Charge-Shielding and the "Paradoxical" Stimulation of Tubulin Polymerization by Guanidine Hydrochloride

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ABSTRACT: Low concentrations of guanidine hydrochloride (GuHCl) increase the rate (and to a lesser degree, the extent) of tubulin polymerization as assessed by light scattering. Maximum enhancement occurs at 120−160 mM GuHCl followed by decreases at higher GuHCl. The latent period is decreased, and there is a 3−4-fold reduction in the critical concentration for polymerization. Electronmicrographs reveal microtubules in the controls and an increasing fraction of total polymers present as aberrant microtubules as the GuHCl concentration is increased from 20 to 100 mM. The GuHCl effect is markedly reduced, but not abolished, in tubulin S (in which the anionic C termini of both monomers have been removed). The GuHCl-induced polymerization has an absolute requirement for GTP and taxol or DMSO, is very sensitive to podophyllotoxin inhibition, and can overcome urea-mediated inhibition of polymerization. Guanidinium analogues mimic the GuHCl effect roughly as a function of the number of potential hydrogen bonds. The anions of the guanidine salts superimpose their inhibitory action on the guanidinium cation effect according to the lyotropic series. At higher GuHCl concentrations (peak effect 500−700 mM), a different polymer (type II) is formed that is GTP and taxol independent, but whose polymerization is retarded but not prevented by podophyllotoxin. Its structure resembles the fibrillar network seen in unfolding intermediates of other proteins. We conclude that both charge and hydrogen-bonding ability are major contributors to the GuHCl-induced promotion of tubulin polymerization, and that charge-shielding is likely to be the basis for this effect.

The mechanism of action of guanidine hydrochloride or urea is not understood in detail. To what extent the effects produced by these compounds can be ascribed to binding to protein or to changes in the properties of water has not been settled (Arakawa & Timasheff, 1984; Makhatadze & Privalov, 1992, for references). The number of binding sites, measured by calorimetry on three small proteins in the unfolded state, is 2.5−3 times greater for urea than GuHCl.[1] It has been suggested that this reflects the hydrogen-bonding propensity of the two denaturants; i.e., guanidinium ion has a greater number of proton donors than urea. However, in special situations (e.g., urea-soaked crystals), urea may form up to five hydrogen bonds (Pike & Acharya, 1994), so this question has not been settled. Enzyme activity is often, but not inevitably, lost before structural changes can be demonstrated, and, frequently, substrates give partial protection in shifting the denaturant concentration curves to higher concentrations. In some enzymes activities are inhibited or lost at rather low concentrations of GuHCl (<0.5 M), perhaps by interacting at the protein surface, by restricted local unfolding, or by interacting with the active site. These include the following: ATPases of myosin subfragment I (Nozais et al., 1992); hexokinase, pyrophosphatase, and glyceraldehyde-3-phosphate dehydrogenase (Garza-Ramos et al., 1992; Liang et al., 1990); papain (Xiao et al., 1993); tetrameric phosphofructokinase (Teschner & Garel, 1989); and cytochrome p450 (Yu et al., 1995). Similar sensitive responses to low urea concentrations have also been described [see Sackett et al. (1994) for references]. Although urea unfolds many of the same proteins affected by GuHCl, it fails to unfold some proteins completely and is generally less effective on a mole for mole basis.

We have recently shown that tubulin, the predominant component of microtubules, can be converted into a number of stable, *locally* unfolded, states by the use of low concentrations of urea. These states are characteristic of the various readily measured functional properties of the tubulin heterodimer (Sackett et al., 1994). The order of urea sensitivities is as follows: decreased rate of polymerization (measured by light scattering at 350 nm) > decreased extent of polymerization ≈ decreased GTPase activity > enhanced fluorescence of a tubulin-bound analogue of colchicine ≈ decreased proteolytic susceptibility toward trypsin and chymotrypsin (on kinetic grounds these latter two phenomena could be ascribed to local tightening of domains) > enhanced ANS (1-anilino-8-naphthalene sulfonate) fluorescence. These effects of urea span a concentration range of <100 mM to ∼1.5 M. The independence of these states was confirmed by the finding that they had different time dependencies. Most of these changes occurred at urea concentrations where no change in intrinsic tubulin fluorescence could be detected. At higher urea concentrations the enhanced fluorescence of the colchicine analogue and ANS was reversed and then abolished, whereas proteolytic susceptibility was markedly increased. These effects were ascribed to *global* unfolding. Several other proteins also show such multiphasic effects

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[1] Abbreviations: GuHCl, guanidine hydrochloride; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether); Mes, morpholinoethanesulfonic acid; C_c, critical concentration for tubulin polymerization.

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during denaturation (Tsou, 1986; Pace & Laurents, 1989; Herold & Kirschner, 1990; Leòn et al., 1991; Hill et al., 1988).

The differences in the potency of urea and GuHCl mentioned above are generally assumed to range from 2 to 3 in favor of GuHCl (Greene & Pace, 1974), but there may be significant deviations from this potency ratio. Because of the ionic nature of GuHCl, this ratio should be dependent on the protein under study. One might reasonably argue that proteins that are significantly stabilized by attractive Coulomb interactions would be destabilized by GuHCl and show a high potency ratio of GuHCl/urea. On the other hand, if repulsion by like charges significantly destabilizes a protein, then GuHCl could stabilize, and the potency ratio would be low (Monera et al., 1994).

Tubulin is an acidic protein with high anionic charge excess at the C-termini of both the α and β monomers. Enzymatic removal (Sackett et al., 1985; Maccioni et al., 1986) or neutralization (Mejillano & Himes, 1991) of these charges markedly changes the properties of tubulin. We were interested to test whether or not low concentrations of GuHCl would mimic the effects of urea on the various tubulin properties described earlier (Sackett et al., 1994) or produce effects ascribable to its cationic nature. To our surprise, low concentrations of GuHCl *promoted* tubulin polymerization considerably and could overcome the inhibition of polymerization produced by urea. This phenomenon, and our attempts to understand its mechanism, are the subject of the present paper.

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 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, 33 °C, for 45 min. Pellets were resuspended in ~1/3 the starting volume with cold 1.0 M sodium glutamate, 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 dithiothreitol 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 (0.1 M morpholinoethane sulfonic acid, 1 mM $MgCl_2$, 1 mM EGTA [ethyleneglycol bis(β-aminoethyl ether)], 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, was dissolved in DMSO; the final DMSO concentration was 0.4−1.0%. Guanidine HCl was from ICN (Ultra Pure Grade) and was stored as a 6 M solution in Mes assembly buffer in fozen pellet form. Guanidine HF, guanidine HBr, and guanidine HI were made from guanidine carbonate (Aldrich) by titration to pH 6.9 with the respective hydrohalic acids. 1,1-Dimethylguanidine HCl was from Chem Service, West Chester, PA. Arcaine and norarcaine were gifts from Dr. Jack Folk, National Institute of Dental Research. All other guanidine derivatives and analogues were from Aldrich Chemical Co., Milwaukee, WI.

