Polyamine-Induced Bundling of F-Actin†

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To better understand the mechanism of actin filament (F-actin) bundling by polyamines, we have measured the onset of bundling as a function of polyamine concentration. Samples were centrifuged at low speeds to separate bundles from unbundled actin, and the relative amounts of actin in the pellet and supernatant were determined via gel electrophoresis, yielding a description of the bundling transition as a function of actin and polyamine concentrations. These experiments were carried out for two different polyamines, spermine (tetravalent) and spermidine (trivalent). We found that the threshold concentration of polyamine needed to bundle actin is independent of both actin concentration and Mg^{2+} concentration over a wide range in Mg^{2+} concentration. We also find that spermine in F-actin bundles is essentially invisible in solution-phase proton NMR, suggesting that it is bound so tightly to F-actin that it is immobilized.

Introduction

F-actin (filamentous actin) is a highly negatively charged and fairly rigid biopolymer that is crucial to cell motility, cellular shape, and cell division. The ability of F-actin to overcome electrostatic repulsions and form bundles in which the filaments are closely packed and nearly parallel is key to its function in these cellular roles. Generally, actin-binding proteins that bind two filaments together are responsible for bundles found in vivo.[1,2] However, multivalent positively charged polyamines such as spermine and spermidine are known to cause bundle formation in vitro[3−6] and are present in vivo during processes such as neuron repair and sperm activation,[7−11] both of which rely in part on actin bundle formation. In the process of cell division, actin bundles are found in the ring canal, which pinches the cell in two. The concentration of polyamines is elevated when the ring canal is present, suggesting that the attractive interactions bringing the filaments together may arise, at least in part, due to the presence of polyamines. These considerations suggest some functional significance of polyamine-driven bundling of F-actin.

More generally, bundling of F-actin by many different multivalent positively charged species, including spermine and spermidine, has been observed extensively in vitro.[3−6,12−36] Until now, the experimental results for multivalent cation-induced bundling of F-actin have been consistent with an electrostatic mechanism known as counterion-mediated attraction. According to this picture, multivalent cations condense along the length of filaments, and anticorrelations in the positions of these condensed ions, arising from their electrostatic repulsions, lead to an effective attraction between filaments if they are close enough and parallel enough.[37−44] This basic mechanism is also consistent with experiments on multivalent cation-induced bundling of DNA, another highly negatively charged, semiflexible biopolymer.[45−51]

Our results suggest that the counterion-mediated attraction picture is incomplete in the case of polyamine-induced bundling of F-actin. First, we find that variation of divalent magnesium ion concentration by a factor of 16 does not perceptibly shift the threshold concentration of tetravalent spermine needed to induce actin bundling, in contrast to predictions from the counterion-mediated attraction picture. Second, we find that spermine is immobilized within actin bundles and is thus invisible in solution-phase proton NMR measurements. Simulations of small tetravalent ions and oppositely charged rods indicate that the multivalent ions should be highly mobile within the bundles under similar conditions.[42] Thus, the counterion-mediated attraction scenario, while consistent with some of our results, does not appear to provide a full description of polyamine-induced bundling of F-actin.

Experimental Methods

Purification of Actin. G-Actin (globular actin) was purified from acetone powder of rabbit skeletal muscle as previously described.[52] Purity of actin was assessed by gel electrophoresis. Actin was stored in G-form at 4 °C for no more than two weeks in buffer G (5.0 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, 25 mM β-mercaptoethanol, pH 8.0). The concentration of actin was determined by absorption at 291 nm using an extinction coefficient of 1.15 for 1.0 mg/mL of actin and a path length of 1 cm.

Polymerization and Bundling of Actin. G-actin was polymerized to F-actin by incubation in buffer F (buffer G plus 2.0 mM $MgCl_2$) at room temperature for approximately 2 h. G-actin was polymerized at a concentration that was twice the desired final actin concentration to allow for later dilution upon addition of the polyamine.

Bundling of the resulting F-actin was initiated by mixing F-actin with a polyamine (spermine^{4+} or spermidine^{3+}) solution.

