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Dissociative Mechanism of F-Actin Thermal Denaturation

V. V. Mikhailova[1], B. I. Kurganov[1], A. V. Pivovarova[1,2], and D. I. Levitsky[1,3]*

[1]*Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr. 33, 119071 Moscow, Russia; fax: (495) 954-2732; E-mail: levitsky@inbi.ras.ru*
[2]*Faculty of Bioengineering and Bioinformatics and* [3]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia*

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Abstract—We have applied differential scanning calorimetry to investigate thermal unfolding of F-actin. It has been shown that the thermal stability of F-actin strongly depends on ADP concentration. The transition temperature, T_m, increases with increasing ADP concentration up to 1 mM. The T_m value also depends on the concentration of F-actin: it increases by almost 3°C as the F-actin concentration is increased from 0.5 to 2.0 mg/ml. Similar dependence of the T_m value on protein concentration was demonstrated for F-actin stabilized by phalloidin, whereas it was much less pronounced in the presence of AlF_4^-. However, T_m was independent of protein concentration in the case of monomeric G-actin. The results suggest that at least two reversible stages precede irreversible thermal denaturation of F-actin; one of them is dissociation of ADP from actin subunits, and another is dissociation of subunits from the ends of actin filaments. The model explains why unfolding of F-actin depends on both ADP and protein concentration.

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Key words: actin, thermal denaturation, differential scanning calorimetry

Actin is found in virtually every eukaryotic cell. Actin filaments have a crucial role in biological motility as the main partners of the myosin-based "motor" systems and as the major constituent of the cytoskeleton. The cyclic interaction of myosin heads with actin filaments, which is accompanied by ATP hydrolysis in the heads, is the basis of the molecular mechanism of a number of events in biological motility, from intracellular transport to muscle contraction. Filamentous actin (F-actin) is a spiral polymer of actin monomers. Monomeric actin (G-actin) is a globular protein with molecular mass of 42 kD. It consists of a single polypeptide chain with a bound ATP and a divalent cation. An important feature of actin is its ability to polymerization upon addition of neutral salts with formation of long polar filaments of F-actin. The polymerization of G-actin into F-actin is accompanied by the hydrolysis of bound ATP followed by slower release of P_i; as a result, each subunit of F-actin contains tightly bound ADP [1].

Differential scanning calorimetry (DSC) is the most effective and commonly employed method to study the thermal unfolding of proteins [2-4]. Previously, this method was successfully used to investigate domain structure of monomeric G-actin. The existence of at least two interacting domains in the G-actin molecule has been proposed from analysis of the G-actin heat sorption curve [5, 6]. This suggestion has been fully confirmed by the three-dimensional atomic structure of the G-actin published in 1990 [7]. It has been established that the G-actin molecule consists of two well distinguishable domains separated by a deep cleft, each domain being subdivided into two subdomains. The DSC method was also successfully used to investigate interaction of F-actin with the cyclic heptapeptide phalloidin (one of the principal toxins of the toxic mushroom *Amanita phalloides*) [8, 9], nucleotides [10], phosphate analogs [9, 11], membrane lipids [12], and various proteins, such as myosin heads and their fragments [13-17], tropomyosin [18-21], troponin [19], and cofilin [22].

In this respect, the interaction of F-actin with cofilin is of a special interest. It has been shown using DSC that this actin-binding protein has two opposite effects on the thermal unfolding of F-actin, depending on molar

Abbreviations: AlF_4^-) aluminum fluoride anion; ANS) 1-anilinonaphthalene-8-sulfonic acid; DSC) differential scanning calorimetry; F-actin) filamentous actin; G-actin) monomeric actin.

* To whom correspondence should be addressed.

ratio cofilin/actin [22]. At saturating concentration, cofilin stabilizes F-actin, i.e., it increases the thermal stability of F-actin, but at sub-saturating concentrations it causes a strong decrease in the F-actin thermal stability. It has been suggested from these intriguing DSC results that cofilin, when it binds to F-actin at sub-saturating concentrations, stabilizes only those actin subunits to which it directly binds, but it destabilizes with a very high cooperativity neighboring regions of the actin filament that are free of cofilin [22].

