nearly constant value. The results obtained with 10% DMSO or 10 μM taxol as polymerization promoters yielded a slope similar to DP 6, although the taxol curve was displaced to higher residue concentrations. Experiments with larger lysine oligomers were not carried out with DMSO. These results suggest that the interaction of oligolysines with tubulin has a charge-cluster component at low DP which is cooperative, and a more global one with residue numbers >7 which is very much less so. The term cooperative as used here differs from the common usage in oxygen−hemoglobin interactions in that the consecutive ligands are linked. This confers major entropic advantages, but does not necessarily imply changes in the binding site for second and subsequent interactions, although these may occur. We merely imply that the first interaction facilitates the second residue interaction and so forth until, in the present case, seven such interactions have occurred.

To assess whether the polymers formed with oligolysines behaved like microtubules, several tests characteristic for microtubules were carried out. Because the oligolysine effect was presumably charge-based, enhanced polymerization rates should be abolished at high ionic strength. Such experiments had to be carried out in DMSO as taxol polymers are poorly reversible by these manipulations. We used CsCl because it has the least intrinsic stimulating effect of the alkali metal cations on tubulin (*20*). As shown in Figure 1, right panel, 300 mM CsCl rapidly and nearly completely depolymerized both controls and microtubules formed in the presence of large concentrations of hexalysine. Moreover, polymers induced by large concentrations of K_4 in 10% DMSO are readily cold-reversible (Figure 1, right panel). Also cold-sensitive were polymers formed with K_5 and K_{12}, oligoarginines R_2-R_5, and spermidine (data not shown). Hexalysine, the only oligomer so tested, did not induce polymerization in the cold. In addition, 30 μM colchicine completely prevented the polymerization induced by hexalysine. It is pertinent to mention here that colchicine binding to tubulin was reported to be enhanced by large polylysines as a function of their degree of polymerization through an effect both on affinity and stability of the binding site (*30*). Although the data are incomplete, this effect occurred at a roughly constant per lysyl residue potency.

We also studied the properties of the fluorescent tribasic tetrapeptide, 7-VKKR-4-trifuoromethyl coumarone ($\lambda_{max(ex)}$ = 338 nm; $\lambda_{max\ (em)}$ = 434 nm under our solvent conditions) with the aim of examining the environment of the fluorophore when bound. The concentration needed to triple the rate of polymerization was 160 μM, but there was only a small enhancement of fluorescence intensity as a function of tubulin concentration without an emission blue shift (data not shown). This suggests that the fluorophore remains solvent exposed. The greater potency of this tribasic peptide, when compared with K_3 or R_3 (see below), suggests that the nonpolar components may have contributed to binding.

Electron Microscopy. It was important to ascertain what fraction of the polymers formed under the influence of oligolysine or oligoarginine were microtubules. When polymers made with hexalysine or hexaarginine (see below) were pelleted in a microfuge and sectioned, normal microtubules were observed for the most part (Figure 5). This was true whether 10% DMSO or 10 μM taxol was used to promote polymerization. Some aberrant tubules such as s or double-s shapes, and some tailed tubules were observed; this was more common with taxol than DMSO, but was not influenced by the presence of hexalysine or hexaarginine. Markham rotation carried out on a few tubules from each group with good cross sections revealed all (4/4 each) to have 13 protofilaments, but no statistical inference can be drawn from this small number. These results should be contrasted to those in which polylysines of high degrees of polymerization are used leading to structureless aggregates (*31*).

To assess the possibility that the oligocations might induce bundling of microtubules, we measured the wavelength dependence of light scattering of polymers formed with or without pentalysine. The slopes of plots of log OD vs log wavelength were identical within the error of the method, sug-

