Cation selective promotion of tubulin polymerization by alkali metal chlorides

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Abstract

A role for charge-based interactions in protein stability at the monomer or dimer level is well known. We show here that such interactions can also be important for the higher-order structures of microtubule assembly. Alkali metal chlorides increase the rate of polymerization of pure tubulin driven by either taxol or dimethyl sulfoxide. The effect is cation selective, exhibiting a sequence $\text{Na}^+ > \text{K}^+ > \text{Li}^+ > \text{Cs}^+$, with optimal concentrations for Na^+ at ~ 160 mM. Hofmeister anion effects are additive with these rate stimulations. Sodium is less potent than guanidinium ion stimulation reported previously, but produces a larger fraction of normal microtubules. Alkali metal cations lower the critical concentration by a factor of ~ 2 , produce cold reversible polymers whose formation is sensitive to podophyllotoxin inhibition, increase the fraction of polymers present as microtubules from ~ 0.9 to 0.99 , and reverse or prevent urea-induced depolymerization of microtubules. In the presence of microtubule-associated proteins, the promotion of polymerization is no longer cation selective. In the polymerization of tubulin S, in which the acidic C termini of both monomers have been cleaved, the cation enhancement is markedly decreased, although selectivity persists.

Because the selectivity sequence is similar to that of the coil/helix transition of polyglutamic acid, we suggest that a major part, although not all, of the cation selective enhancement of polymerization results from shielding of the glutamate-rich C termini of the tubulin monomers.

Keywords: cation selective charge shielding; microtubule-associated proteins; tubulin polymerization rate; tubulin S; urea-induced depolymerization

Tubulin can unfold through a series of stable intermediates when exposed to low concentrations of urea. These intermediate states are characterized by a sequence of functional changes that occur at urea concentrations below those needed to demonstrate even small changes in the intrinsic fluorescence (Sackett et al., 1994). The most sensitive of these functional changes is the inhibition of assembly of tubulin to form microtubules ($\text{IC}_{50} \sim 0.16$ M urea), particularly the *rate* of assembly. Because guanidine hydrochloride (GuHCl) is often a more potent denaturant than urea, it was important to learn whether even lower GuHCl concentrations would mimic the urea effects on polymerization. Unexpectedly, GuHCl had precisely the opposite effect of urea, promoting polymer formation at very low (10–120 mM) concentrations (Wolff et al., 1996). Because both the α and β monomers of nearly all tubulins carry considerable excess anionic charge, particularly at the C termini (Little & Seehaus, 1988), and because the guanidinium ion carries a positive charge, it seemed likely that *this* property of the denaturant accounted for the “paradoxical” action of GuHCl. We

proposed that, at these low concentrations, GuHCl exerted its polymer-promoting action by shielding repulsive anionic charges in the tubulin monomer (Wolff et al., 1996), as had been shown earlier for two-stranded coiled coils (Monera et al., 1994; Kohn et al., 1995b). The effectiveness of GuHCl could be ascribed either to direct charge neutralization or to strong hydrogen bonds resulting from the partial formal charge localized to each of the six hydrogens. The role of hydrogen bonds is emphasized by the finding that certain non-hydrogen bonding organic cations have little polymer-promoting activity.

Preliminary experiments showed that monovalent inorganic cations could mimic the polymer-promoting effects of GuHCl. It has been known for a long time that many enzyme reactions are activated by monovalent cations, sometimes as a function of ionic strength, but, in a number of cases, with considerable cation selectivity, most often for K^+ (Suelter, 1974).

Early work on tubulin polymerization, using microtubule protein preparations containing microtubule-associated proteins (MAPs), revealed inhibition of polymerization by NaCl or KCl at concentrations in excess of 100–150 mM (Haga et al., 1974; Lee et al., 1974; Olmsted & Borisy, 1975; Nishida, 1978; Gupta & Abou-Donia, 1994). These effects were ascribed to interference with the (charge-

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based) interactions of MAPs with tubulin (e.g., Burns et al., 1984). A similar effect occurs with the interaction of basic proteins with tubulin (Erickson & Voter, 1976; Mithieux et al., 1984; Modesti & Barra, 1986). Other properties of tubulin are also sensitive to salt. Thus, the nucleotide content of tubulin is decreased by high salt (Croom et al., 1986), and sodium is preferred for GTPase activity (Caplow et al., 1994). Both near- and far-UV circular dichroic extrema of tubulin are diminished by salt, an effect that may be due to a change in oligomer concentration present in such preparations because it is not seen in pure dimeric tubulin (Martin et al., 1982; Rousset et al., 1983).