The highly cooperative destabilization of F-actin by sub-saturating concentrations of cofilin could not be explained by any existing models that describe the thermal unfolding of proteins interacting with each other. It became clear that to explain this effect, the mechanism of the F-actin thermal denaturation should be elucidated. Earlier we proposed a new model describing in a preliminary form the mechanism of the F-actin thermal denaturation and explaining the highly cooperative effect of destabilization of F-actin by cofilin [22, 23]. An important feature of this model was that the irreversible thermal unfolding of F-actin is preceded by a reversibly stage of dissociation of actin monomers (or short oligomers) from the pointed (or "–") end of the actin filament. It was clear, however, that the thermal denaturation of F-actin is a very complicated process, and many additional experiments are needed to elucidate its mechanism.

In the present study, we have investigated in detail the thermal denaturation of F-actin using DSC. A new "dissociative" mechanism of the thermal unfolding of F-actin has been proposed from the results obtained. The main feature of the proposed model is that the irreversible thermal unfolding of F-actin is preceded by at least two reversible stages: one of them is dissociation of ADP from actin subunits, and another is dissociation of subunits from the ends of actin filaments.

MATERIALS AND METHODS

Actin preparation. Actin was prepared from rabbit skeletal muscle and purified by polymerization–depolymerization [24]. G-Actin in G buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, and 1 mM NaN_3) was used within one week. G-Actin concentration was determined spectrophotometrically using the absorption coefficient at 290 nm $A^{1\%} = 6.3$ cm^{-1}. G-Actin was polymerized by the addition of $MgCl_2$ to the final concentration of 4 mM. *Prior to* experiments, F-actin was diluted to a final concentration (from 0.5 to 2.0 mg/ml) with 30 mM Hepes-KOH, pH 7.3, containing 1 mM $MgCl_2$ and, as a rule, 100 mM KCl. In some cases, F-actin was stabilized by the addition of 1.5-fold molar excess of phalloidin (Sigma, USA), or by forming a complex with aluminum fluoride (AlF_4^-). To obtain the latter, 0.5 mM $AlCl_3$ was added to F-actin in the presence of 2 mM ADP and 5 mM NaF [11].

Differential scanning calorimetry. Calorimetric experiments were performed on a DASM-4M differential adiabatic scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia) with 0.47 ml capillary platinum cells, as described earlier [11, 13-22]. The measurements were carried out at scanning rates of 0.5, 1.0, or 1.8 K/min. The reversibility of the thermal transitions was tested by checking the reproducibility of the calorimetric trace in a second heating of the sample immediately after cooling from the first scan. The thermal denaturation of actin was fully irreversible. Calorimetric traces were corrected for instrumental background and for possible aggregation artifacts by subtracting the scans obtained from the second heating of the samples. The temperature dependence of the excess heat capacity was further analyzed and plotted using Origin 1.16 and Origin 7.0 software (MicroCal Inc., USA).

Temperature dependences of light scattering. Thermally induced aggregation of F-actin was detected by an increase in light scattering at 90° on a Cary Eclipse fluorescence spectrophotometer (Varian, Australia) equipped with temperature controller and thermoprobes. F-Actin was heated with constant rate (1°C/min) from 25 to 80°C, and the light scattering was measured at 350 nm. The temperature dependences of light scattering were measured under the same conditions, at the same protein concentration, and at the same heating rate as corresponding DSC experiments.