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Spermine tetrahydrochloride and spermidine trihydrochloride were purchased from Sigma Aldrich (St. Louis, MO) and dissolved in water to prepare concentrated stock solutions. Prior to the initiation of bundling, an aliquot of the polyamine stock was diluted in reaction buffer to twice the final experimental concentration. F-actin was added to the bundling agent solution using a cut pipet tip (to minimize shearing of the filaments) and mixed very gently by several aspirations ($\leq$100 μL/second) of the solution. The resulting solution was allowed to sit at room temperature for approximately 30 min before storage at 4 °C, overnight.

Addition and Removal of Salts. For samples with a $MgCl_2$ concentration of less than 2.0 mM, actin was first polymerized as described above. To remove the excess $MgCl_2$, the F-actin was dialyzed against a solution consisting of buffer G plus 0.25 mM $MgCl_2$ for approximately 2 days, with three dialysis buffer changes. The actin concentration was measured after dialysis and the protein was diluted as necessary.

To increase the $MgCl_2$ concentration or to add NaCl to the system, concentrated actin was polymerized at a higher concentration than desired as described. Upon completion of polymerization, the actin was diluted with high salt buffer to the appropriate actin and salt concentrations. The F-actin was then allowed to sit on ice for between 30 min and 2 h before the initiation of bundling.

Analysis of Bundle Formation. To determine the extent of bundle formation, a combination of low-speed centrifugation and gel electrophoresis was used. F-actin/bundling agent solutions covering a range of polyamine concentrations were removed from the 4 °C environment and allowed to adjust to room temperature for approximately 30 min, after which they were centrifuged for 30 min at 11 000 g in a Brinkmann Eppendorf 5415C tabletop centrifuge. The supernatant was carefully removed from the pellet; the pellet was then resuspended and homogonized in buffer. A constant amount of bovine serum albumin (BSA) was added to each sample for calibration purposes. The samples were then run on 10% polyacrylamide gels, stained with Coomassie blue and destained until the background on the gel was free or nearly free of stain. The clarity of the background of the gels was not consistent and was accounted for in the analysis of band intensity.

Gel Analysis. The program Scion Image (Scion Corporation, Frederick, MD) was used to quantify the amount of actin in the supernatant and pellet fractions. For each lane on the gel, an area including two bands, one for BSA and one for actin, was selected for analysis, as shown in Figure 1A. The program provided a plot of the stain intensity in the gel as a function of vertical position, as shown in Figure 1B. A baseline was drawn by hand through the noise. The error introduced by "eyeballing" a baseline was negligible relative to the error in the result and was neglected. The relative amount of each protein in a given lane was calculated by measuring the area between the curve and the baseline (Figure 1B). The fraction of bundled actin was found by determining the percentage of actin in the pellet fractions, using eq 1.

$$\frac{(A_{\text{actin}}/A_{\text{BSA}})_{\text{pellet}}}{(A_{\text{actin}}/A_{\text{BSA}})_{\text{supernatant}} + (A_{\text{actin}}/A_{\text{BSA}})_{\text{pellet}}} \times 100\% = \% \text{ Actin}_{\text{pellet}} \quad (1)$$

NMR. To determine where polyamine was present with respect to bundles, NMR proton spectra of spermine and spermidine were measured using a Bruker Avance 600 spectrometer with a frequency of 600.13 MHz and a spectral resolution of 0.2 Hz. To prepare actin samples for NMR, the pellet fraction was resuspended in a volume of buffer equal to the original sample volume, and the pelleted bundles were mechanically broken up and resolubilized via repeated aspirations with a pipet and vortex mixing of the sample. The resulting pellet and supernatant fractions obtained from low-speed centrifugation of bundled samples were then diluted 2-fold with D_2O in an NMR tube. To study bundles dissociated by high ionic strength, 100 mM NaCl was included in the buffer in which the pelleted bundles were resuspended prior to NMR measurements.