Low concentrations of NaCl or KCl have been found in certain cases to *enhance* the extent of polymerization of microtubule protein from brain or *Strongylocentrotus purpuratus* outer doublets (Haga et al., 1974; Kuriyama & Sakai, 1974; Lee et al., 1974; Olmsted & Borisy, 1975; Farrell et al., 1979). This was ascribed to a general ionic strength effect. By contrast, small organic cations enhance polymerization by a highly ion-specific mechanism (Wolff et al., 1996). It was important, therefore, to determine whether the inorganic salt effects on tubulin were due merely to Debye-Hückel screening or also showed specific binding. The latter mechanism finds support in the selective Na^+ dependence of tubulin GTPase activity (Caplow et al., 1994). The results on the rate of polymerization presented below demonstrate that alkali metal cations enhance tubulin polymerization, lower the critical concentration, and increase resistance to urea-induced depolymerization, and that there is considerable selectivity among the alkali metal cations for the polymerization-enhancing effect.

Results

Taxol-driven tubulin polymerization

At the concentrations of pure tubulin used, no polymerization occurs without a promoting agent such as dimethyl sulfoxide (DMSO),

taxol, or MAPs. Because MAPs would themselves be subject to ionic influences, they would complicate interpretation. Consequently, and for comparison with our earlier studies (Wolff et al., 1996), 10 μM taxol and 25.5 $^\circ\text{C}$ were used. Chlorides of alkali cations increase the maximal rate of polymerization of rat brain tubulin in the presence of 10 μM taxol and 0.8 mM GTP in Mes (morpholinoethane sulfonic acid) assembly buffer described in Materials and methods (Fig. 1). Sodium is the most effective cation, followed by $\text{K}^+ > \text{Li}^+ > \text{Cs}^+$. When taxol and GTP were deleted from the polymerization medium, no polymerization occurred with up to 1,000 mM NaCl. In the presence of taxol without GTP, there was a slow rate of polymerization that was increased ~ 9 -fold by 120 mM NaCl (data not shown). At higher salt concentrations, polymerization rates in the complete system may again decrease; this inhibitory effect is least with Na^+ . It is difficult to rule out a contribution from nonspecific ion effects, but this must be small because, e.g., in concentrations comparable to those used above, choline chloride was inhibitory for the polymerization rate of tubulin in the presence of taxol. Similar, selective, cation-induced stimulation of the polymerization rate can be demonstrated in polymerization promoted by DMSO (data not shown). We also tested NH_4Cl and its methylated derivatives (see inset to Fig. 1). These proved to be relatively poor effectors, with progressively decreasing activity with increasing methylation; tetramethylammonium chloride was only inhibitory. This again suggests that a considerable degree of selectivity is involved in this cation effect.

The maximal rate increase under the usual incubation conditions was attained by Na ion and was 6–10 times the control rate; it occurred at concentrations of 120–160 mM added NaCl in different experiments. This is substantially less rate enhancement than achievable with comparable concentrations of GuHCl and some of its congeners (Wolff et al., 1996). Differences between the cations are most apparent at the higher concentrations and appear to be dictated in part by the downward slope in the dose-response curve. This effect occurred in the reverse rank order, i.e., $\text{Cs}^+ > \text{Li}^+ >$

Fig. 1. Selective effect of alkali metal chlorides on the rate of rat brain tubulin polymerization. Reactions were conducted at 25.5 $^\circ\text{C}$ in Mes assembly buffer, pH 6.9, containing 10 μM taxol, 1% DMSO, 0.8 mM GTP, and the salt. After 2 min temperature equilibration, 8.8 μM rat brain tubulin was added to start the reaction and the OD at 350 nm was recorded starting at 30 s. Inset: Comparison of chlorides of ammonium salts with different degrees of methyl substitution indicated by the numbers 0, 1, 2, 3, 4. Note the change in scale. Conditions as above.

$K^+ > Na^+$. Could the nature of the counter ion be involved in this? Because anions tend to have more powerful chaotropic (structure breaking) effects than cations, a comparison was made with different sodium salts (Fig. 2). Sodium salts of anions such as acetate and fluoride have a more pronounced enhancing effect on polymerization than chlorides, whereas NaBr shows only slight stimulation and NaSCN is inhibitory at all concentrations. It has been suggested that the NaF effect in lowering the critical concentration for tubulin polymerization may result from inhibition of protein phosphatases (Lobert et al., 1994); however, this effect is likely to be less important in the present studies because sodium acetate is equally effective.