RESULTS

Effects of nucleotides on the thermal denaturation of F-actin. Our study in this direction was initiated when we observed remarkably lower thermal stability with F-actin polymerized in G buffer and then diluted by 6-7 times to the final concentration of 1 mg/ml by Hepes-KOH, pH 7.3, compared with F-actin diluted to the same concentration by G-buffer (Fig. 1). The difference in maximum temperature between the two heat sorption curves was 3.7°C. In the beginning, we proposed that this effect might be caused by some specific influence of organic buffer (Hepes) on the thermal denaturation of F-actin. To check this assumption, we studied by DSC the thermal unfolding of F-actin diluted in different buffers (phosphate buffer, imidazole-HCl, Hepes, Mops, Tris) of the same molarity (24 mM) and at the same pH (7.3); all these buffer solutions also contained 100 mM KCl and 1 mM $MgCl_2$. It was shown that in all these buffer solutions the thermal unfolding of F-actin was virtually the same as that observed in Hepes-KOH (Fig. 1, curve *1*). Furthermore, it was shown that the thermal stability of F-actin increases with increasing content of G-buffer in the protein solution. It became clear that some component of

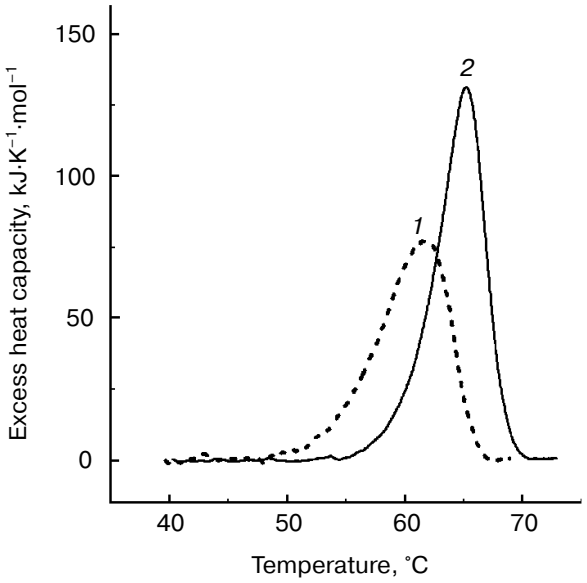


Fig. 1. Temperature dependences of excess heat capacity (ΔC_p) of F-actin in 30 mM Hepes-KOH, pH 7.3, (*1*) and in G-buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, and 1 mM NaN_3) (*2*). Both buffers contained 100 mM KCl and 1 mM $MgCl_2$. Protein concentration was 1 mg/ml. Heating rate was 1 K/min.

G-buffer was responsible for the increase in the thermal stability of F-actin. It was shown by further experiments that this component is a nucleotide (ATP or ADP). This led to special and more detailed studies of the effects of nucleotides on the thermal unfolding of F-actin.

It has been shown that the thermal stability of F-actin strongly depends on concentration of added nucleotide (ADP or ATP). Figure 2a shows that an increase of ADP concentration in solution leads to a pronounced shift of the heat sorption curve of F-actin towards higher temperature. A similar effect was also observed in the case of ATP (data not shown). The transition temperature (T_m) increases by 5-6°C with increasing ADP concentration up to 1 mM and reaches a plateau at higher concentrations of ADP (Fig. 2b). This effect was observed at all heating rates used, from 0.5 to 1.8 K/min (Fig. 2b).

The stabilizing effect of ADP was highly specific, as it was observed only with ADP, but not with other nucleoside diphosphates (IDP, UDP, GDP, CDP); moreover, some of them (UDP, GDP, and CDP) even somewhat decreased the thermal stability of F-actin (Fig. 3). In contrast, the strong stabilizing effect of ATP on F-actin was much less specific, as it was observed, although to a lesser extent, for other nucleoside triphosphates (ITP, UTP, and GTP) (Fig. 3). This suggests that stabilizing effect of ATP significantly differs in its mechanism from the effect caused by ADP. For example, it is not excluded that ATP and other nucleoside triphosphates may interact with some additional nucleotide-binding site on F-actin. The presence of such "second", low-specificity and low-affinity nucleotide interacting site on actin has been postulated in some works [25-28]. Binding of nucleotides in this site occurred at their millimolar concentrations, ITP and CTP being even more effective than ATP [27].