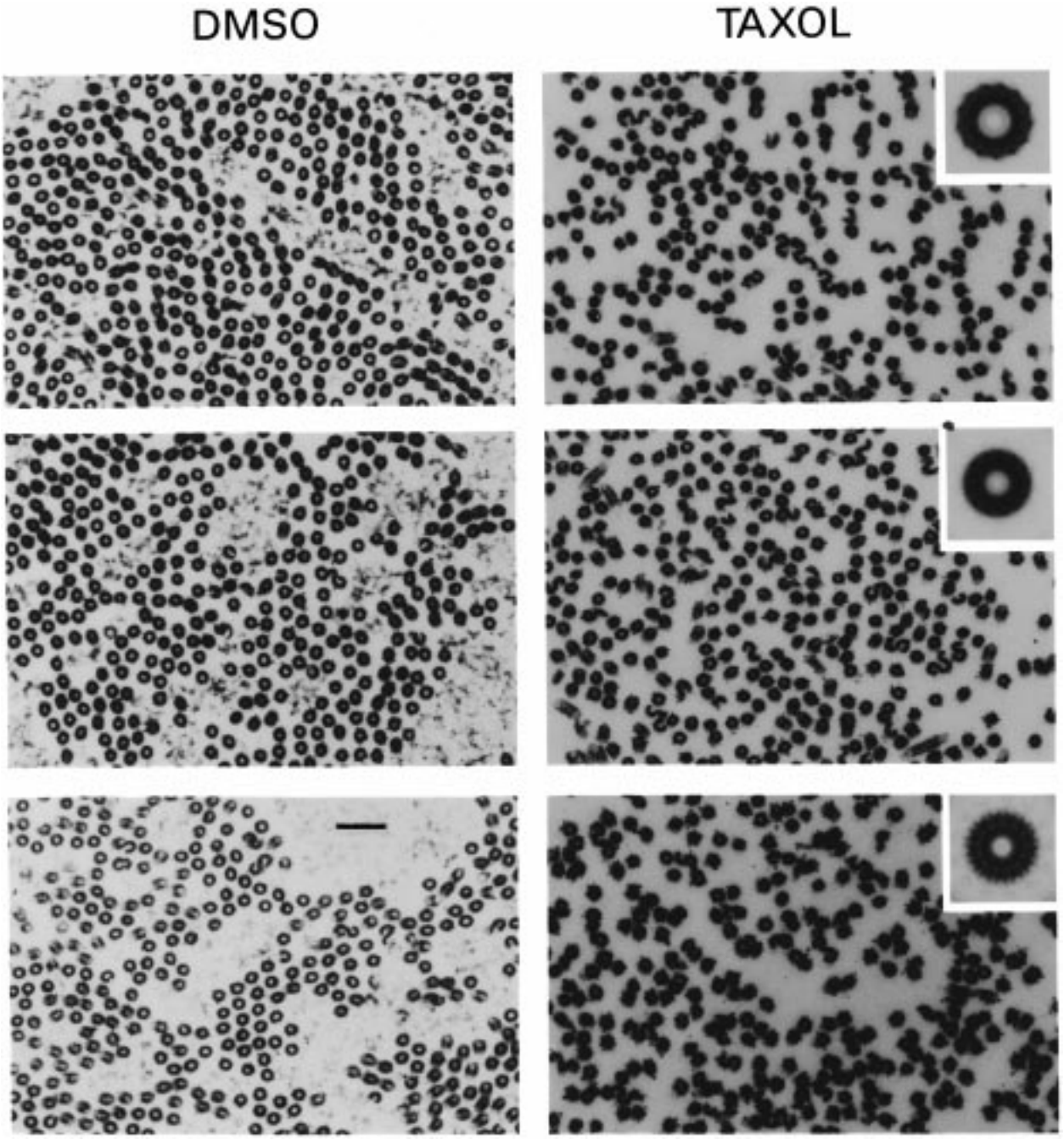

FIGURE 5: Electron micrographs of tubulin polymers formed in the presence of hexalysine (K_6) or hexaarginine (R_6). Polymer pellets were prepared and processed as described in Materials and Methods. Left column; 10% DMSO; right column; 10 μM taxol. Top row, controls; center row, 70 μM hexalysine; bottom row, 12 μM hexaarginine. Bar = 100 nm.

gesting that the nature of the polymer had not changed. In addition, when tubulin in 10% DMSO and 1 mM GTP was incubated with 100 μM pentalysine or 80 μM pentaarginire, negative staining of the preparations revealed only single microtubules but failed to reveal any bundling of microtubules.

To test whether oligolysines would promote tubulin polymerization in the absence of either taxol or DMSO, experiments were carried out with 50 μM tubulin at 37 °C to conserve protein. Under these conditions hexalysine promoted a brisk enhancement of the polymerization rate, yielding a 3-fold increase in the maximal rate at ~7 μM of the oligocation. For pentaarginine the concentrations required to triple the rate under otherwise similar condtions was ~23 μM. At lower tubulin concentrations, where spontaneous asssembly failed to occur, hexalysine nevertheless induced polymer formation, albeit at a higher hexalysine concentration.