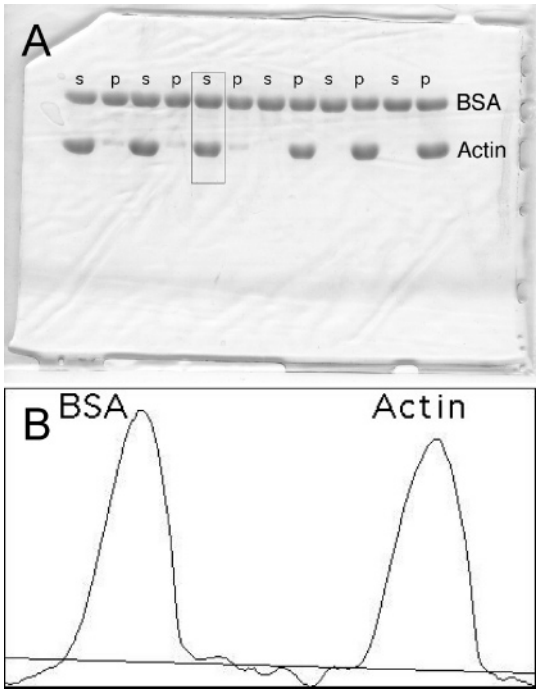

Figure 1. Example of a typical polyacrylamide gel is shown in Figure 1A. The s or p symbols above the vertical lanes refer to the supernatant or pellet fraction, respectively, from a single set of actin/linker concentrations. The polyamine concentration increases from left to right across the gel, yielding a gel with all of the actin in the supernatant lanes on the left and all of the actin in the pellet lanes on the right. The upper row of stained bands is the BSA standard that was added to each sample. The lower bands are the actin in a given sample. If all the actin for a particular experiment was found to be in the supernatant (unbundled), this would be seen in the gel as the absence of a band in the pellet lane. In B, the concentration of stain that remains bound to protein in the gel is plotted as a function of vertical position for a single lane. The two peaks corresponding to BSA and actin are from the boxed part of the gel shown in part A.

Results and Discussion

Bundling Threshold of F-actin. The analysis of actin bundling as a function of actin and spermine concentrations revealed that the threshold concentration of spermine required to bundle F-actin does not depend on the concentration of actin over the range from 4.5 to 36 μM. Instead, the threshold concentration of spermine is constant over almost an order of magnitude in actin concentration, as shown in Figure 2. This is consistent with analogous studies of F-actin bundling by polylysine and cobalt hexamine[27−29,34] and of DNA condensation by spermine.[46,47,49,53] It should be noted that, for an actin concentration of 72 μM, the threshold concentration of spermine increased by 50% or more (data not shown). However, at such high actin concentrations, F-actin filaments are in the nematic phase, where on average they are ordered in one direction but

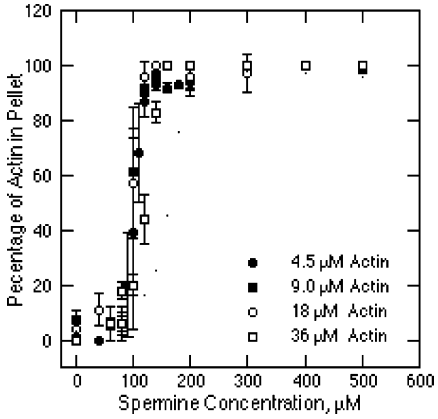

Figure 2. Percentage of actin in the pellet, which corresponds to the amount of actin in the bundled form vs the concentration of spermine for four actin concentrations. It should be noted that not only is the transition quite sharp, but also that the threshold concentration of spermine is independent of the actin concentration over nearly an order of magnitude.