It seems very likely that the stabilizing effect of ATP and other nucleoside triphosphates is caused just by their interaction with the "second" nucleotide-binding site on F-actin. Favoring this assumption is the fact that a rise in ATP concentration from 1 to 5 mM leads to further significant increase in the F-actin thermal stability, whereas a similar rise in ADP concentration has no influence on the thermal stability (Fig. 4). The most pronounced changes in the thermal stability of F-actin occur just at low concentrations of added nucleotide (0.1-0.2 mM), and under these conditions the effects of ADP and ATP

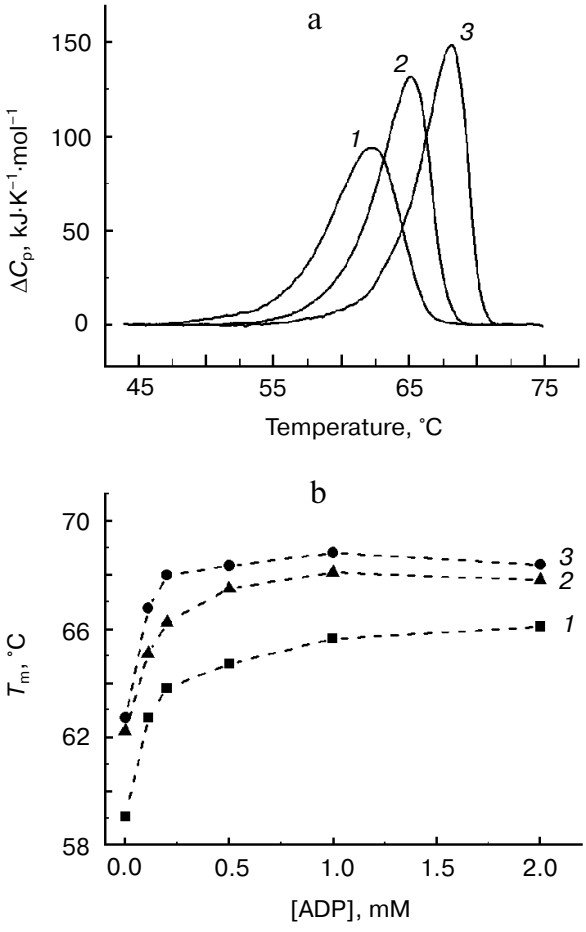


Fig. 2. Effect of ADP on the thermal denaturation of F-actin. a) Heat sorption curves of F-actin in the absence of added ADP (*1*) and in the presence of 0.11 or 1.0 mM ADP (*2* and *3*, respectively). Protein concentration was 1.5 mg/ml. Other conditions: 30 mM Hepes-KOH, pH 7.3, 2 mM $MgCl_2$. Heating rate was 1 K/min. b) Dependence of the maximum temperature (T_m) of the thermal transition of F-actin on concentration of added ADP at different heating rates: 0.5 (*1*), 1.0 (*2*), and 1.8 (*3*) K/min.

are very similar (Figs. 2b and 4). It is difficult to explain these effects by some interaction of ADP or ATP with additional nucleotide-binding site on F-actin, which needs much higher concentrations of nucleotide. It seems most likely that these effects, that are observed at low concentrations of ADP or ATP, are caused by their binding with highly specific, high-affinity sites in actin subunits, whereas additional stabilization of actin filaments, that is observed at high concentrations of ATP, is caused by ATP binding to low-affinity sites, which are able to bind ATP and other nucleoside triphosphates, but not ADP.

It seems to us that the most likely explanation of the stabilizing effect of nucleotides on the thermal unfolding of F-actin is as follows. We propose that irreversible thermal unfolding of F-actin is preceded by a reversible stage of dissociation of nucleotide (ADP) from specific nucleotide-binding sites in actin subunits. It is well known that actin lacking bound nucleotide is very unstable and it easily denatures [29] unless protected by high concentrations of sucrose, which permit actin to retain stability and ability to form filaments of F-actin [30, 31]. Under normal conditions, at room temperature, ADP is tightly bound in F-actin subunits, and it is unable to exchange with free nucleotides. Reversible dissociation of ADP from actin subunits probably occurs only upon heating, just before irreversible denaturation of the protein. The presence of free nucleotide (ADP or ATP) in solution prevents actin-bound ADP from dissociation, and this can explain the nucleotide-induced increase in the thermal stability of F-actin.