Charge Spacing. An attempt was made to ascertain the optimal spacing between lysyl residues, i.e., the charge density, by introducing intervening alanyl residues for K_6. As shown in Figure 6, increasing the sequence distance between lysyl residues led to decreasing potency of the peptides in the order K_6, $(AK)_6$ and $(AAK)_6$. Clearly optimal effectiveness (spacing) occurs for uninterrupted lysyl sequences, suggesting interaction with closely spaced carboxylates, presumably the C termini, i.e., separation of the lysyl residues makes interaction with vicinal Glu residues more difficult while not necessarily hindering reactions away from

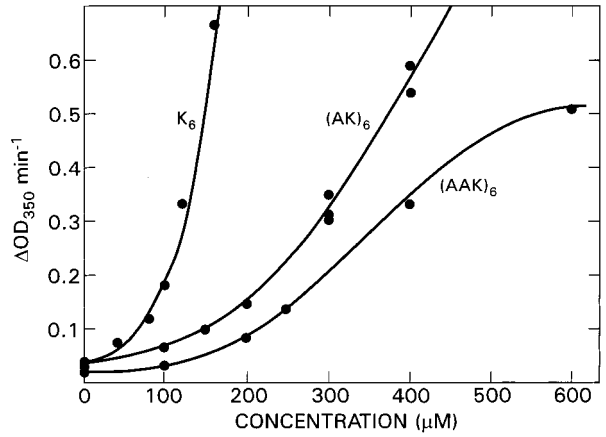

FIGURE 6: The effect of intercalating alanyl residues between the lysyl residues of hexalysine on the promotion of microtubule assembly. Rat brain tubulin (25 μM) in Mes assembly buffer containing 0.8 mM GTP and 10% DMSO at 25.5 °C. Data are calculated and plotted as for Figure 2.

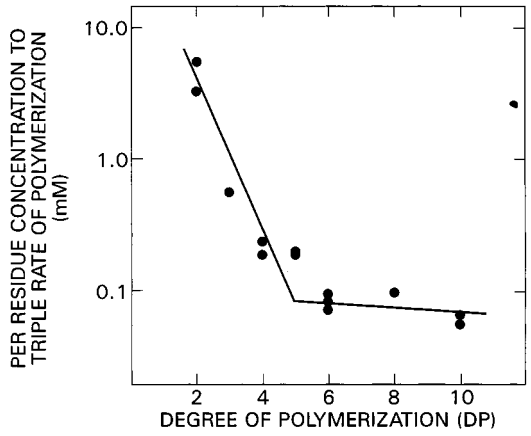

FIGURE 7: The concentrations required to attain a 3-fold increase in the rate of tubulin polymerization calculated on a per arginyl residue basis and plotted as a function of the DP for arginines. The per arginyl residue concentration is calculated from data in Figure 3 by multiplying the oligomer concentration by the DP. Tubulin (12 μM) in Mes assembly buffer with 0.8 mM GTP and 10 μM taxol at 25.5 °C.

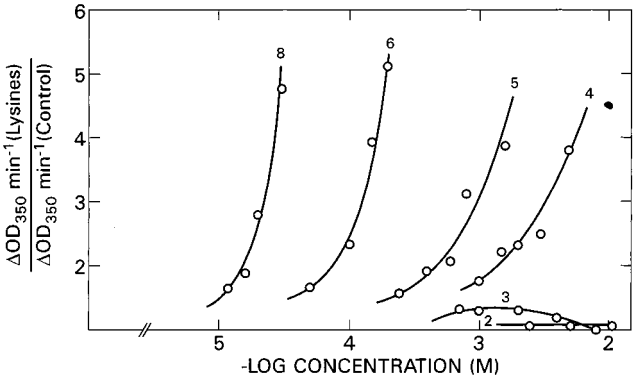

FIGURE 8: Oligolysine-promoted assembly of tubulin S as function of the concentration of oligolysine of different degrees of polymerization. The reaction mix contains ~1.5 μM tubulin S in Mes assembly buffer with 0.8 mM GTP, 10 μM taxol at 25.5 °C. Because different protein preparations were used in some cases, all values are presented as the ratios of the maximal polymerization rate in the presence of lysine oligomers to the maximal control polymerization rate.

the C termini. The helix-forming potential of the alanyl residues might contribute to the differences in polymerization promotion; however, CD spectra of these peptides in aqueous solution (kindly performed by Dr. Peter McPhie) showed little secondary structure. From available data we cannot, of course, say anything about charge distribution in the folded protein. The effect of charge density may also explain the high potency of melittin 15−26 (see Figure 2) because its four basic amino acids are adjacent.