It should be noted that in Figure 1 at concentrations of ≤ 50 mM, the effect of added salts on the rate of increase in OD_{350} differed only slightly for the different alkali metal chlorides. To test whether there was real selectivity at these low concentrations, we argued that the absence of a chaotropic anion might enhance any selectivity differences, if any were there. When alkali metal acetates were added at 30 or 40 mM (Table 1), a clear difference between cations could be shown in the polymerization rates, exhibiting the same selectivity noted above. Thus, the rank order of cation selectivity $Na^+ > K^+ > Li^+ > Cs^+$ is present over the whole concentration range where polymerization is enhanced.

Cation and anion effects are additive (von Hippel & Schleich, 1969), thus it is reasonable to propose that, for carboxylate-based repulsive interactions, the upward (polymerization enhancing) effect is dominated by the cation, whereas the downward (polymerization inhibiting) slope at higher concentrations is largely determined by the chaotropic potency of the anion and, to a lesser extent, by the modulating effect of the cations. Thus, the balance between cation stimulation and chaotropic anion inhibition will determine both the height of the rate peak and its position with respect to concentration.

Fig. 2. Effect of different sodium salts on tubulin polymerization. Incubations as in Figure 1. Note logarithmic concentration scale.

Table 1. Effect of alkali metal acetates on tubulin polymerization^a

Concentration (mM)	$\Delta OD_{350}/min$				
	Control	Cs^+	Li^+	K^+	Na^+
30	0.026	0.039	0.063	0.071	0.082
40	0.033	0.058	0.108	0.111	0.169

^aRat brain tubulin 9.5 μM , taxol 10 μM , GTP 0.8 mM, 25.5 °C.

Because the Mes assembly buffer used contained ~ 74 mM Na^+ ion, it was important to learn what the alkali metal cation response would be in the presence of a “neutral” buffer counterion. The usual sodium substitute, choline chloride, could not be used because it was inhibitory. Consequently, we examined different methylamine chlorides (Fig. 1, inset). Of these, dimethylamine chloride proved to have the least effect on the rate of polymerization when added to the standard polymerization system. Mes was, therefore, titrated to pH 6.9 with dimethylamine (DMAMes) and used to compare the effects of alkali metal chlorides. As shown in Figure 3, the selectivity sequence was the same as seen in standard Mes buffer, i.e., $Na^+ > K^+ > Li^+ > Cs^+$. However, the concentration required for peak response to added salts was increased by

Fig. 3. Alkali metal chloride-induced enhancement of tubulin polymerization in the presence of 0.1 M dimethylamino-Mes buffer, pH 6.9, 10 μM taxol, 1% DMSO, 0.8 mM GTP, 25.5 °C, and 2 min temperature equilibration. Higher tubulin concentrations were required and 18.4 μM tubulin was added to start the reaction. OD_{350} readings were started at 30 s.

Table 2. Effect of alkali metal chlorides on the polymer properties of rat brain tubulin

Salt	Concentration (mM)	Critical concentration (mg/mL)	Fraction microtubules	Scattering coefficient OD ₃₅₀ /mg/mL
Control		0.39	0.90	0.231 ± 0.013
NaCl	100	0.19	0.97	0.277 ± 0.011
KCl	100	0.21	0.97	0.279 ± 0.012
LiCl	100	0.29	0.95	0.289 ± 0.009
CsCl	50	0.31	0.99	
CsCl	100			0.256 ± 0.005

approximately 50–80 mM. In the same vein, we compared the alkali metal chlorides in 10 mM Mes assembly buffer and found that selectivity persisted despite the lower polymerization rates. These data are presented in conjunction with those on microtubule protein (Fig. 6D). We thus felt justified in using standard Mes buffer for most of the experiments.

Although the maximal rate enhancement was 6–10-fold, the steady state OD₃₅₀ was increased only approximately twofold. This is confirmed by measurements of the critical concentration under the same conditions (10 μ M taxol, 25.5 °C, 0.8 mM GTP in Mes assembly buffer). The critical concentration was reduced from a control value of $\sim$ 0.4 mg/mL to $\sim$ 0.2 with 100 mM NaCl or KCl, 0.29 with 100 mM LiCl, and 0.31 with 50 mM CsCl (Table 2).

Criteria for microtubules

That the polymers formed in the presence of added alkali metal chlorides were microtubules or microtubule-like was ascertained by three criteria: cold reversibility of polymerization, podophyllotoxin sensitivity, and electron microscopy. Because taxol-promoted polymers are hyperstabilized, reversibility by cold was examined in the presence of 10% DMSO as polymerization promoter. Cold reversibility was 89% for controls and 82% in the presence of

160 mM NaCl. This degree of cold reversibility is greater than that observed with GuHCl-promoted tubulin polymers (Wolff et al., 1996), consistent with the greater degree of stabilization that is obtained with GuHCl. Rewarming led to repolymerization at nearly the starting rate.