Dependence of F-actin thermal denaturation on protein concentration. It is known that, upon thermal denaturation of many oligomeric proteins and enzymes, the maximum temperature of the thermal transition remarkably increases with the rise of protein concentration. According to recent views, such dependence of T_m on protein concentration denotes the presence of reversible stage of dissociation of subunits from the oligomer before their irreversible denaturation [32, 33]. We have applied this approach to F-actin and shown that the T_m value strongly depends on protein concentration: the T_m value increases by more than 3°C when the concentration of F-actin is increased from 0.5 to 2.5 mg/ml (Fig. 5).

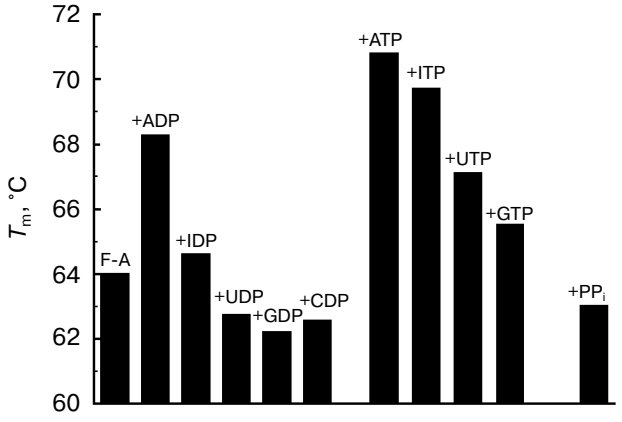


Fig. 3. Comparison of the effects of different nucleoside diphosphates (ADP, IDP, UDP, GDP, and CDP) and nucleoside triphosphates (ATP, ITP, UTP, and GTP), as well as inorganic pyrophosphate (PP_i), on the maximum temperature of the F-actin thermal transition (T_m). F-A, F-actin in the absence of added nucleotides. Concentration of actin was 1 mg/ml, concentration of all nucleotides 2 mM, and PP_i 5 mM. Heating rate was 1.8 K/min.

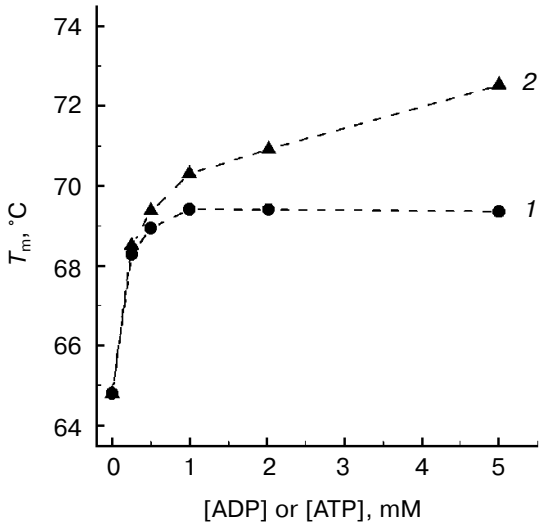


Fig. 4. Dependence of the maximum temperature (T_m) of the thermal transition of F-actin on concentration of added nucleotide—ADP (*1*) or ATP (*2*). Protein concentration was 1.5 mg/ml. Other conditions: 30 mM Hepes-KOH, pH 7.3, 2 mM $MgCl_2$, 100 mM KCl. Heating rate was 1.8 K/min.

It should be noted that in our experiments the area under DSC curve increased in proportion to the increase in protein concentration (Fig. 5), with no appreciable changes in the calorimetric enthalpy (ΔH_{cal}), which was equal to 700 ± 50 kJ/mol at all protein concentrations used. This is in contradiction with recently published DSC results of Gicquaud et al. [34] showing that the calorimetric enthalpy of F-actin drastically decreases with increasing concentration of the protein. It seems most likely that at high protein concentrations, the authors observed overlapping of two calorimetric peaks, i.e., an endothermic peak corresponding to the thermal unfolding of F-actin, and an exothermic peak reflecting precipitation of denatured and aggregated protein in the calorimetric cell. In the present work, special capillary construction of the measuring cells of the DASM-4M calorimeter almost fully prevented the artifacts caused by precipitation of denatured protein [35].