Oligoarginines. Lysine and arginine are often considered to be conservative substitutions in the primary sequence of proteins. However, these two basic amino acids are different in a number of respects: (1) arginine can form more hydrogen bonds, has a different cationic charge distribution, and is more commonly involved in complex salt bridges; (2) the Cα-N distance is 5.64 Å in lysine and 6.50 Å in arginine; (3) arginine is a poorer proton donor; (4) exchange of these two amino acids in the active sites of enzymes often leads to drastic changes in catalytic activity (*32*, *33*). It was, therefore, important to compare oligolysines with oligoarginines for their effects on tubulin polymerization. As with lysine, monoarginine increased the rate of polymerization at millimolar concentrations but did not achieve a tripling of the maximal rate. As shown earlier (*21*), decarboxylation of arginine to agmatine, increasing the charge by one, caused a large potency increase. Figure 7 provides data on the arginyl residue concentrations required to increase polymerization rates under the influence of oligoarginines with DP2 to DP10 (data collected as for Figure 3 and then corrected for DP as in Figure 4). Data are again presented as the equivalent monomer concentrations required to triple the maximal rate. Although arginyl residues were somewhat more potent, the effect of these oligomers was generally similar to that obtained with oligolysines and the slopes are the same within experimental error. The division between cooperatively and virtually noncooperatively interacting arginyl residues resembles that for lysyl residues, but the break in the curve occurs at DP = 5. It is not clear at present whether this is the result of size and charge distribution or the fact that these experiments were carried out with a different tubulin preparation. The comparison nevertheless suggests that two of the carboxylates neutralized by oligolysines in a cooperative manner are not equally accessible to oligoarginines. With DP ≥ 5, i.e., two fewer than seen for the oligolysines, the concentration dependence for excess arginyl residues does not change significantly.

Tubulin S. To determine if the extreme C termini are the high-charge density sites for oligocation ineeraction, these regions were cleaved by limited proteolysis with subtilisin, removing seven and eight coded acidic amino acids from α and β tubulin, respectively, as well as an unspecified number of post-translationally added Glu residues (*1*−*4*). Such charge reduction is readily demonstrated by electrophoresis of the nondenatured protein (*5*). We have shown previously that this procedure also markedly reduces the efficacy of guanidinium or alkali metal cations in promoting microtubule assembly (*20*, *21*). Figure 8 shows the response of tubulin S to oligolysines K_2−K_8. It is apparent that the polymerization enhancement is markedly attenuated at low DP when compared to uncleaved tubulin, as shown in Figure 3. In contrast to intact tubulin, dilysine and trilysine promoted virtually no enhancement at these concentrations and a tripling of the rate could not be attained; larger concentrations

were inhibitory. Significant enhancement was attained with tetralysine and larger oligomers. Interestingly, the potency of the longer oligolysines for tubulin S approached those for uncleaved tubulin as a function of increasing DP, suggesting that charge-based interactions of the oligolysines elsewhere in the tubulin molecule must also occur. The ratios of the oligolysine concentrations required to triple the polymerization rate for tubulin S to the concentrations required for native tubulin were $K_4 = 7.3$, $K_5 = 6.3$, $K_6 = 1.6$, and $K_8 = 1.8$. The location of such residual interactions can only be surmised, but must presumably involve roughly vicinal anionic charges. Significant excess anionic charge remains in the newly created C termini of tubulin S; thus, e.g., in $\alpha_{(411-438)}$, there is still a net negative charge of -9, including three pairs of vicinal acidic amino acids, whereas in $\beta_{(410-433)}$ the net negative charge is -6, with two pairs of vicinal acidic amino acids. These may account for the residual cooperativity in tubulin S, because larger oligomers may span less closely spaced carboxylates, but this needs to be independently tested. We conclude then that removal of the C termini from α and β tubulin leads to a marked reduction in the ability to respond to smaller oligolysines.

