Noncooperative Stabilization Effect of Phalloidin on ADP.BeF_x- and ADP.AlF_4-Actin Filaments†

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ABSTRACT: Actin plays important roles in eukaryotic cell motility. During actin polymerization, the actin-bound ATP is hydrolyzed to ADP and P_i. We carried out differential scanning calorimetry experiments to characterize the cooperativity of the stabilizing effect of phalloidin on actin filaments in their ADP.P_i state. The ADP.P_i state was mimicked by using ADP.BeF_x or ADP.AlF_4. The results showed that the binding of the nucleotide analogues or phalloidin stabilized the actin filaments to a similar extent when added separately. Phalloidin binding to ADP.BeF_x- or ADP.AlF_4-actin filaments further stabilized them, indicating that the mechanism by which phalloidin and the nucleotide analogues affect the filament structure was different. The results also showed that the stabilization effect of phalloidin binding to ADP.BeF_x or ADP.AlF_4-bound actin filaments was not cooperative. Since the effect of phalloidin binding was cooperative in the absence of these nucleotide analogues, these results suggest that the binding of ADP.BeF_x or ADP.AlF_4 to the actin modified the protomer-protomer interactions along the actin filaments.

Actin is one of the main components of the cytoskeleton and plays important roles in the motility of eukaryotic cells (*1–7*). The actin monomer can bind a nucleotide in complex with a divalent cation in the cleft between the two main domains of the protein (Figure 1) (*8*). During polymerization, the ATP is hydrolyzed to ADP and P_i[1] (*9–16*). The ADP.P_i state is transient as the inorganic phosphate product is released from actin after polymerization. For the characterization of the short-lived ADP.P_i state, nucleotide analogues such as ADP.BeF_x or ADP.AlF_4 can be applied (*17–19*).

The effect of the binding of ligands to actin filaments is often cooperative; i.e., binding of the ligands induces allosteric conformational changes in the actin protomers distant from the bound protomer (*20–24*). In most of the cases, the biological function of the cooperative behavior of actin filaments is unclear. In a special case, it was proposed that cooperative interactions could play an important role in the regulation of muscle contraction (*25*). We suggested recently that the cooperative behavior of actin filaments could provide the structural bases for information channels in living cells, through which the different actin-binding effectors can express their full effect even under substoichiometric binding conditions (*26*).

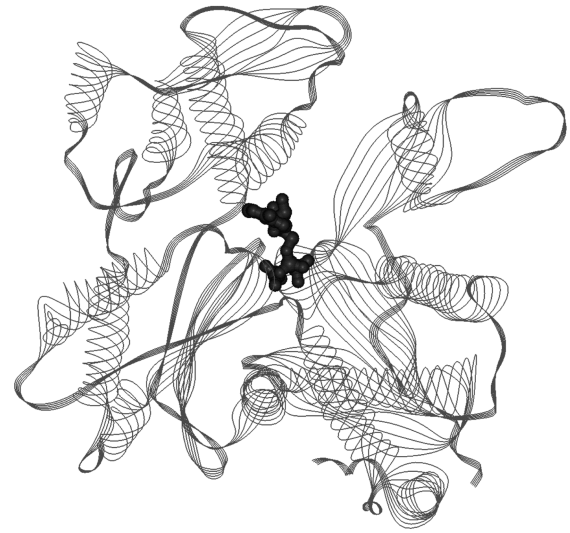

FIGURE 1: Ribbon model of the α-skeletal actin monomer from rabbit skeletal muscle. ATP (represented with spheres) in the nucleotide-binding cleft is shown (Protein Data Bank entry 1NWK).