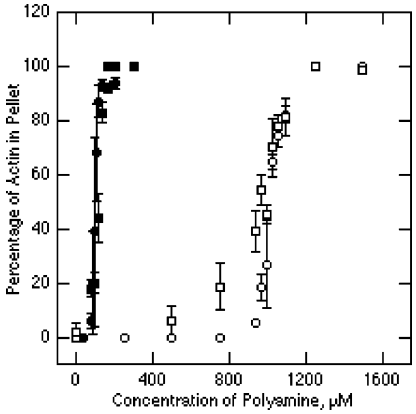

Figure 3. Percentage of actin in the pellet, which corresponds to the amount of actin in the bundled form vs the concentration of polyamine. Closed symbols refer to samples that were bundled by spermine; open symbols refer to actin bundled by spermidine. Squares correspond to 36 μM actin, and circles represent 4.5 μM actin. Note that the threshold concentration of spermidine is also roughly independent of actin concentration and that the threshold concentration of spermidine is much higher than the spermine threshold concentration.

are not close packed and aligned as stongly as they are in bundles. Orientational order in the nematic phase may cause weak attractions between filaments to increase, leading to the formation of bundles, even without addition of any bundling agent.

We repeated these experiments with spermidine, a bundling agent that is structurally similar to spermine but which has a lower valence (+3). Our results (Figure 3) show that the threshold concentration of spermidine is much higher than that of spermine, but that for both polyamines the threshold concentration is independent of the actin concentration (at least between 4.5 and 36 μM). Given an electrostatic picture of actin bundling, one would expect that a higher concentration of trivalent vs tetravalent linkers would be necessary, which is clearly consistent with our results.

The existence of a polyamine threshold concentration independent of actin concentration is at least partially consistent with arguments of Burak, Gil, and Andelman[53] based on a counterion-mediated attraction scenario. Only polyamines that are localized near the actin filaments (i.e., condensed polyamines) can mediate attractions between filaments. Bundling can occur once there are enough condensed polyamines. The existence of

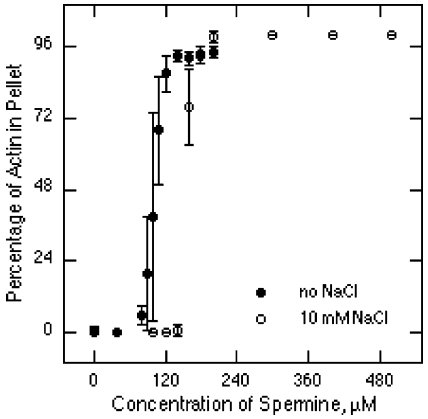

Figure 4. Percentage of actin in the pellet, which corresponds to the amount of actin in bundled form vs spermine concentration for actin bundled in both the absence and presence of NaCl. Addition of 10 mM NaCl increases the threshold concentration of spermine by about 50%.

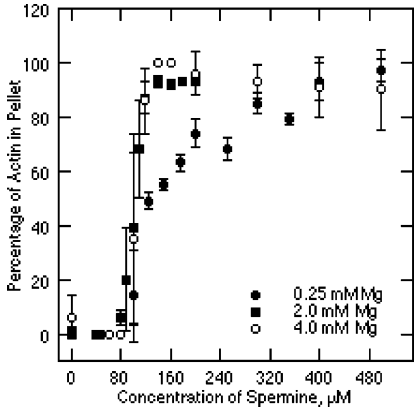

Figure 5. Percentage of actin in the pellet, which corresponds to the amount of actin in the bundled form vs the concentration of spermine for several $MgCl_2$ concentrations. Note that the spermine concentration corresponding to the onset of bundling is not changed by addition or removal of $MgCl_2$ over a factor of 16 in $MgCl_2$ concentration.

a finite polyamine threshold concentration for bundling can be explained as follows: Even in the limit of zero filament concentration, the polyamine concentration in solution required to yield enough condensed polyamines is nonzero because of the competition between entropy and the attraction of the polyamines to filaments. According to this counterion-mediated attraction picture,[42,54] the polyamines must bind weakly (through nonspecific electrostatic interactions) to the actin. The fact that the threshold concentration for the trivalent polyamine, spermidine, is higher than that for the tetravalent one, spermine (Figure 3), is qualitatively consistent with this scenario. However, the dependence of the bundling threshold on actin concentration that we observe is weaker than one would expect from such arguments.