Thus, dependence of maximum position of the peak of F-actin thermal denaturation on protein concentration (Fig. 5) suggests that reversible fragmentation of actin filaments and/or dissociation of subunits from the ends of

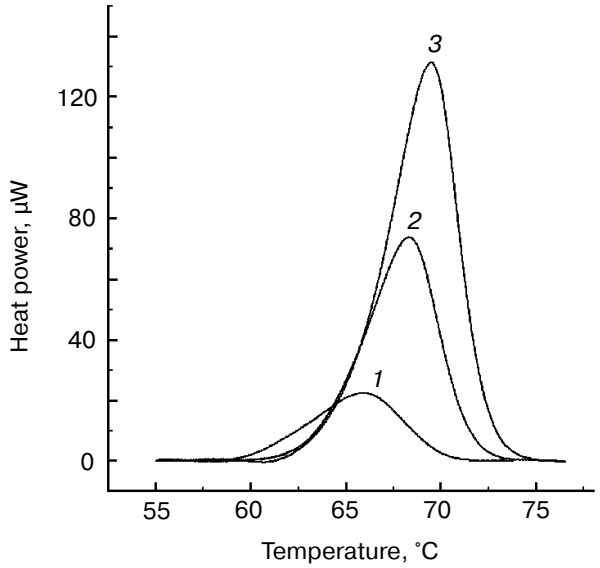


Fig. 5. DSC curves of F-actin obtained at different protein concentrations: 0.5 (*1*), 1.5 (*2*), and 2.5 (*3*) mg/ml. Other conditions: 30 mM Hepes-KOH, pH 7.3, 2 mM $MgCl_2$, 2 mM ADP. Heating rate was 1.8 K/min.

the filaments precedes the irreversible thermal denaturation of the protein. To check this assumption, we studied the T_m dependences on protein concentration for actin in its different states: monomeric G-actin, filamentous F-actin, and F-actin stabilized by phalloidin or aluminum fluoride (AlF_4^-). The DSC curves for these actin states are presented in Fig. 6a. It is seen that actin polymerization, i.e., transformation of monomeric G-actin into F-actin filaments, leads to significant changes in the thermal unfolding of the protein. These changes are expressed in a significant increase in the denaturation temperature, in a considerable increase in calorimetric enthalpy (the area under the peak), and in a sharp change in the peak shape (the peak becomes much narrower thus indicating a significant increase in the cooperativity of thermal denaturation) (Fig. 6a). Specific binding of phalloidin to F-actin significantly increases the temperature of F-actin thermal denaturation shifting the thermal transition by 14-16°C to a higher temperature (Fig. 6a) [8, 9, 18]. A similar and even more pronounced effect of F-actin stabilization is observed in the presence of AlF_4^- (Fig. 6a). In this case, the stabilization of F-actin is caused by formation of a stable ternary complex of actin with ADP and P_i analogs – aluminum fluoride or beryllium fluoride, which mimics the actin-ADP-P_i intermediate state that is formed in the nucleotide-binding sites of actin subunits during actin polymerization [9, 11].

The dependences of the T_m value on protein concentration for all above described states of actin are presented in Fig. 6b. It is seen that in the case of F-actin the T_m value increases by almost 3°C when the protein concentration is increased from 0.5 to 2.0 mg/ml. A similar dependence of the T_m value on protein concentration was demonstrated for F-actin stabilized by phalloidin, whereas it was much less pronounced in the presence of AlF_4^-. As to monomeric G-actin, in this case, as expected, the T_m value was independent of concentration of the protein (Fig. 6b). These results confirm our assumption on the presence of a reversible stage of fragmentation of actin filaments or their dissociation into separate monomers (or short oligomers) just before irreversible thermal denaturation of the protein.