DISCUSSION

The fact that charge repulsion is one of the important regulatory factors for tubulin polymerization is now well established. A large part of that regulation resides in the carboxyl termini of both α and β tubulin, which are very rich in both coded and post-translationally added Glu residues (*5*−*8*, *29*). Early work by Krimm and co-workers (*34*, *35*) suggested that, at neutral pH, the Glu residues are charged and are likely to be in the form of an extended helix rather than a random coil. In the extended helix there are ∼2.8 residues per turn and the rotational angle between side chains is slightly greater than 120°. Charge separation is a function of the number of side chain CH_2 groups and is 3.6 Å for glutamates and 5.7 Å for lysines, assuming a fully extended side chain. However, more recent calculations with poly-L-(Glu)20 have estimated 47% right-handed α-helix at physiologic ionic strength (*37*). The occasional Asp or uncharged amino acid intercalation in the C termini, as well as the fact that post-translational glutamylation forms a branched protein, would cause imperfections in the calculated structure predictions and there is disagreement regarding the secondary structure of the C termini of β-tubulin. With β-(400−445), CD experiments revealed largely random coil with <5% α-helix or β-sheet, irrespective of ionic strength (*36*). The findings do not, however, agree with results from electron crystallography of tubulin (3.7 Å resolution) that assign an α-helix (H12) to residues β408−β423 (*38*). The extreme C termini could not be visualized in this work, and the question of their structure remains unresolved.

Reduction of the repulsive charges at the C termini by a variety of covalent methods promotes assembly characterized by an increased rate and extent of polymerization, and by reduction of the latent period and critical concentration (*5*, *6*, *8*). Three types of noncovalent manipulations of the anionic charge repulsions in α and β tubulin also promote assembly. The simplest of these uses monovalent cations, with selectivity $Na^+ > K^+ > Li^+ > Cs^+$ (*20*) characteristic for the coil−helix transition in polyglutamate, and is consistent with the Glu-rich C termini of the two tubulin monomers. Monovalent cations capable of forming H bonds, as exemplified by guanidine-HCl and its congeners, are more potent promoters of tubulin polymerization, but also produce aberrant microtubule forms (*21*). Pairing of the anionic charges of tubulin with appropriately spaced, but linked, oligocations is shown here to be another effective method for charge reduction. With oligolysines, an increase in molar potency with increasing molecular mass was expected simply on the basis of increasing residue concentration. However, at low degrees of polymerization a marked cooperative *per residue* increase in the assembly promoting potency was readily demonstrated.

Assuming that the promotion of polymerization is a function of binding of the oligolysines, the finding in Figure 4, that the increase in *per residue* potency shows a break after DP = 7 for K_7 followed by a nearly constant per residue potency at $K > 7$, suggests that this number may represent a 1:1 stoichiometry for oligolysine binding to a carboxylate-rich region, hence the binding site seen by this method contains seven Glu or Asp residues that are within reach of a single lysine oligomer. Larger oligomers can only saturate the seven anionic charges at this site but do not recruit additional Glu or Asp residues to the site; they may, of course, bind at other loci with lower anionic charge density. For oligoarginines the binding site would be comprised of five anionic side chains; at present we have no explanation as to why this number is smaller than for oligolysines. These data shed no light on whether the anionic site is on α or β tubulin or both monomers, or are proximal because of a protein fold. Similar types of results have been obtained by direct binding measurements of oligolysines ($z = +8$) to poly(dT) which occurred with a 1:1 stoichiometry with phosphate residues, and only 1 octavalent cation is bound to $dT(pdT)_{10}$ (*39*). In view of the regularly spaced charges in polynucleotides, it is surprising that the details of oligolysine binding and the associated ΔH and $T\Delta S$ are highly nucleotide dependent. There is also a change in the mode of peptide interaction when the charge, *z*, exceeds +4-(occasionally +5) (*40*); for purposes of comparison $z \sim DP$. This resembles the change in interaction seen as a function of DP seen in tubulin; however, for tubulin, with its asymmetric charge distribution, simple averaging of anionic charges over the whole protein molecule could lead to erroneous interpretation.