Effect of Ionic Strength on the Bundling Transition. The effect of ionic strength on the location of the bundling transition was tested by varying the concentration of mono- and divalent salts. When the ionic strength was increased by addition of NaCl, the threshold concentration increased, as shown in Figure 4. However, varying the $MgCl_2$ concentration does *not* change the threshold concentration of spermine, as shown in Figure 5, which is in contrast to expectations. The counterion-mediated attraction picture predicts that, upon increasing $MgCl_2$ concentration, the threshold concentration of spermine needed to induce bundling should increase due to competition between spermine and Mg^{2+} for condensation along the filaments Alternatively,

if actin bundling were initiated at some specific screening length, the threshold concentration of spermine should decrease upon addition of $MgCl_2$ (or any other salt for that matter). Our finding (Figure 5) that the addition or removal of $MgCl_2$ does not change the threshold concentration of spermine required for bundling is not consistent with either of these predictions.

The range over which $MgCl_2$ can be varied is necessarily limited. If the $MgCl_2$ concentration is too high, then $MgCl_2$ alone can bundle actin. On the other hand, if the $MgCl_2$ concentration is too low, the filaments depolymerize. High-speed centrifugation results show that unbundled actin at 100 μM spermine and at 0.25 mM $MgCl_2$ is predominantly in monomeric form (data not shown). However, even at 0.25 mM $MgCl_2$, where filaments had partially depolymerized, we saw no shift in the onset of bundling, even though the spermine had not only to bundle the actin, but also to polymerize the monomers in solution in this case. Thus, variation of the $MgCl_2$ concentration by a factor of 16 had no effect on the threshold concentration of spermine needed to bundle actin, even when some of the spermine was needed to polymerize monomers into filaments.

Solution-Phase Proton NMR Measurements. In addition to quantifying the fraction of actin in bundle form, we attempted to measure the fraction of the polyamines in the bundles. Because of the presence of amines in the protein and our inability to remove 100% of the protein from solution, we were unable to use standard analytical techniques to measure the concentrations of the polyamine (these techniques all measure the total amine concentration). Consequently, solution-phase proton NMR was used to determine the location of polyamines with respect to the bundles and the solution containing the bundles. These measurements served only to identify the presence of polyamine and did not allow us to measure the relative amounts.

To identify where the polyamines were, we separated the supernatant and pellet fractions, resuspending the pellet in buffer without any additional polyamines, and then used NMR spectroscopy to search for spermine or spermidine in each fraction. Figure 6A shows that spermine was detected only in the supernatant fraction of the 36 μM actin bundled by 140 μM spermine. This result was also seen for 4.5 μM actin bundled by 140 μM spermine (data not shown). The upper and lower spectra correspond to the supernatant and pellet fractions, respectively. For reasons of clarity, the spermine standard is not shown here. The two peaks seen in the supernatant fraction, which are not detectable in the pellet fraction, correspond to spermine. We repeated these experiments with spermidine, and the results are shown in Figure 7A. Here, we find that, for 36 μM actin bundled by 1.25 mM spermidine, spermidine was visible in the pellet fraction (lower trace), although far more apparently appeared in the supernatant fraction (upper trace). For 4.5 μM actin bundled by spermidine, no spermidine was seen in the pellet fraction by NMR if we rinsed the pellet with spermidine free buffer before resuspension. These results for spermine and spermidine suggest two possibilities: (1) spermidine binds more tightly to actin than spermine and pellets with the actin bundles while spermine does not, or (2) both spermine and spermidine are present in the pellet fraction, but spermine is sufficiently immobilized in actin bundles as to be invisible to proton NMR, while spermidine is not.