Rat brain tubulin S was prepared from rat brain tubulin diluted to 2.0 mg/mL in Mes assembly buffer and 1 mM GTP (final concentration) by addition of subtilisin BPN in a weight ratio of 1:100 and incubated at 30 °C for 70 min. The reaction was stopped with 0.01% phenylmethanesulfonyl fluoride in DMSO, incubated at 37 °C for 10 min to polymerize the tubulin S, and centrifuged at 70000*g*, 33 °C, for 30 min. The pellet was resuspended in Mes assembly buffer, placed in ice for 45 min, and centrifuged at 4 °C for 30 min. The supernatant solution was assayed for protein by the bicinchoninic acid (BSA) method (Pierce Chemical Co., Rockford, IL) and drop-frozen and stored in liquid nitrogen.

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 carried out 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 (26 °C) reagents. In some experiments polymerization was carried out in 10% DMSO (final) and 0.8 mM GTP starting the reaction 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 min), and, in some cases, samples were cooled on ice for at least 5 min stirring with 2 × 10 strokes up and down a pipette tip, in order to measure the extent of cold depolymerization.

Scattering coefficients were determined after incubation of tubulin for 1 h at 25.5 °C in the taxol system. After measurement of the OD_{350}, samples were centrifuged for 5 min in a 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 $\log OD = n \log \lambda$.

Formation of large polymers was measured by centrifugation. Tubulin solutions (10 μM) in Mes assembly buffer, 10 μM taxol, and 0.4 μM GTP, containing varying concentrations of GuHCl were incubated at room temp (23 °C) or on ice, followed by centrifugation in a microfuge at 13000*g* at 23 or 4 °C, respectively. Aliquots of the supernatant solutions were carefully removed and assayed for protein by the Bradford method.

Critical Concentration. To determine the effect of guanidine HCl on the critical concentration (C_c) for polymerization, different concentrations of tubulin were incubated in the 10% DMSO system at 36−37 °C for 30 min in the absence or presence of different concentrations of GuHCl. Samples were then centrifuged for 5 min in an A-100/18 rotor at room temperature in an airfuge run at 30 psi (~150000*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

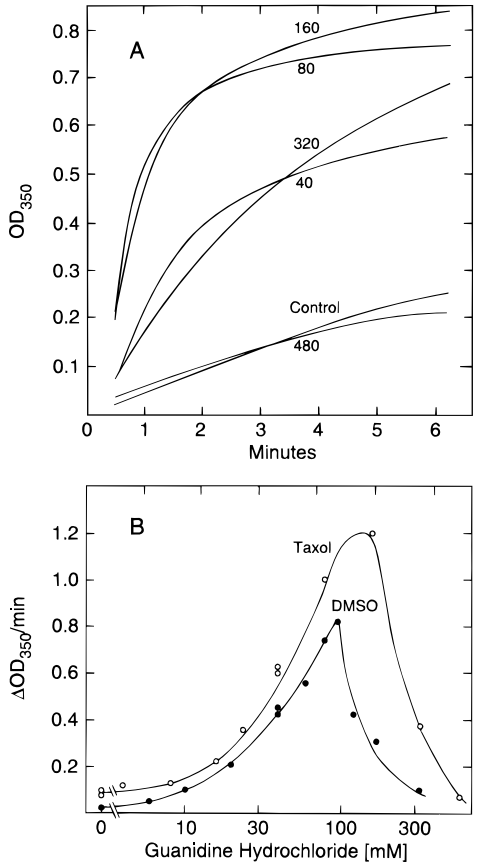

FIGURE 1: Effect of GuHCl on the rate of polymerization of rat brain tubulin at 25.5 °C. (A) Taxol system: tubulin = 7.5 μM; the numbers on the curves are mM GuHCl. (B) The "maximal" rates of increase in OD_{350} as a function of GuHCl concentration. (○) Taxol system with 7.5 μM tubulin. (●) DMSO system with 25 μM tubulin.

of tubulin in the taxol system at 25.5 °C in the absence or presence of added GuHCl. Following polymerization, polymers were pelleted in a Beckman airfuge at 178000*g* for 5 min at room temperature. Pellets were processed using a protocol modified from Kim et al. (1979); pellets were fixed overnight in Mes assembly buffer containing 0.5% glutaraldehyde and 1% tannic acid, and postfixed 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.

RESULTS

Type I Polymers. The effect of GuHCl on the polymerization of tubulin (measured as light scattering at 350 nm) was examined in the presence of two different promoters of polymerization: (a) 10 μM taxol with 7.5 or 8.8 μM rat brain tubulin, or (b) 10% DMSO and 25 μM rat brain tubulin. To permit comparison with our earlier urea experiments (Sackett et al., 1994), most of the reactions were carried out at 25.5 ± 0.5 °C. This predicated higher tubulin concentrations, especially in the DMSO system. Results of a typical experiment using the taxol system are depicted in Figure 1A. For clarity only selected concentrations are shown. Reproducible rate increases could be obtained with GuHCl concentrations <10 mM. Such low concentrations probably indicate a relatively high affinity for the ion and need not necessarily imply the same molecular interactions that lead to denaturation at high concentrations. There was a gradual increase in the rate of development of the OD, attaining a maximum at 120−160 mM GuHCl. This amounted to an average ~25 ± 8-fold increase in rate over the controls under these conditions. Because of the lag period, the term maximal rather than initial rate has been preferred. At higher GuHCl concentrations the maximal rates fell off sharply. Results with the taxol and DMSO systems gave generally similar results (Figure 1B), although the DMSO system showed a somewhat greater sensitivity toward GuHCl in most experiments. The lag was reduced commensurate with the increase in rate, although quantitation of this effect was difficult because of the rapid rates. At low GuHCl concentrations (0−40 mM) there was a ~3-fold decrease in the latent period going from ~45 to ~14 s. We will term the polymers formed under these conditions type I polymers which are microtubule-like and are the focus of the present study. For contrast, we give a brief description of a very different GuHCl-induced tubulin association, called type II polymers, which are reticulated aggregates not resembling microtubules and which are formed under very different conditions (see below).