Previous studies have shown that the binding of phalloidin stabilizes the structure of actin filaments (*27–33*) and one bound phalloidin can stabilize seven neighboring protomers (*26*). In this work, we characterize the effect of phalloidin on the thermal stability of actin filaments in complex with different nucleotide analogues (ADP.BeF_x or ADP.AlF_4) by using differential scanning calorimetry (DSC). The toxin was applied at various phalloidin:actin concentration ratios. The results show that the stabilizing effect of phalloidin binding on ADP.BeF_x- or ADP.AlF_4-bound actin filaments was not cooperative, indicating that the binding of ADP.BeF_x or

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[1] Abbreviations: P_i, inorganic orthophosphate; ADP.P_i, adenosine 5′-diphosphate in complex with inorganic orthophosphate; BeF_x, collective nomination of ADP.$BeF_2(OH)^- \cdot H_2O$ and ADP.$BeF_3^- \cdot H_2O$ complexes; ADP.BeF_x or ADP.AlF_4, ADP in complex with Be or Al fluoride, respectively; MOPS, 3-(*N*-morpholino)propanesulfonic acid, used as the pH buffer; EGTA, ethylene glycol tetraacetic acid; T_m, melting temperature.

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ADP.AlF_4 to actin substantially modifies the interaction between neighboring protomers along the actin filaments.

MATERIALS AND METHODS

Chemicals. KCl, $MgCl_2$, $CaCl_2$, MOPS, EGTA, $AlCl_3$, and NaF were purchased from SIGMA-Aldrich (Budapest, Hungary). ATP, ADP, and β-mercaptoethanol were obtained from MERCK (Darmstadt, Germany). NaN_3 and $BeSO_4$ were purchased from Fluka (St. Gallen, Switzerland).

Protein Preparation. Skeletal actin was prepared from acetone powder obtained from rabbit muscle (*12, 34*). After purification, the calcium-bound actin monomers were stored in a 2 mM MOPS buffer (pH 7.3) with 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM β-mercaptoethanol, and 0.005% NaN_3. The actin monomer concentration was determined by absorption photometry using 0.63 mg^{-1} mL cm^{-1} as the extinction coefficient at 290 nm (*35*). Actin-bound calcium was exchanged for magnesium by incubating the samples with 0.2 mM EGTA and 0.1 mM $MgCl_2$ for 5 min (*36*). Actin polymerization was initiated by the addition of 2 mM $MgCl_2$ and 100 mM KCl. ADP.BeF_x-actin filaments were prepared in a similar way as described by Levitsky and colleagues (*37*); $BeSO_4$ (0.03–3 mM) and NaF (0.1–10 mM) were added to the samples, and actin was polymerized at room temperature for 3 h. Note that since the presence of ADP, $BeSO_4$, and NaF in the solution leads to formation of either ADP.$BeF_2(OH)^- \cdot H_2O$ or ADP.$BeF_3^- \cdot H_2O$, we refer to them collectively as ADP.BeF_x. ADP.AlF_4-actin filaments were prepared the same way as the ADP.BeF_x except for the addition of 3 mM $AlCl_3$ instead of $BeSO_4$. To prepare phalloidin-bound actin filaments, phalloidin at the desired concentrations was added to actin prior to polymerization and the samples were incubated for 12 h at 4 °C. The analogues were added at the same time as polymerization buffer 1 h before the toxin to prepare the actin filaments, and the samples were incubated overnight.

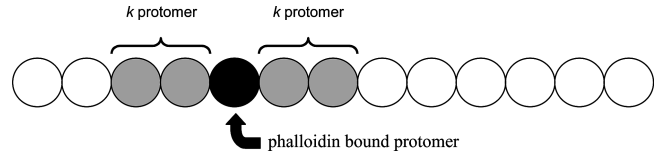
DSC Measurements. Calorimetric experiments were performed with a SETARAM Micro DSC-II calorimeter. The temperature range was 0–100 °C with a heating rate of 0.3 K/min, and the actin concentration was 69 μM (~3 mg/mL). Data were analyzed with MicroCal Origin. Experimental buffer with no protein content was used as a reference. In each case, the samples were heated twice. The second heating measurement indicated full irreversible denaturation of the actin during the first run.