To distinguish between the two possibilities, we dissolved the bundles by adding 100 mM NaCl. By 90° light scattering, we have observed that 100 mM NaCl dissolves spermidine-bundled actin completely and rapidly (data not shown). This amount of salt, however, is not enough to cause the filaments

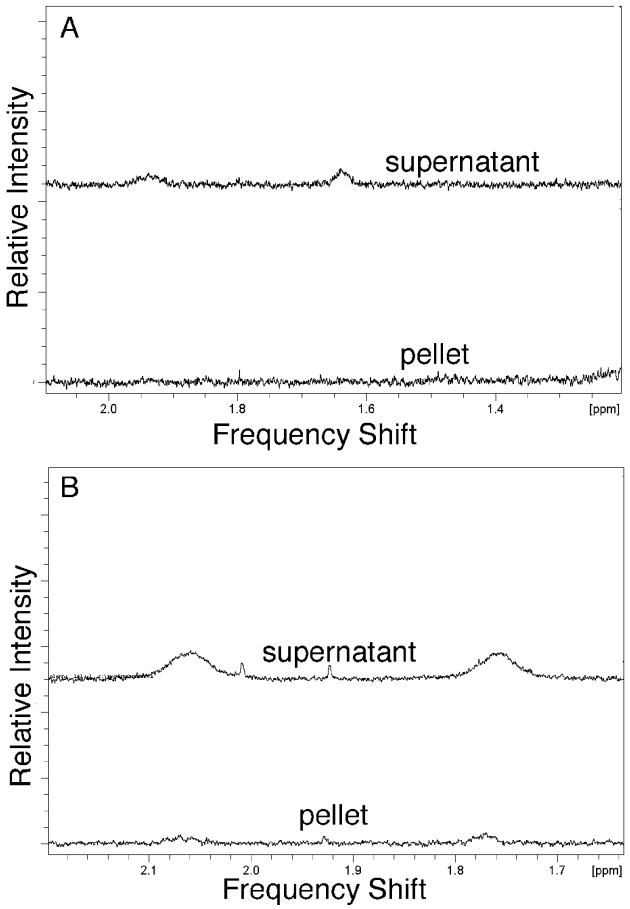

Figure 6. (A) Solution-phase proton NMR spectra (8 scans each) of the supernatant (upper trace) and pellet (lower trace) fractions resulting from pelleting 36 μM actin bundled by 140 μM spermine. The two peaks visible in the supernatant spectrum correspond to spermine and are not visible in the spectrum of the pellet fraction. (B) The supernatant (upper trace) and pellet (lower trace) spectra (128 scans each) for 36 μM actin bundled by 140 μM spermine with the pellet resuspended in buffer F plus 100 mM NaCl. Peaks corresponding to spermine are present in both spectra.

to dissociate into monomers. Thus, by adding 100 mM NaCl to the buffer used to resuspend the pelleted actin bundles, we ensured that the actin would not rebundle and that the polyamine, if present in the bundles, would be detectable by NMR. This experiment revealed that spermine is in fact present in the bundled fraction of actin, as shown in Figure 6B. The two peaks in both the uppper (supernatant) and lower (pellet) spectra correspond to spermine. Again, the spermine standard has been omitted for clarity. The peaks in 6B have moved upon the addition of 100 mM NaCl by $^1/_{10}$ ppm or less. This is insignificant because no attempt was made to include a water-soluble standard to measure the absolute chemical shift. Instead, water was assigned a chemical shift of 4.8 ppm even though slight changes in pH, buffer component concentrations, or temperature can change the location of this peak. Similarly, Figure 7B shows that spermidine is present in both the supernatant (upper trace) and pellet (lower trace) fractions of the sample. These results indicate that spermine binds more strongly than spermidine to bundled actin; indeed, it is apparently bound so tightly that it is immobilized in the bundles. Note that these results taken alone are consistent with the counterion-mediated attraction picture, which would predict that the tetravalent spermine would be more strongly bound than trivalent spermidine. However, immobilized multivalent cationic species have not been observed in other counterion-mediated