Are these relatively nonspecific cationic interactions with tubulin involved in intracellular regulation of the tubulin-microtubule equilibrium? While the basic patches of the classical microtubule-associated proteins are known to contribute to the promotion of assembly with the attending sensitivity to ionic strength, other properties of these proteins also contribute. Likewise, whether basic proteins of the cell can regulate assembly purely on a charge basis remains to be examined. On the other hand, spermine and spermidine are the most cationic small molecules in the cytoplasm. Total cell concentrations of these compounds is ∼1 mM, well within the range where they might affect the state of tubulin polymerization. However, the bulk of these compounds is bound to RNA, DNA, or other cellular anions, or is sequestered in vacuoles (*41*, *42*), and probably <10% of the total is freely available. The high concentration of tubulin in many cells and the high charge density may, however, effectively compete with some of the other anions, and

participation of these polyamines in microtubule assembly is possible. Deprivation of polyamines has significant effects on the cytoskeleton in some cells. Thus, CHO cell polyamine auxotrophs lose actin filaments and microtubules but not intermediate filaments (*43*), whereas in keratinocytes the microtubule network is rearranged (*44*). In other cells, actin is the primary cytoskeletal target for polyamine action (*45*, *46*). It is not clear as yet whether these are primary cation effects. Thus, cellular polycations could contribute to microtubule assembly, but direct proof is lacking.

We conclude then that lysine oligomers interact with tubulin by two distinct mechanisms depending on the DP. When DP $\leq$ 7 (or 5 for arginines), the primary locus of interaction is one of high charge density, most likely at the extreme C termini as judged from the sequence, removal of the C termini, and the loss of potency upon increasing the separation of lysyl residues. The oligolysine interaction is cooperative insofar as the per lysyl residue potency increases much faster than by simple additivity. This is also expected from thermodynamic consideration of the succesive decrease in the entropy penalty for binding of linked charges. Tubulin also exhibits less reactive cooperative interactions not in the extreme C termini. Comparable conclusions apply to the polyamines, although the per charge differences are expected to differ from oligolysines because of differences in chemical properties. Such cooperative interactions may also be expected at other oligoanionic sites, but may easily be missed if the DP of the polycation is substantially greater than the number of clustered anions to be neutralized (i.e., when $z^{n+} \gg z^{n-}$). The lysines in excess of seven no longer interact cooperatively, and the potency of the polymer becomes an approximately linear function of the total lysyl residue concentration.