Earlier studies with urea revealed significant time dependence for the effects induced by moderate urea concentrations (Sackett et al., 1994). Such time dependence also occurred with GuHCl over a period of several hours. However, since we wished to avoid these complications in the present studies, all polymerization effects of GuHCl were evaluated without preincubation, i.e., polymerization was initiated by addition of tubulin to the otherwise complete reaction mixture. In addition, total time of exposure of tubulin to these salts was minimized in most experiments by observing *rates* of polymerization rather than steady-state levels of polymer, which would require long and variable periods of incubation.

We measured the critical concentration (C_c) for polymerization using the DMSO system, pelleting in an airfuge, and determining the protein content of the pellet. The weighted mean values ± standard deviation from multiple experiments are depicted in Figure 2. Under these incubation conditions the control C_c was 1.3 mg/mL. Despite the sizeable standard deviations, it is clear that this value was reduced by a factor of 3−4 by GuHCl but could not be reduced below a C_c of ~0.3 mg/mL with higher concentrations of GuHCl.

Cold and shearing depolymerization of the type I polymers was measured with both the DMSO and taxol systems. Tubulin samples were allowed to polymerize in DMSO at 25.5 °C, and the cuvettes were then immersed in ice water for 5 min and mixed twice with 10 passes up and down a pipette tip at the beginning and end of the cooling time. The reduction in OD_{350} was expressed as percent of the maximum attained at 25.5 °C. In the controls the decrease in OD_{350} produced by cold exposure varied from 68 to 98%. The reason for this variability is not known. GuHCl-promoted polymers invariably depolymerized to a lesser extent with average values in the 55% range. Upon rewarming, polymerization resumed at a somewhat slower rate than the inital polymerization. The reduced reversibility is similar to that observed when assembly is promoted by polycations (Anderson et al., 1985; Lopez et al., 1985).

There was an absolute requirement for added GTP in GuHCl-induced polymerization, and no polymerization occurred with 40, 80, or 120 mM GuHCl when no GTP was

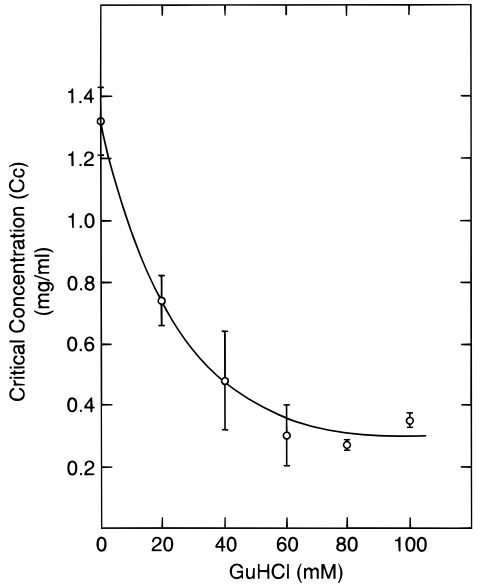

FIGURE 2: Critical concentration for tubulin polymerization as affected by GuHCl. Starting tubulin concentrations varied from 8−48 μM; incubation was at 36−37 °C for 30 min. Microtubules were pelleted in an airfuge, and the protein contents of the pellets was determined. The results are means ± standard deviations of 3−7 experiments.

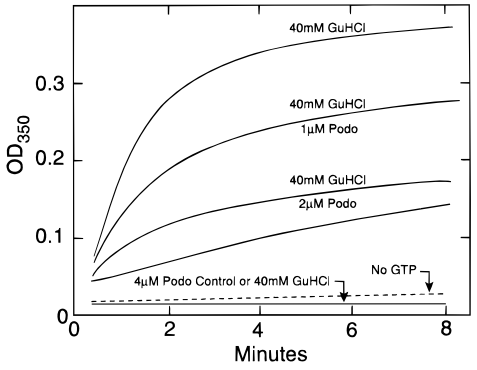

FIGURE 3: Effect of podophyllotoxin on the rate of GuHCl-induced polymerization of tubulin. Taxol system: 8.8 μM tubulin, 25.5 °C, DMSO concentration = 0.4%. Podo = podophyllotoxin. Note that the lowest curve depicts three different conditions: (a) control with 4 μM podophyllotoxin; (b) 40 mM GuHCl with 4 μM podophyllotoxin; and (c) 40 mM GuHCl in the absence of added GTP. The dashed line refers to polymerization in the absence of added GTP. The unlabeled curve is the control.

added (Figure 3). Controls showed a very slow increase in OD_{350} in the taxol system in the absence of added GTP (dashed line in Figure 3). In an additional attempt to understand the nature of the polymer formed under the influence of GuHCl, we studied the effect of podophyllotoxin on the changes in absorption. This drug is a well known mimic of many of the effects of colchicine (Sackett, 1993) but has a more rapid onset of action. In the taxol system excess (120 μM) podophyllotoxin totally prevented polymerization of tubulin in the absence or presence of 24 or 40 mM GuHCl (data not shown). Colchicine had a similar effect. When the concentration of podophyllotoxin was reduced to 4 μM, there was still complete inhibition, and even 1 μM drug led to a marked reduction in the GuHCl-induced polymerization (Figure 3). This inhibition persisted for at least 65 min, the longest period studied. It might be argued that podophyllotoxin merely stabilizes tubulin against GuHCl effects, However, since podophyllotoxin was effective at low substoichiometric levels, it is unlikely that this would protect the bulk (>3/4) of the unliganded tubulin. Rather, it is likely that the podophyllotoxin binding site remained functional in the presence of GuHCl. When podophyllotoxin was added after plateau values in OD_{350} had been attained, even large excesses (120 μM) of the drug failed to induce decreases in OD_{350} beyond the level that can be attained with cold (data not shown). Similar podophyllotoxin sensitivities were also seen in the DMSO system (data not shown) and have been recorded under other conditions (Sackett, 1993).

As a further test of the nature of the type I polymer formed, the ratio between OD_{350} and the pelleted protein content was determined after a 1 h incubation at 25.5 °C. For controls with taxol but no GuHCl, the mean ± SD of the OD_{350}/mg of pellet protein was 0.32 ± 0.028 (n = 13); it was 0.41 ± 0.056 (n = 10) and 0.56 ± 0.046 (n = 10) for samples incubated in the taxol system with 20 and 100 mM GuHCl, respectively. Similar changes in the scattering coefficient were observed in the DMSO system measuring 0.23, 0.30, and 0.45, respectively. The OD/mg increased by ~50% while the rate increased manifold, and the latter is thus not due to an increase in scattering coefficient. When scattering was measured as a function of wavelength from 320 to 500 nm, the difference in n, the slope of plots of log OD vs log λ, was relatively small: for control polymers (±SE) n = −2.93 ± 0.051, with 20 mM GuHCl n = −2.78 ± 0.041, and with 100 mM GuHCl n = −2.84 ± 0.033. These values should be compared with a calculated value of −3 for microtubules (Berne, 1974).