We applied the model described recently (*26*) to analyze the phalloidin concentration dependence of the DSC data obtained with actin filaments and to define the degree of cooperativity along the filaments. The model assumes that phalloidin can stabilize the conformation of the protomer to which it is bound. In the case of cooperative binding effects, phalloidin can also stabilize adjacent actin protomers along the actin filament (Scheme 1). Using this model, the number of actin protomers stabilized by one phalloidin can be determined by fitting the following equation to the phalloidin concentration dependence of the relative contribution of actin populations unaffected by phalloidin (A):

$$A = (1 - p)^{2k+1} \quad (1)$$

where p is the probability that an actin protomer in the filament binds phalloidin and k is the cooperativity factor.

Scheme 1: Model for the Analysis of the DSC Data (eq 1)[a]



[a] Circles represent the actin protomers in a filament. The phalloidin is bound to the protomer shown as a black circle and stabilizes its conformation. Neighboring actin protomers (gray circles; k in both directions) could potentially be stabilized by the phalloidin provided that the conformational changes can propagate along the actin filament by allosteric protomer-protomer interactions. The protomers represented by white circles are not affected by the binding of phalloidin. Fit of eq 1 to the experimental data provides the value for k, and thus, the number of actin protomers stabilized by one phalloidin molecule can be calculated as $2k + 1$.

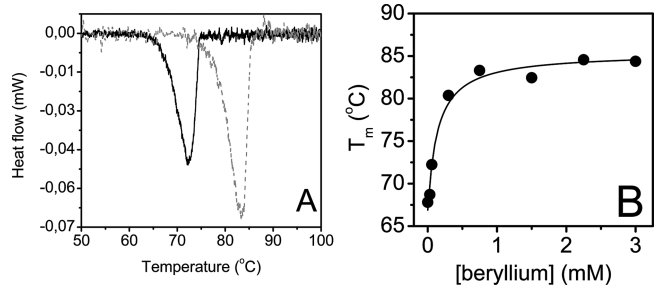


FIGURE 2: Effect of ADP.BeF_x on the thermal stability of actin filaments. Panel A shows the DSC curve obtained when the actin filaments were polymerized after the addition of 0.06 mM $BeSO_4$ and 0.2 mM NaF (black solid line) or 3 mM $BeSO_4$ and 10 mM NaF (gray dashed line). The actin concentration was 69 μM. The value of T_m was determined to be 72.2 or 84.8 °C, respectively. (B) The panel shows the T_m values as a function of the beryllium (added $BeSO_4$) concentration. Hyperbola fit (solid line) to the plot gave a half-saturation beryllium concentration of 0.14 ± 0.05 mM.

The value of A can be determined from the analysis of the DSC curves by determining the under-curve area of the transition characteristic of the actin not affected by phalloidin. In this study, we approximated the under-curve area by using Gaussian fits. The value of p can be calculated as the ratio of applied phalloidin concentration to actin concentration. Thus, $1 - p$ is the probability that an actin protomer does not bind phalloidin. The value of k can be determined by fitting eq 1 to the experimental data, and then the number of actin protomers affected by one phalloidin molecule is calculated to be $2k + 1$ (*26*).

RESULTS AND DISCUSSION

We carried out differential scanning calorimetry (DSC) experiments to characterize the cooperativity of the stabilizing effect of phalloidin on ADP.BeF_x- and ADP.AlF_4-actin filaments. To achieve this aim, we first characterized the effect of phalloidin and nucleotide analogues separately. Our experiments showed that the binding of ADP.BeF_x, ADP.AlF_4, or phalloidin stabilized the structure of actin filaments, in agreement with previous studies (*37, 38*). In the presence of 3 mM $BeSO_4$ and 10 mM NaF, the T_m was greater (84.8 °C) than in the absence of them (64.1 °C) (Figure 2A), in agreement with the results from the Levitsky group (*37*). In control experiments, T_m values were measured at different beryllium concentrations ([$BeSO_4$]/[NaF] = 3/10)