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REFERENCES

1. Eddé, B., Rossier, J., LeCaer, J. P., Desbruyeres, E., Gros, F., and Denoulet, P. (1990) *Science 247*, 83−85.
2. Alexander, J. E., Hunt, D. F., Lee, M. K., Shebanowitz, J., Michel, H., Berlin, S. C., Macdonald, T. L., Sundberg, R. J., Rebhun, L. I., and Frankfurter, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4685−4689.
3. Bouchet, D., Larcher, J.-C., Gros, F., and Denoulet, P. (1994) *Biochemistry 33*, 12471−12477.
4. Redeker, V., Rossier, J., and Frankfurter, A. (1997) *Mol. Biol. Cell 8*, 46a.
5. Sackett, D. L., Bhattacharyya, B., and Wolff, J. (1985) *J. Biol. Chem. 260*, 43−45.
6. Maccioni, R. B., Serrano, L., Avila, J., and Cann, J. B. (1986) *Eur. J. Biochem. 156*, 375−381.
7. Melki, R., Kerjan, P., Waller, J. P., Carlier, M. F., and Pantaloni, D. (1991) *Biochemistry 30*, 11536−11544.
8. Mejillano, M. R., and Himes, R. H. (1991) *J. Biol. Chem. 266*, 657−664.
9. Jacobs, M., Bennett, P. M., and Dickens, M. J. (1975) *Nature 257*, 707−709.
10. Behnke, O. N. (1975) *Nature 257*, 709−710.
11. Levi, A., Cimino, M., Mercanti, D., Chen, J. S., and Calissano, P. (1975) *Biochim. Biophys. Acta 399*, 50−60.
12. Erickson, H. P., and Voter, W. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2813−2817.
13. Murphy, D. B., Vallee, R. B., and Borisy, G. G. (1976) *Biochemistry 16*, 2598−2605.
14. Lee, J. C., Tweedy, N., and Timasheff, S. N. (1978) *Biochemistry 17*, 2783−2790.
15. Burton, P. R. (1981) in *Cell and Muscle Motility* (Dowben, R. M., and Shay, J. W., Eds.) Vol. 1, pp 289−333, Plenum Publ. Co., New York.
16. Serrano, L., Avila, J., and Maccioni, R. B. (1984) *Biochemistry 23*, 4657−4681.
17. Mithieux, G., Alquier, C., Roux, B., and Rousset, B. (1984) *J. Biol. Chem. 259*, 15523−15531.
18. Modesti, N. M., and Barra, H. S. (1986) *Biochem. Biophys. Res. Commun. 136*, 482−489.
19. Littauer, U. Z., Giveon, D., Thierauf, M., Ginzburg, I., and Ponstingl, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7162−7166.
20. Wolff, J., and Knipling, L. (1996) *Protein Sci. 5*, 2020−2028.
21. Wolff, J., Knipling, L., and Sackett, D. L. (1996) *Biochemistry 35*, 5910−5920.
22. Satoh, M., Komiyama, J., and Ijima, T. (1982) *Biopolymers 21*, 1927- 1931.
23. Sackett, D. L., Knipling, L., and Wolff, J. (1991) *Protein Expression Purif. 2*, 390−393.
24. Hamel, E., and Lin, C. M. (1984) *Biochemistry 23*, 4173−4184.
25. Anderson, P. J., Bardocz, S., Campos, R., and Brown, D. L. (1985) *Biochem. Biophys. Res. Commun. 132*, 147−154.
26. Kuznetsov, S. A., Gelfand, V. I., Rodionov, V. I., Rosenblatt, V. A., and Gulyaeva, J. G. (1978) *FEBS Lett. 95*, 343−346.
27. Owen, Z., and Shai, Y. (1997) *Biochemistry 36*, 1826−1835.
28. Lee, J. C., and Timasheff, S. N. (1977) *Biochemistry 16*, 1754−1764.
29. Arakawa, T., and Timasheff, S N. (1984) *J. Biol. Chem. 250*, 4979−4986.
30. Roychowdhuri, S., Banerjee, A., and Bhattacharyya, B. (1983) *Biochem. Biophys. Res. Commun. 113*, 384−390.
31. Miyatake, K., and Flavin, M. (1983) *Int. J. Biochem. 15*, 1305−1312.
32. Musafa, B., Buchner, V., and Arat, D. (1995) *J. Mol. Biol. 254*, 761−770.
33. Vacca, R. A., Giannatassio, S., Graber, R., Sandmeier, E., Marra, E., and Christen, P. (1997) *J. Biol. Chem. 272*, 21932−21937.
34. Tiffany, M. L., and Krimm, S. (1968) *Biopolymers 6*, 1379−1382.
35. Krimm, S., and Mark, J. E. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 1122−1129.
36. Reed, J., Hull, W. E., Ponstingl, H., and Himes, R. H. (1992) *Biochemistry 31*, 11888−11895.
37. Evans, J. S., Chan, S. I., and Goddard, W. A., III (1995) *Prot. Sci. 4*, 2019−2031.
38. Nogales, E., Wolf, S. G., and Downing, K. H. (1998) *Nature 391*, 199−203.
39. Zhang, W., Bond, J. P., Anderson, C. F., Lohman, T. M., and Record, M. T., Jr. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2511−2516.
40. Mascotti, D. P., and Lohman, T. M. (1993) *Biochemistry 32*, 10568−10579.
41. Pegg, A. E., and McCann, P. P. (1982) *Am. J. Physiol. 243*, C212−C221.
42. Davis, R. H., Morris, D. R., and Coffino, P. (1992) *Microbiol. Rev. 56*, 280−290.
43. Pohjanpelto, P., Virtanen, I., and Hölttä, E. (1981) *Nature 293*, 475−477.
44. Pomidor, M. M., Ruhl, K. K., Zheng, P., Song, Y., Jänne, O. A., Tuan, R. S., and Hickock, N. J. (1995) *Exp. Cell Res. 221*, 426−437.
45. Sunkara, P. S., Rao, P. N., Nishioka, K., and Brinkley, B. R. (1979) *Exp. Cell Res. 119*, 63−68.
46. Parkkinen, J. J., Lammi, M. J., Agren, U., Tammi, M., Keinanen, T. A., Hyvönen, T., and Eloranta, T. O. (1997) *J. Cell. Biochem. 66*, 165−174.

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