Electron Microscopy. Electron micrographs of steady-state microtubule pellets (178000g) were obtained after 1 h incubation at 25.5 °C in the taxol system without or with 20 or 100 mM GuHCl. Sections from the three groups are depicted in Figure 4. Figure 4A shows polymers produced with no added GuHCl. Both cross-sectioned and longitudinally sectioned polymers are seen. The great majority are normal microtubules. Figure 4B is a section of pellets formed in the presence of 20 mM GuHCl, and Figure 4C is a section from a 100 mM GuHCl pellet. Imperfectly assembled tubules are seen in both Figure 4B and 4C and increase as a function of the GuHCl concentration. These include "C" forms (open microtubules), crenelated ribbons ("S" and "ω" shapes of varying lengths), and "σ" forms (microtubules with an associated ribbon). All of these are microtubule variants, but rings or random aggregates were not observed. Quantitation was carried out from areas in which microtubules were cut in straight cross-section assuming that a similar distribution was present throughout the pellet. The fraction of polymers present as normal microtubules decreased from 94% to 50% to 30% in pellets with no, 20 mM, and 100 mM GuHCl, respectively. The distributions are listed in the legend to Figure 4. The difference is mostly accounted for by an increase in crenelated ribbons at 20 mM GuHCl. Despite a further increase in these forms at 100 mM, most of the difference between 20 and 100 mM is due to an increase in "σ" forms. Microtubule variants of this type have been observed in a number of polymerization systems that do not contain MAPs (Carlier & Pantaloni, 1978; White et al., 1987; Serrano et al., 1988; Mejillano & Himes, 1991). The changes in polymer forms are consistent with the changes seen in the scattering coefficient noted above. The microtubule variants

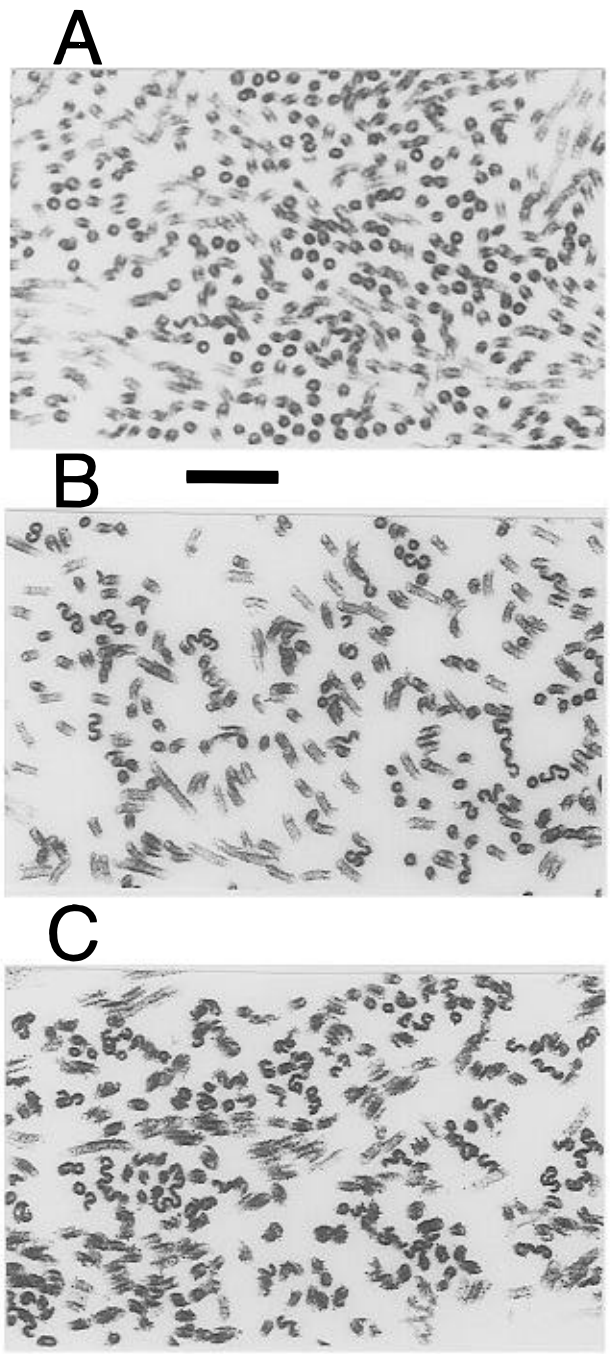

FIGURE 4: Electron micrographs of sections of pelleted polymers produced at different concentrations of GuHCl. Taxol-driven polymerization for 30 min at 25.5 °C, followed by 5 min centrifugation in an airfuge at 30 psi at room temperature. (A) Control polymers: 192 cross sections counted, yielding 94% microtubules, 2% "C" tubules, 3% crenelated ribbons, and 1% "σ" forms. (B) 20 mM GuHCl: 127 cross sections counted, yielding 49% microtubules, 13% "C" tubules, 30% crenelated ribbons, and 8% "σ" forms. (C) 100 mM GuHCl: 218 cross sections counted yielding 31% microtubules, 9% "C" tubules, 36% crenelated ribbons, and 24% "σ" forms. The bar between A and B represents 200 nm for all three panels.

seen after DMSO treatment also show more OD per mg of protein (Himes et al., 1977)

Reversal of the Urea Effect. In our earlier study we showed that polymerization of tubulin was abolished at ≤0.5 M urea (Sackett et al., 1994), a surprisingly low concentration by the usual denaturation standards. The apparently anomalous effect of GuHCl suggested the possibility that the effect

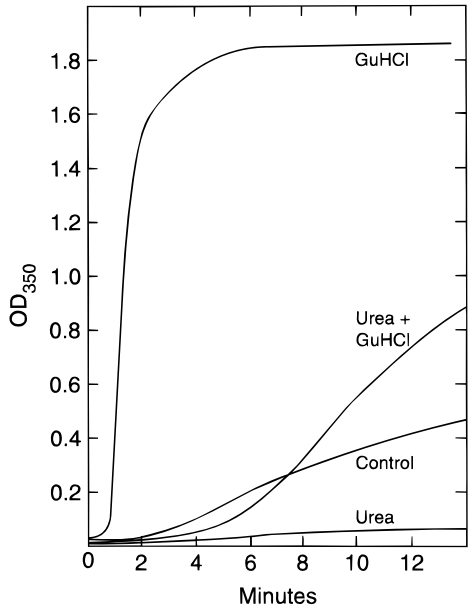

FIGURE 5: Reversal of urea inhibition of tubulin polymerization by GuHCl. DMSO system: samples were incubated at 25.5 °C and contained 25 μM rat brain tubulin, 0.8 mM GTP, 300 mM urea, and 100 mM GuHCl.

of these two denaturants might not be additive, but rather that GuHCl might counteract the inhibition produced by urea. As shown in Figure 5 using the DMSO system, 100 mM GuHCl readily overcame the inhibitory effect of 300 mM urea. The lag period was less efficiently reversed than elongation. While the reversal of the urea effect was sometimes greater than shown in Figure 5, it was not possible to attain the same rates of increase in light scattering that occured in the absence of urea. Similar results were obtained with the taxol system. Moreover, the converse observation was also made: i.e., it took more urea to inhibit tubulin polymerization in the presence of a fixed concentration of GuHCl.