Podophyllotoxin (10 μ M) completely inhibited control and 160 mM NaCl-enhanced polymerization in the presence of 10 μ M taxol when added at the start of the reaction. On the other hand, and as expected, steady-state taxol polymers were depolymerized by only 21% when podophyllotoxin was added to both controls and NaCl-treated preparations. These drug effects resemble those observed previously with GuHCl (Wolff et al., 1996).

Electron micrographs of sections from pellets of microtubules prepared in the absence or presence of alkali metal chlorides are depicted in Figure 4. From cross-section counts of $>2,000$ (1,544 for Li⁺) the predominant polymers were, in all cases, normal microtubules—amounting to 90, 97, 97, 95, and 99% of the total cross sections counted for control, 100 mM Na⁺, K⁺, Li⁺, and 50 mM Cs⁺ tubules, respectively. It is noteworthy that all of the salts increased the fraction of polymers present as microtubules, particularly in the case of CsCl. The largely normal appearance of the microtubules is consistent with the cold depolymerization data and the small change, if any, in scattering coefficient detailed above. The remainder of the polymers consisted of aberrant microtubule forms, consisting primarily of “s” forms of the same contour length as microtubules, but with orientation of one protofilament reversed. There were minor contributions from σ , ω - and vermiform-shaped structures. The presence of a minority of aberrant forms in taxol microtubules has been noted previously (Parness & Horwitz, 1981); these forms appear to be less stable and can be diminished by pH changes (Ringel & Horwitz, 1991) or by alkali metal chlorides, as shown here. It is important to note that such aberrant microtubule forms are substantially less abundant with the alkali metal cations than after exposure to GuHCl (Wolff et al., 1996). In agreement with the high percentage of normal microtubules are the scattering coefficients obtained under the same incubation conditions (Table 2). Because the non-microtubule polymers constitute $<10\%$ of the total and are mostly of the same size cross-section, they would not be expected to influence the scattering coefficient to a large extent. Except for the controls, all values are within ± 2 standard deviations of the mean value of 0.266.

Fig. 4. Electron micrographs of pelleted polymers produced by tubulin assembly in 10 μ M taxol, 0.8 mM GTP, at 25.5 °C for 1 h. Controls at left; 50 mM CsCl at right. Polymers were pelleted by 5-min centrifugation at room temperature at full speed in a Beckman airfuge, fixed, stained, and sectioned as described in Materials and methods.

Effect on urea

We had shown earlier that the polymer-promoting action of GuHCl could overcome the polymerization-inhibiting action of urea (Wolff et al., 1996). In a similar fashion, alkali metal cations can overcome the depolymerizing action of urea, as shown in Figure 5. As shown in the top panel, 400 mM urea decreased the rate of polymerization >90%. The additional 40 mM NaCl restored polymerization to the rate in the absence of additions. Another tubulin preparation required 80 mM added NaCl to restore the control rate (data not shown). Control polymerization rates (in the absence of urea) were increased, as shown also in Figure 1. Alternatively, as shown in the lower panel, 160 mM NaCl increased markedly the urea concentration required to reduce the rate of polymerization to one half, from an IC_{50} of ~ 150 mM urea, at which concentration there is no detectable effect when NaCl is present, to an IC_{50} of ~ 350 mM in the presence of NaCl. These results recall the stabilization against urea or thermal denaturation by sodium ion of ribonuclease T1, a small, very acidic protein ($pI = 3.8$), with excess repulsive interactions at pH 7 (Pace & Grimsley, 1988; Mayr & Schmid, 1993). It is also of interest here that K^+ ion stabilized charge-repulsed coiled coils against urea unfolding (Monera et al., 1993, 1994).

Fig. 5. Effect of NaCl on urea-induced inhibition of tubulin polymerization. Top: 9 μ M tubulin in Mes assembly buffer containing 10 μ M taxol, 0.8 mM GTP, and 400 mM urea where indicated. Added NaCl is indicated on curves in mM. Bottom: Incubation conditions as above but NaCl concentration was fixed and urea varied. Lower curve, no NaCl; upper curve, 160 mM NaCl. Note logarithmic ordinate scale.