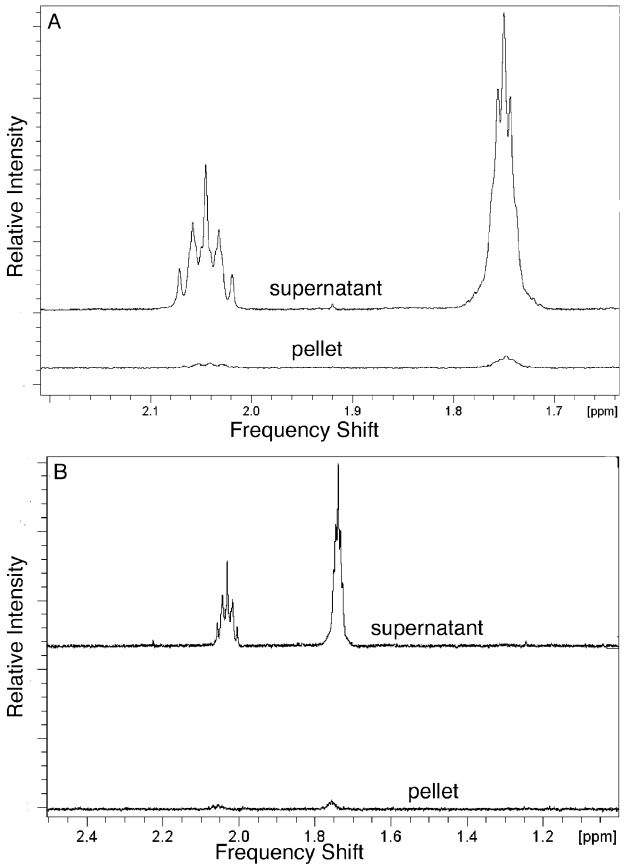

Figure 7. (A) Solution-phase proton NMR spectra (128 scans each) of the supernatant (upper trace) and pellet (lower trace) fractions resulting from pelleting 36 μM actin bundled by 1.25 mM spermidine. The two peaks visible in both spectra correspond to spermidine. (B) the supernatant (upper trace) and pellet (lower trace) spectra (8 scans each) for 36 μM actin bundled by 1.25 mM spermidine with the pellet resuspended in buffer F plus 100 mM NaCl. Peaks corresponding to spermidine are present in both spectra.

attraction systems. Recent X-ray scattering experiments show that divalent ions such as Mg^{2+}, Ba^{2+}, and Ca^{2+} are mobile in actin bundles.[12] Simple models of trivalent and tetravalent ions and charged rods have been studied by numerical simulations; in those models, the multivalent ions are mobile within bundles.[42] We do not know whether spermine is also immobilized on free actin filaments or whether it is only immobilized within bundles of filaments. One possibility is that spermine can move along free filaments but freezes in position when filaments aggregate into bundles. If spermine were immobile even on free filaments, one would expect counterion-mediated attractions to be highly suppressed because the spermine would not be able to adjust its position to maximize charge correlations between filaments.

Discussion

In describing our results, we have indicated the extent to which they are consistent with predictions of the counterion-mediated attraction picture at a qualitative level. Here, we analyze our results somewhat more quantitatively in the context of this scenario.

Burak, Gil, and Andelman[53] have predicted that the concentration of ions of valence z, c_z, required to bundle charged rods should vary linearly with the concentration of actin, c_a: $c_z = \alpha c_a + \beta$, where α is the number of condensed multivalent ions per monomer in the bundle, and β is the threshold concentration of multivalent counterions required for bundling in the limit of zero actin concentration. This model was intended to explain DNA condensation but was also used by Tang and Janmey[28] to fit data for the actin-bundling threshold upon addition of polylysine. The calculations of Burak et al. treat the electrostatic interactions of DNA with salts and counterions within a cell model, where the DNA chains are modeled as rigid rods and do not overlap in space. The monovalent and multivalent salts are also assumed to have the same co-ion. Given that these assumptions should also be reasonable for F-actin, one might expect that this model's predictions would agree with our experimental data

Our data show that the dependence of c_z on c_a is minimal. We can, however, estimate what the limiting values of α and β are by using the linear prediction of Burak et al. From this, we find that $\beta = 92$ μM for spermine, and $\beta = 1050$ μM for spermidine. Additionally, we find that $\alpha \leq 0.7$ for spermine. Within the context of the model, this would imply that there are no more than 0.7 spermine ions bound per actin monomer. This means that only a relatively small fraction of the charge on actin (less than 25% of the actin charge) could be neutralized by condensed spermine. For spermidine, we cannot make any predictions about the degree of condensation because the two data points we have yield a negative value (-1.6) for α.