Type II Polymers. A second and quite different effect on tubulin association is observed at high GuHCl concentrations, concentrations at which type I polymer has depolymerized. This aggregation occurs in the absence of taxol, DMSO, or GTP and has a slower rate of development of OD_{350} than is seen in type I polymerization at low GuHCl concentrations. Although they do not resemble microtubule-derived structures, we have termed these type II polymers because they appear to contain structure. These aggregates form only at >300 mM GuHCl and with a substantially longer lag period than seen with the taxol system (data not shown). The increase in the rate peaks at 700−800 mM, followed by a falling off to 1000 mM GuHCl (Figure 6A); it should be noted that light scattering increased nearly linearly for at least 1 h. Cold reversibility was negligible. Such aggregates have also been observed after incubation, centrifugation, and determination of the supernatant protein as shown in Figure 6B, where the protein values have been normalized to the protein concentration in the absence of GuHCl—note the change in concentration scale. It is of interest that the same process still occurs at 0 °C, although to a smaller extent. The minimum supernatant protein concentration occurred at ∼500 mM GuHCl, followed by a return to control levels at higher GuHCl concentrations. Such guanidine-dependent extrema have also been observed in other systems but at

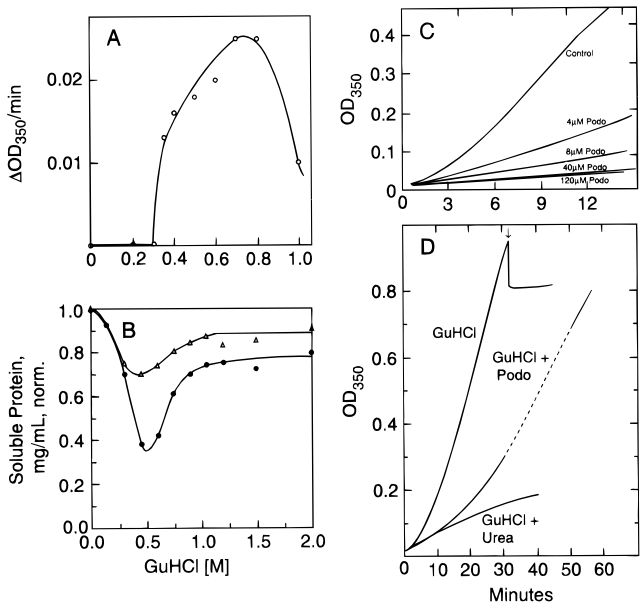

FIGURE 6: Type II polymerization of tubulin. (A) Dependence on GuHCl concentration. Tubulin = 7.5 μM, 25.6 °C. (B) Formation of pelletable protein by high GuHCl concentrations. Tubulin (10 μM) incubated 20 min at 23 °C (●) or on ice (△) and pelleted at 23 or 4 °C for protein determination respectively. Values are normalized to zero GuHCl, i.e., 5.2 and 2.9 μM respectively. The somewhat greater GuHCl sensitivity in B as compared to A derives from the 30 min incubation time used in B but not in A. (C) Effect of podophyllotoxin (Podo) on type II polymer formation. Tubulin (7.5 μM) 25.6 °C, and 600 mM GuHCl was present throughout, and podophyllotoxin was added at the beginning. (D) Recovery from podophyllotoxin inhibition of GuHCl-induced polymerization, and effect of urea. Tubulin = 8.8 μM, 25.5 °C, 640 mM GuHCl, 4 μM podophyllotoxin, 640 mM urea; arrow, 1.28 M urea (corrected for the effect of dilution).

higher GuHCl concentrations (Horowitz & Criscimagna, 1986; Havel et al., 1986; Mitraki et al., 1987).

It was important to distinguish between nonspecific aggregates for type II polymers from aggregates akin to inclusion bodies (Mitraki & King, 1989). The latter are considered to be off (un)folding pathway intermediates with considerable residual structure that are trapped in a conformational energy minimum different from that of the native protein.

Several findings suggest that the type II polymers are not completely nonspecific aggregates. First, high GuHCl concentrations redissolve the aggregate as measured by light scattering depicted in Figure 6A or by direct measurement of supernatant protein (Figure 6B). Second, podophyllotoxin reduces OD_{350} development at respectably low concentrations of podophyllotoxin (Figure 6C). It is possible that, at these concentrations, podophyllotoxin may stabilize tubulin against GuHCl. The aggregates are less sensitive to podophyllotoxin than the type I polymer formed in the taxol system (see Figure 3). However, unlike the taxol/low GuHCl system, there is a gradual resumption of light scattering despite the continued presence of podophyllotoxin, reaching control rates by ~1 h at 25.5 °C (Figure 6D); scattering continues to increase thereafter for considerable periods. Podophyllotoxin leads to a similar inhibition in the increase in light scattering produced by guanidinium sulfate in the absence of taxol or DMSO (data not shown). Finally, equimolar concentrations of urea (640 mM) markedly reduce the effect of GuHCl on type II tubulin association (Figure 6D). However, twice that concentration of urea produced only a very small decrease in OD_{350} once the aggregate had formed (arrow in Figure 6D).

Electron micrographs of these aggregates show rare microtubules with abundant aggregates, which, upon closer inspection, reveal a network of filaments and particles of different size not unlike structures seen in aggregates produced by gentle denaturation of lactic dehydrogenase (Zettlmeissl et al., 1979).