Microtubule protein

The above selective cation effects using pure tubulin and taxol are at variance with those presented many years ago using preparations containing MAPs (Haga et al., 1974; Lee et al., 1974; Olmsted & Borisy, 1975). It was possible that the nonselective screening reported in these studies was a result of the presence of MAPs. Therefore, we looked for cation selectivity in the polymerization of microtubule protein preparations (containing MAPs). The results are depicted in Figure 6A. It is obvious that the rates of polymerization could be increased by adding various salts to nearly the same peak value, and then decreased at higher concentrations. In contrast to the taxol system, there was little selectivity for one cation over another. To elucidate whether MAP2 or tau proteins might be responsible for the marked reduction in cation selectivity for polymerization, we assessed the effects of MAP2 and tau on taxol-driven tubulin polymerization, comparing the two most disparate cations of Figure 3, Na^+ and Cs^+ . It is apparent from Figure 6B that the addition of MAP2 to rat brain tubulin diminished markedly the difference between the Na^+ and Cs^+ effects on polymerization. The ratio of the Na^+ to the Cs^+ effect at 120 mM salt was reduced from a value of ~ 3.8 to 1.9 by addition of MAP2 to a mole ratio of 0.25 (MAP2/tubulin) and approaches the ratio observed with microtubule protein (MTP in Fig. 6B) in the absence of taxol (~ 1.2). Similar results were obtained with tau protein, although the required mole ratios were higher (data not shown).

To investigate the role of the residual Na^+ ion present in the Mes buffer, we studied polymerization of microtubule protein in 10 mM Mes buffer containing 1.0 mM Mg^{++} ion. Under these conditions (Fig. 6C), no polymerization occurred in the absence of added salt. When 80 mM alkali metal chlorides were added (arrow), all four cations stimulated the rate of polymerization to essentially the same extent. That is, no selectivity was observed. This should be contrasted with taxol-driven tubulin polymerization under otherwise the same conditions (Fig. 6D). Here the same cation selectivity observed as with regular-strength buffer (0.1 M Mes, as in Fig. 1) could be reproduced readily. Hence, neither residual Na^+ ion nor ionic strength make major contributions to the observed differences between microtubule protein and tubulin. This suggests that the MAPs shield those anionic groups responsible for the cation selectivity, and any residual cationic shielding is of the Debye-Hückel type. This led us to investigate the reduction of excess charge by the use of tubulin S.

Tubulin S

When C-terminal negative charges are removed or neutralized by esterification, the net negative charge is reduced and the critical concentration for polymerization is markedly lower. Shielding of such charges by guanidinium or alkali metal chlorides also lowers the critical concentration and increases markedly the rate of polymerization. When the C termini are removed, GuHCl is a much less effective promotor of polymerization (Wolff et al., 1996). If the alkali metal cations' shielding is of a similar nature, then removal of a portion of the excess negative charge on tubulin by limited hydrolysis with subtilisin should diminish their effect on the rate of polymerization. To facilitate comparison with the above experiments, tubulin S was studied in the presence of taxol and GTP and at 25.5 °C. In regular Mes assembly buffer, additional Na^+ or K^+ ions stimulated the rate of tubulin S polymerization by no more than 20% (data not shown). In marked contrast, when the buffer is DMAMes, substantial enhancement of the polymerization

Fig. 6. Effect of alkali metal chlorides on the polymerization of microtubule protein or tubulin + MAP₂. **A:** Rat brain microtubule protein (1.6 mg/mL) in Mes assembly buffer containing 0.8 mM GTP and 80 mM salts as indicated. Maximal rates at 25.5 °C are compared. $\blacktriangle$, Na⁺; $\blacksquare$, K⁺; $\circ$, Li⁺; $\bullet$, Cs⁺. **B:** Effect of MAP₂ on the relative effectiveness of cation-induced rat brain tubulin polymerization. Mes assembly buffer containing 10 μ M taxol, 0.8 mM GTP, and 120 mM NaCl or CsCl, and varying concentrations of beef brain MAP₂. **C:** Selectivity for alkali metal chlorides at low ionic strength on microtubule protein polymerization; 10 mM Mes assembly buffer fortified to 1.0 mM MgCl containing 10 μ M taxol, 0.8 mM GTP, and 0.8 mg/mL microtubule protein; 80 mM salts were added at the arrow. Note that no polymerization occurred until salts were added. **D:** Selectivity for alkali metal chloride-induced promotion of polymerization of tubulin at low ionic strength. Conditions as in C, except that 9 μ M tubulin was used.

rate occurs with the addition of Na⁺ or K⁺ and lesser enhancements are produced by Li⁺ or Cs⁺ (Fig. 7). The pattern of stimulation is similar to that seen with intact tubulin (see Fig. 5), and the selectivity remains Na⁺ > K⁺ > Li⁺ > Cs⁺, but the extent of stimulation is clearly less than with intact tubulin. The results with tubulin S suggest that there are additional shielding-sensitive, but still selective, repulsions in the tubulin monomers not at the extreme C termini, and that, under these incubation conditions, tubulin S is less responsive to salts than native tubulin.