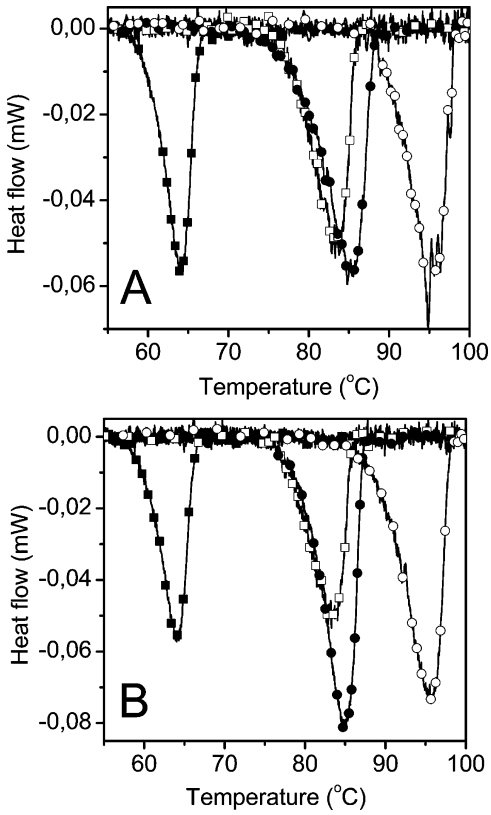


FIGURE 3: Effect of phalloidin on ADP.BeF_x-actin (A) and ADP.AlF_4-actin (B) filaments: calorimetric curves for (■) actin in the absence of nucleotide analogues and phalloidin, (●) actin in the presence of nucleotide analogues, (□) actin with bound phalloidin, and (○) actin in the presence of nucleotide analogues and phalloidin. The corresponding T_m values for actin, actin with phalloidin, actin with nucleotide analogue, and actin with nucleotide analogue and phalloidin were 64.1, 82.3, 84.8, and 95.5 °C, respectively, in panel A. For panel B, the corresponding values are 64.1, 82.3, 84.7, and 95.9 °C.

(Figure 2B). The half-value of the maximal effect was achieved at 0.14 mM $BeSO_4$, showing that the concentrations of 3 mM $BeSO_4$ and 10 mM NaF used in the subsequent experiments were sufficiently high to ensure appropriate conditions to reveal the effect of ADP.BeF_x on the actin filaments.

It has been shown previously that phalloidin can stabilize the structure of the actin filaments (*27–33*). In agreement with these observations, we found that the T_m value shifted to 82.3 °C when phalloidin was added to actin (69 μM) in a 1:1 concentration ratio (Figure 3). Note that due to the high affinity of phalloidin for actin [K_D = 36 nM (*30*)] and considering the applied actin concentration (69 μM) most of the added phalloidin (>99%) bound to actin in these experiments.

Effect of Phalloidin on ADP.BeF_x- and ADP.AlF_4-Actin Filaments. We measured the thermal stability of ADP.BeF_x-actin filaments after phalloidin binding. Previously, it was shown that the binding of phalloidin to ADP-actin filaments polymerized from ATP-actin monomers increased the value of T_m from 64.1 to 82.3 °C. In the case of ADP.BeF_x-actin filaments, the phalloidin, added in a 1:1 concentration ratio, could further stabilize the structure of ADP.BeF_x-actin filaments as in the presence of phalloidin the T_m was greater (95.5 °C) than in the absence of it (84.8 °C) (Figure 3A). When similar experiments were carried out with ADP.AlF_4-actin filaments, the DSC data showed that ADP.AlF_4 stabilized the actin filaments (T_m = 84.7 °C) and the binding of phalloidin could further increase the thermal stability of ADP.AlF_4-actin filaments (T_m = 95.9 °C) (Figure 3B).