Other Guanidinium Compounds. We were interested to learn what properties made guanidine HCl such an effective promoter of tubulin polymerization. Could the size of the cation be of importance? We found that the alkali metal cations could mimic the guanidine effects in many respects but were substantially less effective. This will be the subject of another report. What parts of the guanidino group were necessary for promoting enhanced light scattering in tubulin preparations? A number of derivatives and analogues of guanidinium (all as chlorides) were tested for their effects on the rate and extent of formation of type I polymer. The results are depicted in Table 1. The maximal rates, the concentrations to attain these, and the approximate maximal-fold increase are listed, as are the concentrations required to achieve 3-fold stimulation of the rates. Because control values varied between experiments and with different preparations of tubulin, the 3-fold increases are only roughly comparable. Single substitutions as in aminoguanidine or 1-methylguanidine caused only modest reductions in potency. However, replacement of a second H with a methyl group as in 1,1-dimethylguanidine led to marked reduction of potency, and a 3-fold increase in rate was never attained. Tetramethylguanidine HCl showed no stimulation at all and was only inhibitory. It is of interest that Gordon and Jencks (1963) found this compound to have poor denaturing activity. These findings suggest that hydrogen bonding may play an important part in the low-concentration effect of guanidine hydrochloride. Reduction of the number of hydrogen bonds as in formamidine HCl led to a less potent cation (Table 1). We were somewhat surprised that the maximal rates obtained with arginine were so low, never achieving 3-fold stimulation. Part of this appears to be due to the carboxyl group of arginine because agmatine (decarboxylated arginine) proved to be a potent promoter of polymerization. Bisguanidino compounds such as arcaine or norarcaine induced the highest maximal rates and fold stimulation. The four or three carbon spacing appeared to make little difference, but two guanidinium groups are clearly more effective than one. It is of interest in this regard that DAPI (4′,6-diamidino-2-phenylindole), a dication related to the above compounds, binds to tubulin with micromolar affinity and leads to a small drop in the critical concentration for polymerization. The authors (Bonne et al., 1985) suggest that this binding occurs at the C-termini of tubulin.

The concentration at which peak rate enhancement occurs varies considerably from one analogue to the next. However, the absence of plateaus in the rate enhancement might imply that there are two competing processes at work in the creation and location of the peak values: (1) promotion of polymerization at low concentrations of guanidinium cations, presumably based on charge shielding and hydrogen-bonding capacity, and (2) reversal of this enhancing effect at somewhat higher GuHCl concentrations (but well below

Table 1: Promotion of Polymerization by Guanidine Analogues

compounds (as hydrochlorides)	maximal rate of polymerization[a] (ΔOD_{350}/min)	concentration (mM) at peak	concentration (mM) for 3-fold increase	fold increase[a] (maximal)
guanidine	0.51	90−120	18−34	20−30
aminoguanidine	0.24	160	13	32
1-methylguanidine	0.13	30	4−5	13
1,1-dimethylguanidine	0.019	20−25		2.4
tetramethylguanidine	inhibitory			
formamidine	0.11	60	37	11
arginine	0.025	35		2.3
agmatine	0.31	20	∼1	44
norarcaine (1,3-diguanidino propane)	0.75	15	∼1	58
arcaine[b] (1,4-diguanidino butane)	0.67	>25	∼2	>83

[a] Mean and standard deviation of 24 control determinations on different days and with different tubulin preparations was 0.037 ± 0.027. In any one experiment controls replicated well; however, direct comparison of the fold stimulation between compounds cannot be made. [b] Peak values were not attained.

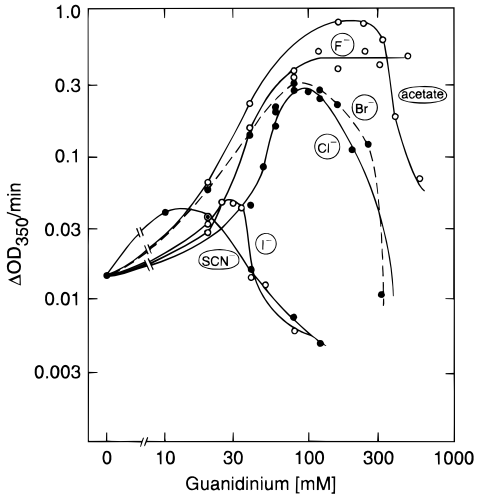

FIGURE 7: Polymerization of tubulin by different guanidinium salts. Taxol system with 8.8 μM tubulin at 25.5 °C. Note that both scales are logarithmic.

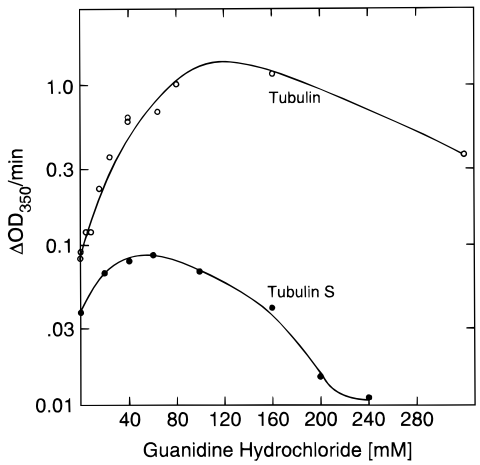

FIGURE 8: Comparison of the GuHCl effect on tubulin and tubulin S. Taxol system with 7.5 mM tubulin at 25.4 °C; tubulin S = 2.05 μM. Note that the ordinate scale is logarithmic.

those for aggregate formation), by a mechanism not immediately apparent. It is known that the two ions of neutral salts are independently effective and are additive for denaturation (von Hippel & Schleich, l969), and the relative effectiveness of different anions remains the same with guanidinium derivatives of different potencies (Castellino & Barker, 1968). Thus, chaotropic salts of guanidine make good denaturants, whereas kosmotropic salts have markedly reduced denaturing activity. Therefore, one possibility to be investigated was the role of the anion at these lower salt concentrations [on the basis of its chaotropic properties (Collins & Washabaugh, 1985; Robinson & Jencks, 1965)]. To this effect, we compared a number of guanidinium salts of monovalent anions. Figure 7 shows that this additive effect is also operative at the low concentrations employed in this study where charge appears to play the dominant role. Kosmotropic anions such as acetate or fluoride promote very high rates of polymerization and fall off only at much higher concentrations than guandine HCl, if at all. By contrast, the iodide and thiocyanate guanidinium salts show only marginal stimulation, and the small "peak" obtained is shifted to much lower concentrations. It thus appears that, at the low concentrations with which we are here concerned, the position of the peak and its height represent a balance between the cation-driven polymerization and the anion-driven depolymerization. We cannot, of course, exclude a guanidinium contribution to the depolymerization phase at these concentrations.