The threshold concentration β of polyamine required to bundle actin in the zero concentration limit should depend on the effective binding energy ϵ of the polyamine to actin, which should in turn depend linearly on the valence z of the polyamine, $\epsilon_z = z\epsilon_0$, where ϵ_z is the binding energy of the z-valent polyamine and ϵ_0 is the binding energy per polyamine charge. In the low concentration limit, the competition of entropy and binding energy ϵ yields the perhaps naïve prediction $\beta\lambda^3 \propto \exp(-\epsilon_z/kT)$, where λ is the thermal de Broglie wavelength, $\lambda = h/\sqrt{2\pi mkT}$, of the polyamine, m is the mass and kT is the thermal energy. We find that β_4 (for spermine) is 92 μM and β_3 (for spermidine) is 1050 μM. To estimate the binding energy of spermine and spermidine, which are chemically similar, we found ϵ_0 by taking the ratio $\beta_4\lambda_4{}^3/\beta_3\lambda_3{}^3$, and setting this equal to the corresponding exponential ratio. We then solved for ϵ_0. This approach yields the binding energy of spermine for the buffers we used as roughly $\epsilon_4 \approx 8\ kT$, while that of spermidine is $\epsilon_3 \approx 6\ kT$, i.e., $\epsilon_0 \approx 2\ kT$. These numbers are in reasonable agreement with the estimated binding energy of cobalt hexamine with DNA.[55]

We would also expect that the threshold polyamine concentration β should depend sensitively on the concentrations of additional (not including the polyamine) positively charged ions in solution. In the case where the additional positive ions are Mg^{2+}, there is competitive binding of the divalent magnesium ions and tetravalent spermine. In that case, one would roughly expect to find[56] $\beta \approx c_2{}^2$, where c_2 is the concentration of Mg^{2+} for a fixed monovalent ion concentration. This would predict that, for magnesium concentrations varying by a factor of 16, β should change by a factor of 250. This clearly does not agree with our finding that β is independent of magnesium concentration over that range. However, this analysis neglects the fact that Mg^{2+} alone can induce actin bundling at concentrations of 6−8 mM. One might, therefore, expect β for spermine to first increase with Mg^{2+} concentration and then to decrease, eventually reaching zero when the Mg^{2+} concentration increases to 6−8 mM. Even taking this effect into account, the constant behavior we observe in β is surprising.

Given that the Burak model does not give a physically reasonable estimate of the number of polyamines bound to the

F-actin, it appears that a more detailed model will need to be developed to properly describe polyamine-induced actin bundling. This model may need to incorporate more experimental details to account for the differences between the F-actin and DNA systems.

Conclusions

Many of the results discussed are consistent with the scenario of counterion-mediated attractions with the polyamine acting as a multivalent counterion. The concentration of spermine needed to bundle F-actin is lower than the threshold concentration of spermidine, consistent with the higher valence of spermine compared to spermidine. The threshold concentration of polyamine increases with added monovalent salt, as one would expect. The dependence of threshold concentration on actin concentration is also consistent with predictions of the counterion-mediated attraction picture. However, there are also striking differences. First, the threshold concentration of spermine needed to induce actin bundling is independent of $MgCl_2$ concentration as it is varied by a factor of 16. Second, spermine is immobilized in actin bundles. If counterions were immobilized on filaments, it is difficult to see how correlations between their positions (which lead to filament−filament attractions) could develop. In these respects, the behavior of the polyamine/actin system is different from that of a purely electrostatic system, even though there is no evidence of specific binding of polyamines to F-actin. Further studies are needed to elucidate the nature of F-actin bundle formation by polyamines.

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