Discussion

For most proteins, electrostatic interactions are considered for their potentially positive contributions to stability of the protein or of protein/protein interactions. Much of this may be achieved by minimization of repulsive interactions (Spasov et al., 1994). Microtubules differ in that excess polymer stability hinders function, and some level of reversibility is desirable for normal microtubule dynamics. This is achieved to a substantial extent by the very acidic C termini on both α and β tubulin, formed in part from the primary sequence, and in part by posttranslational γ glutamylation (see Redeker et al., 1994 for refs); when the acidic C termini are removed or neutralized, the equilibrium is shifted markedly toward

Fig. 7. Tubulin S (2.1 μ M) was incubated at 25.5 °C in DMAMes buffer containing 10 μ M taxol, 0.8 mM GTP, and increasing concentrations of alkali metal chlorides.

polymer (Sackett et al., 1985; Serrano et al., 1988; Mejillano & Himes, 1991; Melki et al., 1991). Several types of repulsive interactions have been shown in the present study to exert effects on tubulin polymerization depending on the type of polymerization promoter employed: (1) cation-selective interactions in taxol-driven tubulin polymerization; (2) less responsive, but still selective cation interactions in tubulin S; and (3) nonselective cation effects in the presence of MAPs.

When *in vitro* studies of microtubule assembly first became feasible, NaCl and KCl were found to inhibit polymerization at concentrations in excess of 80 to 150 mM, whereas lower concentrations sometimes stimulated polymerization (Haga et al., 1974; Kuriyama & Sakai, 1974; Lee et al., 1974; Olmsted & Borisy, 1975; Farrell et al., 1979). Because Na^+ and K^+ were equipotent, these findings were interpreted on the basis of an ionic strength optimum. Subsequently, the inhibitory effects were reinterpreted in terms of nonspecific shielding of attractive electrostatic interactions between anionic regions on tubulin and cationic patches on MAPs (Littauer et al., 1986; Melki et al., 1991).

Our results with taxol-polymerized tubulin and organic cations (Wolff et al., 1996), and the present findings with alkali metal cations, show that there is marked cation selectivity for increasing the rate of polymerization. Three components appear to contribute to this selectivity: (1) a background influence of the anion depending on its chaotropic potential; (2) promotion of polymerization with cation selectivity $\text{Na}^+ > \text{K}^+ > \text{Li}^+ > \text{Cs}^+$; and (3) an *apparent* inhibitory cation effect at high concentrations with a reverse rank order to that promoting polymerization, i.e., $\text{Cs}^+ > \text{Li}^+ > \text{K}^+ > \text{Na}^+$. This effect may be exerted on a different tubulin region or may merely reflect the greater ability of $\text{Na}^+ > \text{K}^+ > \text{Li}^+ > \text{Cs}^+$ to counteract the chaotropic effect of increasing chloride concentrations. An identical selectivity sequence has been obtained for the activation of thrombin (DiCera et al., 1995) and for coil-helix transitions in polyglutamic acid (Satoh et al., 1982). The electrostatic contribution for an interhelical Glu–Glu repulsion in coiled coils has been calculated to be +0.5 kcal/mol (Kohn et al., 1995a). It is of interest that cation-promoted association can also be seen in the monomer–monomer interaction of tubulin: 0.3 M NaCl leads to an increase in the association constant between α and β monomers in both tubulin and tubulin S (Panda et al., 1992).

There is no simple linear relationship of the above potency sequence with either the crystal radii or hydration enthalpies of the cations. The most successful theory accounting for ion selectivity is that of Eisenman (1961, 1962), which is based on the balance between the electrostatic attraction of the fixed charge and the exchanging ion on the one hand, and the hydration enthalpy of that ion (which provides a thermodynamic penalty for desolvation) on the other. The equation governing this relation (in an ion exchange context) for the gain in the free energy, $\Delta G_{1/2}$, of the selecting ion exchange reaction is:

$$\Delta G_{1/2}^\circ = \left(\frac{e^2}{r_f + r_2} - \frac{e^2}{r_f + r_1} \right) - (\Delta G_2 - \Delta G_1),$$