If charge shielding plays a role in the guanidinium effect, then GuHCl should be substantially less stimulatory in tubulin S, in which the highly acidic C-termini of both α and β tubulin have been removed by limited proteolysis with subtilisin (Sackett et al., 1985; Maccioni et al., 1986). This tubulin derivative has a lower C_c; hence lower concentrations were used than for unmodified tubulin. The taxol system was used for comparison purposes. GuHCl still enhanced light scattering with tubulin S, peaking at lower GuHCl concentrations than unmodified tubulin. Direct comparisons are not possible as different amounts of protein were used. However, maximum enhancement was by less than a factor of 3, whereas unmodified tubulin was enhanced by a factor of 13−14 in this experiment and up to 25 in others (Figure 8). These results suggest that other anionic repulsions, not removed by limited subtilisin treatment, may also be involved in the regulation of tubulin polymerization. Whether or not this second effect stems from charge−charge shielding or charge−dipole shielding is not known.

DISCUSSION

Guanidine hydrochloride is usually considered a chaotropic agent, but it is sometimes forgotten that guanidinium is also a cation. This significant property distinguishes it from the other common chaotropic agent, urea. Differences between

the effects of low concentrations of urea and GuHCl on proteins are, therefore, unlikely to be due to their common chaotropic action; rather, they may be due to the charge on the guanidinium ion. The present results showing increased rates and extent of tubulin polymerization under the influence of low concentrations of GuHCl are consistent with the argument that anionic charge repulsion is one factor that controls polymerization and that the guanidinium ion can modulate this repulsion. This effect is due both to an increased rate of nucleation (decreased latent period) and an increase in the rate of elongation; the latter appears to be the bigger effect. This postulated effect of charge has previously been shown by entirely different approaches: (1) the removal of the acidic C-termini from both monomers promotes microtubule assembly and lowers the critical concentration (Sackett et al., 1985; Bhattacharyya et al., 1985; Maccioni et al., 1986); (2) the promotion of tubulin polymerization by MAPs, thought to be due to their basic domains (Littauer et al., 1986; Melki et al., 1991), or by synthetic polycations (Erickson & Voter, 1976; Burton, 1981; Mithieux et al., 1984); (3) esterification or amidation of the C-terminal carboxyl groups promotes assembly and lowers the C_c (Mejillano & Himes, 1991). In this respect it is of interest that lowering the pH used for polymerization of microtubule protein causes similar changes in turbidity development (Gaskin et al., 1974).

Several other examples exist of the apparently paradoxical effect of GuHCl on protein stability and/or activity. Thus, *Escherichia coli* alkaline phosphatase activity is increased 3−4-fold by low GuHCl concentrations but not by urea. This stimulation is thought to be caused by dissociation of the homodimer and the consequent abolition of negative cooperativity (Rao & Nagaraj, 1991). Both lactate dehydrogenase and α-glycerol-phosphate dehydrogenase activities are stimulated by low GuHCl concentrations (Garza-Ramos et al., l992; Fernandez-Velasco et al., 1992; Ma & Tsou, 1991). β-Lactoglobulin is stabilized against urea denaturation by guanidinium and acetamidinium chlorides at low concentrations (Pace & Marshall, 1980). Ribonuclease T1 is stabilized by <0.3 M GuHCl against thermal and urea-induced unfolding (Mayr & Schmid, 1993). Guanidine HCl (<0.5 M) can replace the guanidino group of Arg127 in a R127M mutant of carboxypeptidase and restore activity by stabilizing the enzyme−substrate transition state (Phillips et al., 1992). Finally, the intrinsic fluorescence or quantum yield of aspartate aminotransferase or cytochrome oxidase, respectively, were increased at <1.0 M GuHCl (Herold & Kirschner, 1990; Hill et al., 1988). The mechanisms, where understood, by which the observed effects were obtained may be different, but most are consistent with a charge-based interaction. A number of the stimulatory effects of GuHCl occur with monomeric proteins, whereas in some proteins the effect is exerted on dimers or tetramers, in which the relation between the constituent monomers is altered to bring about the stimulatory effect. A striking analogy of such higher order structural changes produced by GuHCl is seen with melittin, whose tetramerization requires the shielding of its basic amino acids by high salt concentrations (Hagihara et al., 1992). As might be expected with excess cationic charge (+5 per melittin monomer), the nature of the counter-*anion* is of greatest importance in this case, and the ClO_4^- salt is more effective than Cl^- salt. This is equivalent to the tubulin/guanidinium case but with charges reversed. Perhaps the clearest demonstration of the role of thermodynamic destabilization by like charges stems from synthetic two-stranded coiled coils whose hydrophobic contacts were kept constant, but whose charged contacts were varied between strongly attractive and strongly repulsive interactions (Monera et al., 1994). Urea sensitivity was greatest with maximal repulsion and least when maximal attractive interactions had been introduced into the coils. These differences were abolished when GuHCl was used. The potency ratios (GuHCl/urea) varied from 0.47 to 2.47, respectively. The conclusion was that urea measures the sum of the hydrophobic and electrostatic effects, whereas GuHCl measures primarily interactions not including charge. Electrostatic interactions appear to control the association of specific helices, accounting, e.g., for the preference for heterodimer formation in some DNA binding proteins because homodimers would be electrostatically destabilized (O'Shea et al., 1993). It should be noted that the GuHCl concentrations effective on tubulin were substantially lower than those used by Monera et al. (1994).

While the higher order structural details of tubulin are not yet known, we interpret the present results in a similar vein: the high urea sensitivity of the polymerization reaction (Sackett et al., 1994) and the enhanced polymerization at very low GuHCl concentrations point to an important role of charge repulsion in regulating the association of dimers to form polymers. It is these interactions that we propose here to be influenced by low GuHCl concentrations. The fact that there is a persistent, though smaller, enhancing effect of GuHCl in tubulin S polymerization suggests that the remaining excess anionic charges not removed by subtilisin (Sackett et al., 1985) also contribute to repulsion.

Certain properties of the type I polymer differ from those of normal microtubules. There is a greater light scattering yield per mg protein in the GuHCl polymers, and electron micrographs show microtubules plus incompletely assembled tubules of several sorts. Such forms have also been seen after polymerization of tubulin with DMSO or glutamate (Himes et al., 1977; Hamel et al., 1982), and an increase in scattering coefficient occurs with decreasing pH (Gaskin et al., 1974). There is, nevertheless, an increased amount of polymer formed as shown by the 3-fold decrease in critical concentration; the increase in the *rate* of change in light scattering is also greater than can be explained solely on the different optical properties of the GuHCl polymer. Previous studies with tubulin polymerization produced by charge reduction using C-terminal cleavage (Sackett et al., 1985; Maccioni et al., 1986) have also shown variations in microtubule structures. Properties diagnostic of microtubule assembly persist in these GuHCl polymers. Podophyllotoxin binding remains intact and produces substoichiometric inhibition of polymerization (Figure 6). More importantly, there is an absolute taxol and GTP requirement when the GuHCl concentrations are low, and a substantial, though lesser, degree of cold depolymerization persists. Finally, direct examination shows these polymers to be microtubule variants.