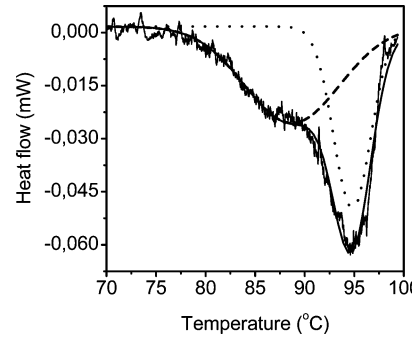


FIGURE 4: Analysis of the DSC curve obtained with ADP.BeF_x-actin filaments at a substoichiometric phalloidin concentration. The phalloidin:actin protomer concentration ratio was 0.8:1. This figure shows the experimental data (solid line) and the two Gaussian curves (dashed and dotted lines) obtained by the decomposition of the experimental curve. The sum of the two Gaussians is also indicated with a solid line. The first Gaussian curve gave an estimate for the T_m of 84.8 °C, while the second peak gave a value of 95.5 °C.

To interpret these observations, we considered that if the nucleotide analogues and phalloidin use the same mechanism to stabilize the structure of actin filaments, then the addition of phalloidin could not further increase the thermal stability of nucleotide analogue-saturated actin. According to the calorimetric results, the binding of phalloidin resulted in further stabilization of the ADP.BeF_x- and ADP.AlF_4-actin filaments [~84 and ~95 °C, respectively (Figure 3], indicating that the nucleotide analogues and the phalloidin stabilized the filaments via different molecular mechanisms.

Lack of Cooperativity in ADP.BeF_x- and ADP.AlF_4-Actin Filaments. Previous studies provided evidence that the effect of phalloidin on actin filaments was cooperative (e.g., ref (*26*)). In this case, cooperative binding meant that one phalloidin molecule could stabilize the conformation of more than one actin protomer; i.e., the stabilization effect of phalloidin propagated along the filaments by allosteric interactions to protomers distant from the phalloidin-bound protomer. In this work, we tested how the nucleotide analogues alter the protomer-protomer interactions along actin filaments by examining the effect of phalloidin on ADP.BeF_x- or ADP.AlF_4-actin filaments at different substoichiometric phalloidin concentrations, i.e., at various phalloidin:actin protomer concentration ratios.

At substoichiometric phalloidin concentrations, the DSC curves could be decomposed into two peaks (Figure 4). To approximate the contribution of these peaks to the heat absorption curves, we applied Gaussian fits. We applied a similar approach successfully in a previous study for the analysis of complex DSC curves (*26*). The contribution of the lower- and higher-temperature transitions in the DSC curves was quantified by determining the integral of the corresponding Gaussian curves (under-curve areas). The T_m values for the lower- and higher-temperature Gaussian peaks fell into the range of 83–85 and 94–96 °C, respectively (Figure 5), and were in the same range as those determined without the Gaussian fits. By comparing these values to those obtained with the nucleotide analogues in the absence of

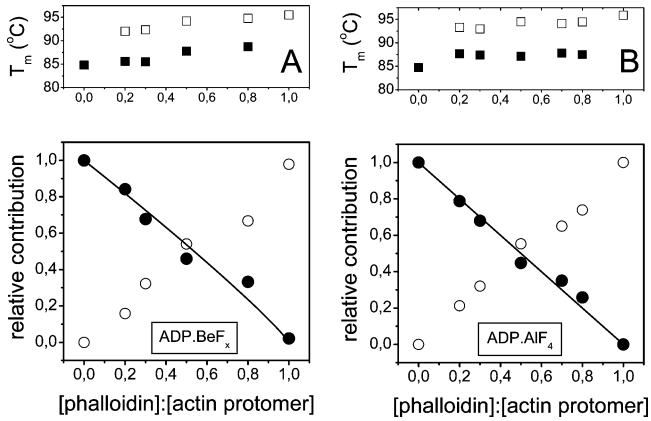


FIGURE 5: Phalloidin concentration dependence of the DSC transitions resolved from the analysis of the data for ADP.BeF_x-actin (A) and ADP.AlF_4-actin (B) filaments. The relative contributions of the actin unaffected by phalloidin (●) or stabilized by the binding of phalloidin (○) were determined by decomposing the DSC curves to two Gaussian curves (Figure 4) and calculating the under-curve areas. The lower-temperature transitions corresponded to the unaffected actin, while the higher-temperature Gaussian curve describes the phalloidin-stabilized actin. Fit of eq 1 to the data corresponding to the unaffected actin (solid lines) gave k values of 0.05 ± 0.1 and -0.01 ± 0.03 for ADP.BeF_x- and ADP.AlF_4-actin filaments, respectively. The top insets show the melting temperatures of actin filaments unaffected by phalloidin (■) or stabilized by the binding of phalloidin (□) at the given actin:phalloidin concentration ratios.