where e is the electronic charge; ΔG_1 and ΔG_2 are the standard free energies of hydration of cations 1 and 2; and r_f , r_1 , and r_2 are the radii of the fixed negative charge, the entering cation, and the leaving cation, respectively. The more strongly the ion binds, the greater the selectivity for it. For a weak fixed negative field, which has greater difficulty in overcoming the hydration energy of incoming cations, the sequence is $\text{Cs}^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+$. By

contrast, for a strong fixed negative field (often associated with a relative small negative ion; carboxylate is considered small relative to other polyatomic anions), the reverse sequence is observed, i.e., $\text{Li}^+ > \text{Na}^+ > \text{K}^+ > \text{Cs}^+$. Intermediate rank order of sequences can exist depending on the balance of the electrostatic and hydration forces (Eisenman, 1962). Reichenberg (1966) has provided a very instructive numerical example (for anion exchange), demonstrating such rank order shifts in selectivity sequences. Although there may be overlapping fields on the one hand, and partial contributions from several fields (depending on the spacing) on the other, the rank order of selectivity provides information on the field strength of the fixed charge.

Various negative fields can contribute to cation binding in proteins. These include: formal negative charges, as in carboxylates or phosphates; polarized bonds as in $>\text{C}=\text{O}$, where oxygen carries a -0.44 electron charge (Pauling, 1960), or $-\text{OH}$ of serine or threonine; helix dipoles; and cation- π electron interactions. In most cases, carbonyl and carboxyl contributions predominate, although charge- π interactions appear to be the dominant selecting force in some cases (Raine et al., 1995; Dougherty, 1996). Water can mediate between the electrostatic fields in all of these cases, affecting primarily the magnitude rather than the rank order of the selectivity (Eisenman, 1961, 1962).

The large excess of coded and posttranslationally added carboxylates in tubulin, particularly at the C termini, can be expected to be in the anionic form under the pH conditions of polymerization, see, e.g., the relatively unperturbed pK_a values determined recently by NMR titration of individual carboxylates in thioredoxin (Qin et al., 1996). A few tubulin carboxylates may be “neutralized” in salt bridges, but the bulk are not. The excess glutamates at the C termini suggested a comparison with carboxylate exchange resins or with polyglutamic acid. Carboxylate ion exchange resins show a selectivity ($\text{Li}^+ > \text{Na}^+ > \text{K}^+$) consistent with a strong negative fixed field (Gregor et al., 1956). On the other hand, promotion of the coil-helix transition of polyglutamic acid by cations follows the same selectivity sequence as tubulin polymerization, i.e., $\text{Na}^+ > \text{K}^+ > \text{Li}^+ > \text{Cs}^+$ (Satoh et al., 1982). Not surprisingly, this order is sensitive to the polarity of the environment, becoming $\text{Na}^+ > \text{Li}^+ > \text{K}^+$ when the solvent dielectric constant is reduced (Satoh et al., 1991). Krimm and coworkers suggested for polyglutamic acid at neutral pH that repulsive forces would dictate the formation of an extended helix of ~ 2.5 residues per turn to obtain minimum energy structures (rather than random coils) (Krimm & Mark, 1968; Tiffany & Krimm, 1968). This, in turn, suggests a charge distribution extending well beyond the cylinder constituting the microtubule (Sackett, 1995). It is possible that the clear zone observed frequently around microtubules in electron microscope pictures may be the result of such charge distribution (Stebbins & Bennett, 1975).

The possibility that taxol influences the charge distribution should be kept in mind. For example, taxol-assembled microtubules show a different distribution of protofilament numbers per microtubule than reference microtubules and have a slightly increased subunit spacing in the lattice (Arnal & Wade, 1995), and they are thought to have decreased rigidity (Dye et al., 1993; Venier et al., 1994), possibly changing the exposure of the C-terminal carboxylates of both monomers for cation interaction. Whatever the contribution of taxol-induced structural changes may be, the alkali metal-induced enhancement of the rate of polymerization may be ascribed to their interaction with and shielding of glutamates (or aspartates) abundantly present in tubulin.

We conclude that both guanidinium congeners (Wolff et al., 1996) and alkali metal cations promote tubulin polymerization (particularly the rate) by cation-selective shielding of repulsive negative charges in tubulin. The polymers generated in the presence of alkali metal chlorides show a larger fraction of normal microtubules than with GuHCl, and they are cold and podophyllotoxin sensitive. The diminished shielding response when the acidic C termini are not operative supports the shielding interpretation; cation selectivity is still present in these preparations. The C-terminal carboxylates may owe their greater effectiveness and selectivity to the high density of the carboxylates. On the other hand, in the presence of MAPs, the residual shielding is no longer significantly cation selective. The difference between MAP-containing tubules and those made with tubulin S suggests that MAPs interact with regions of the protein other than the C-terminal carboxylates.