An entirely different type of tubulin association is seen when large concentrations of GuHCl are used (called type II polymer). These aggregates form at GuHCl concentrations that virtually completely depolymerize type I polymers and

Table 2: Comparison of the Properties of Microtubules with Type I and Type II Polymers

function	control	polymer I	polymer II
GuHCl concentration for peak rate		~120 mM	600−700 mM
GTP	required	required	not required
reversibility	65−95%	partial	poor
podophyllotoxin effect	inhibits polymerization	inhibits polymerization	delays polymerization
OD_{350}/mg of protein	0.32 ± 0.028	0.56 ± 0.046[a]	
n[b]	−2.93	−2.84	
GuHCl effect on urea		reverses	reverses

[a] 100 mM GuHCl. [b] n = slope of plot of log OD vs log λ between 320 and 500 nm.

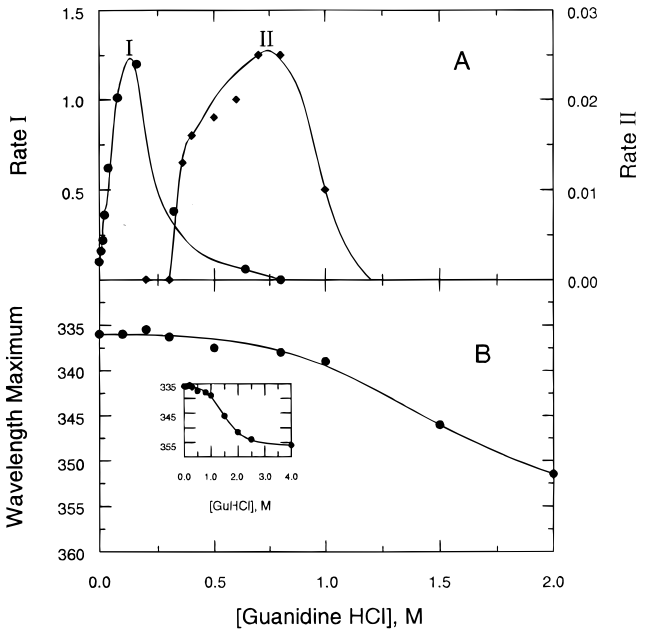

FIGURE 9: Summary of GuHCl effects on tubulin polymerization/aggregation compared with effects on tryptophan fluorescence. (A) The rates of polymerization to microtubules and related polymers (type I) (left curve and left ordinate scale) and to apparently nonrandom aggregates of tubulin termed type II (right curve and right ordinate scale, with lower ΔOD/min). (B) Fluorescence emission spectra (excitation at 295 nm) of 2 μM tubulin following 15 min incubation with increasing concentrations of GuHCl. The GuHCl scale is the same for panels A and B. At concentrations yielding maximal production rates of type I polymer, there is no change in emission maximum, whereas a red-shift begins at concentrations yielding type II polymer. The inset in B depicts the full denaturation profile.

there is no GTP or taxol requirement. They show some structure (to be reported elsewhere) that is not obviously related to microtubules but is similar to previously reported inclusion bodies and folding intermediates (Zettlmeissl et al., 1979). In addition, they retain sensitivity to urea depolymerization and partial sensitivity to podophyllotoxin. A comparison of these two types of GuH Cl-induced association is presented in Table 2. A further comparison between the two polymers, in this case related to tryptophane fluorescence, is provided in Figure 9. It is apparent that type I polymers assemble and disassemble at GuHCl concentrations well below those required to lead to type II polymers. Note the differences in scale between of OD for the two polymers (Figure 9A). Assembly and disassembly of type I polymers occurs in the complete absence of any change in the λ_{max} of tryptophane emission, attesting to its native character and consistent with the formation of microtubule-like polymers. By contrast, a small red-shift occurs at GuHCl concentrations that bring about type II polymer formation (Figure 9B). This change is small compared to those seen with global denaturation occuring at high GuHCl concentrations (Figure 9B, inset). We suggest, therefore, that type II polymerization results from local denaturation by GuHCl.

What is the nature of the interaction between the guanidinium ion and tubulin leading to the formation of type I polymer? Charge density and/or delocalization, ion size, and hydrogen bonding are obvious possibilities to be examined. By comparing urea and GuHCl, charge appears to be a necessary factor, but it is clearly not sufficient because cations such as choline, tetraphenylphosphonium, some alkylammonium salts, and tetramethyl guanidine are ineffective in this system. These may owe some of their poor activity to size. However, we have no information as to whether or not there are significant size constraints for small cations at the repulsive anionic loci of tubulin that may contribute to selectivity as occurs, e.g., in ion channels. It should be noted that the shielding effect on synthetic coiled coils shows a preference for GuHCl over K^+ (Monera et al., 1993).

The apparent requirement for nitrogen-bound hydrogens in the active polyatomic cations suggested the participation of hydrogen bonding in the interaction of imminium and related compounds. With the exception of aminoguanidine, potency can be related to the number of hydrogen bonds a cation can make, and progressive substitution of these hydrogens by methyl groups leads to progressive decreases in the rate of development of light scattering by tubulin solutions and the extent of light scattering. On the basis of a large enthalpy of binding, Makhatadze and Privalov (1992) concluded that (at denaturing concentrations) both urea and GuHCl interact with proteins primarily by hydrogen bonding rather than electrostatic or hydrophobic interactions. They suggested interactions mostly with polar residues utilizing 2 hydrogen bonds/urea and 4−5 hydrogen bonds/GuHCl, but it is difficult to understand how such a mechanism alone can account for the diametrically opposite effects of these two agents at low concentrations. On the other hand, the partial formal charge located to the guanidinium hydrogens will provide greater hydrogen bond energies. Finally, it is not clear that the nature of the interactions of GuHCl with surface polar groups is necessarily the same as with internal residues, and the low concentrations of GuHCl effective under our conditions suggests that it is these surface residues which may be involved. This proposal would envision two quite different classes of interaction for GuHCl: a surface interaction occurring at low (≤300 mM) concentrations which requires both charge and hydrogen bonding for maximal effect, and an interaction at higher GuHCl concentrations with the unfolded protein that would depend primarily on hydrogen bonding.

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