phalloidin (84.8 °C) and at saturating phalloidin concentrations (95.5 °C), we interpret the lower-temperature transitions as the contribution of actin unaffected by phalloidin, while the higher-temperature peaks are interpreted as being characteristic of actin protomers stabilized by the phalloidin. The relative contribution of the lower-temperature peak decreased linearly with an increase in phalloidin concentration in the case of either the ADP.BeF_x-actin (Figure 5A) or ADP.AlF_4-actin (Figure 5B) filaments. In correlation with this observation, the relative contribution of the higher-temperature peak followed a linear, increasing tendency (Figure 5).

We applied the method described previously (*26*) (eq 1) to analyze the phalloidin concentration dependence of the stabilization effect. The relative contributions of actin populations were determined at different actin:toxin concentration ratios. In a previous work, we studied the cooperative binding of phalloidin to actin filaments polymerized from ATP-actin monomers (*26*). Considering that the hydrolysis of ATP by the actin protomers and the subsequent phosphate release step is fast compared to the treadmilling of actin, the filaments polymerized from ATP-actin monomers contain mostly ADP-actin protomers. In the absence of nucleotide analogues, the results showed that the binding of one phalloidin molecule could stabilize seven protomers in the actin filaments [$k = 3$ (*26*)], indicating allosteric interactions between adjacent actin protomers. When the same equation (eq 1) was applied in this study in the case of the lower-temperature transition curves in Figure 5, the value of k was found to be zero within the limits of experimental error for both ADP.BeF_x- and ADP.AlF_4-actin filaments (0.05 ± 0.1 and -0.01 ± 0.03, respectively). These results indicated that only one actin protomer ($2k + 1 = 1$) was stabilized by the phalloidin binding in these filaments, and thus, the effect of binding of phalloidin to these filaments was not cooperative. The comparison of this finding to the observation made in the absence of BeF_x and AlF_4 suggests that the binding of the phosphate analogues to the filaments diminished the cooperative nature of the binding of phalloidin to the actin filaments.

CONCLUSIONS

In this study, we find that the effect of phalloidin and the nucleotide analogues on actin was superimposed, indicating that the mechanism by which phalloidin and the nucleotide analogues stabilized the filament structure was different. We also observed that the phalloidin-induced stabilization of the actin filaments, which is cooperative in ADP-actin filaments in the absence of nucleotide analogues, became noncooperative in the presence of ADP.BeF_x or ADP.AlF_4. It appears that there are allosteric interactions between the nucleotide-binding sites and phalloidin-binding sites in actin filaments, and these interactions are modified by the binding of ADP.BeF_x or ADP.AlF_4 to the nucleotide-binding pocket in the actin protomers. As a result, the effect of the binding of phalloidin on the stability of the actin filaments becomes different; the thermal stability increases, but the cooperativity disappears. The disappearance of the cooperative behavior in ADP.BeF_x- and ADP.AlF_4-actin filaments in the case of phalloidin binding indicates that care should be taken when the biological role of these long-range interactions is considered in the case of other actin-binding ligands or proteins. A complete understanding of the biological function of the long-range allosteric interactions along the actin filaments has not yet been achieved. The different nucleotide states of the actin filaments can represent the aging process of the filaments. The fact that the different nucleotide states are correlated with the conformational properties of the protein suggests the functional involvement of the ATP hydrolysis and these nucleotide states in the timing of the actin-related intracellular events, and thus in the regulation of the actin network in living cells.

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