Materials and methods

Materials

Rat brain tubulin was prepared from microtubule protein (Sackett et al., 1991) by sequential polymerization in 1.6 M Mes, pH 6.9, and 1.0 M sodium glutamate (Hamel & Lin, 1984) as follows: solid, neutralized Mes was added to microtubule protein containing 1 mM dithiothreitol (DTT), 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 DTT. The mixture was incubated for 1 h at 37 °C and centrifuged in a Beckman Ti45 rotor at 30,000 rpm, 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 rotor at 35,000 rpm. The supernatant solution was brought to 0.1 mM DTT and 1 mM GTP, incubated at 37 °C for 45 min, and centrifuged in a Ti70 rotor for 30 min at 33 °C. Pellets were resuspended in Mes assembly buffer, 1 mM MgCl₂, 1 mM EGTA (ethyleneglycol bis-β aminoethyl ether N,N'-tetraacetic acid), pH 6.9, sonicated, incubated on ice for 45 min, and centrifuged in a Ti70 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 (NCI), was dissolved in DMSO; the final DMSO concentration was 1.0%. MAP₂ was a gift of Dr. Ernest Hamel (NCI).

Rat brain tubulin S was prepared from rat brain microtubule protein diluted to 4.0 mg/mL in 0.1 M MOPS buffer, pH 6.0, 1 mM MgCl₂, and 1 mM GTP by addition of subtilisin BPN in a weight ratio of 1:50 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 18,000 × g at 4 °C for 20 min. The supernatant was diluted with 1/2 volume of glycerol, adjusted to 1 mM MgCl₂ and 1 mM GTP, incubated at 37 °C for 45 min to polymerize the tubulin S, and centrifuged at 18,000 × g, 33 °C for 20 min. The pellet was resuspended in 1/2 strength Mes assembly buffer, assayed for protein by the bicinchoninic acid method (Pierce Chemical Co., Rockford, Illinois), and drop-frozen and stored in liquid nitrogen. Some frozen preparations of tubulin S were cloudy when thawed and required re-centrifugation.

Polymerization methods

Both taxol- and DMSO-driven polymerization were employed using turbidity development at 350 nm in 250 μL cells at 25.5 ± 0.1 °C

in thermostated cell holders of a Cary 219 instrument. In most studies, polymerization was conducted in 10 μM taxol and 0.8 mM GTP, and the reaction was started by addition of 7.5 or 8.8 μM tubulin to prewarmed (27 °C) reagents. In 10% DMSO (final), reactions were started with 25 μM tubulin. Rates of polymerization were measured starting at 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 (~30–40 min), followed by cooling on ice for 5–10 min in order to measure the extent of cold depolymerization. Sodium-free Mes assembly buffer was prepared by titrating Mes to pH 6.9 with dimethylamine.

Scattering coefficients were determined after incubation of tubulin for 1 h at 25.5 °C in the taxol system. After measurement of the OD₃₅₀, samples were centrifuged for 5 min in an A100/18 rotor in a Beckman airfuge at 30 psi at room temp, and the protein in the pellet was determined. The dependence of light scattering on the wavelength was determined after 30 min polymerization of tubulin in the taxol system at 25.5 °C over a range from 320 to 500 nm. Results were analyzed according to the relation $\log OD = a + n \log \lambda$.

Critical concentration

Different concentrations of tubulin were incubated in 10 μM taxol at 25.5 °C for 60 min in the absence or presence of alkali metal chlorides. Samples were then centrifuged for 5 min in an A-100/18 rotor at room temperature in an airfuge run at 30 psi (~150,000 × g). Pellet and supernatant solution were separated, the pellet was dissolved in 0.3 M NaOH, and protein was determined by the bicinchoninic acid method.

Electron microscopy

Electron microscopy was performed on sectioned pellets of polymers prepared by 1-h incubation of tubulin in the taxol system at 25.5 °C in the absence or presence of added alkali metal chlorides. Following polymerization, polymers were pelleted as above. Pellets were processed using a protocol modified from Kim et al. (1979); pellets were fixed overnight in Millonig's phosphate buffer, pH 7.4, containing 0.5% glutaraldehyde, 1% tannic acid, and post-fixed with 1% osmium tetroxide for 1 h, dehydrated, embedded in Spurr's medium, and stained en bloc with 2% uranyl acetate for 1 h. Silver sections of about 75 nm were prepared and stained with lead citrate.

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