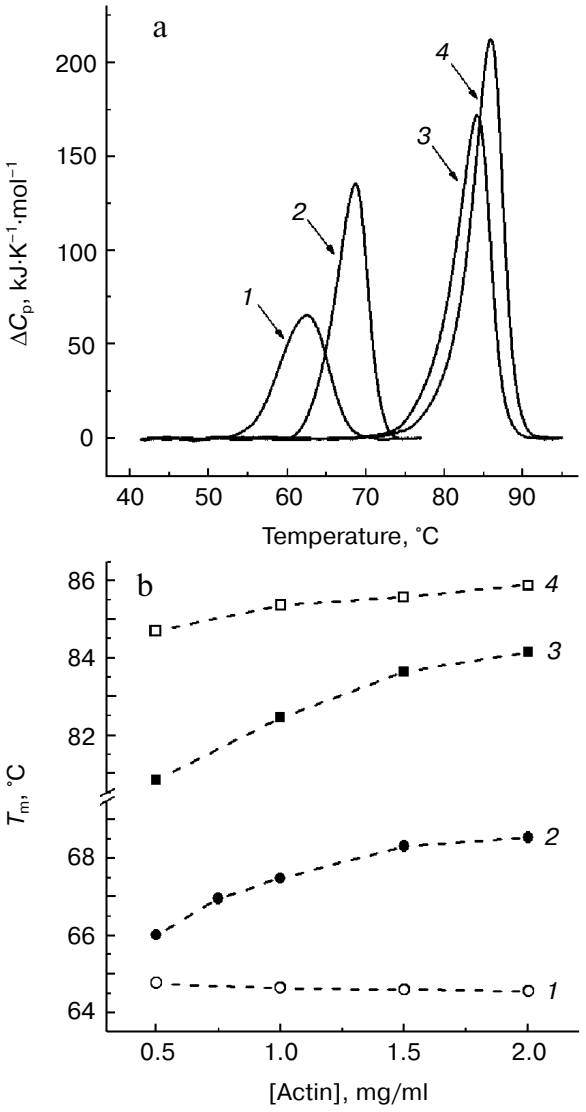


Fig. 6. DSC curves (a) and T_m dependences on protein concentration (b) for G-actin (*1*), F-actin (*2*), and F-actin stabilized by phalloidin (*3*) or by aluminum fluoride (AlF_4^-) (*4*). Conditions: G-buffer (*1*) or 30 mM Hepes-KOH, pH 7.3, containing 2 mM $MgCl_2$, 2 mM ADP (*2*), as well as either 45 μM phalloidin (*3*) or 0.5 mM AlF_4^- (5 mM NaF and 0.5 mM $AlCl_3$) (*4*). Concentration of actin was 1.5 (a) and 0.5-2.0 mg/ml (b). Heating rate was 1.8 (a) or 1.0 K/min (b).

Thermally induced aggregation of F-actin. Thermal denaturation of actin is accompanied by aggregation of the protein. Denaturation of monomeric G-actin is known to lead to formation of so-called "inactivated actin", which is represented by stable homogeneous associates consisting of a limited number of unfolded protein molecules [36-39]. In the case of F-actin, the thermally induced aggregation is accompanied by significant increase in light scattering (Fig. 7b). We studied the temperature dependences of the thermal unfolding of F-actin (using DSC) and its aggregation (by measuring the increase in light scattering) under exactly the same conditions and at the same heating rate (Fig. 7). A very good correlation was found between thermal denaturation (Fig. 7a, curve *1*) and aggregation (Fig. 7b, curve *1*) of F-actin. In the presence of ADP both curves, the denaturation curve and the aggregation curve, similarly shifted to a higher temperature (Figs. 7a and 7b, curves *2*). It is interesting to note that the protein solution was clear up to achieving the maximum of light scattering; the following decrease in light scattering at higher temperatures can be explained by increased turbidity of solution due to formation of large aggregates of denatured protein, which are inclined to precipitation.

Thus, these results mean that during heating the denatured actin molecules immediately undergo aggregation.

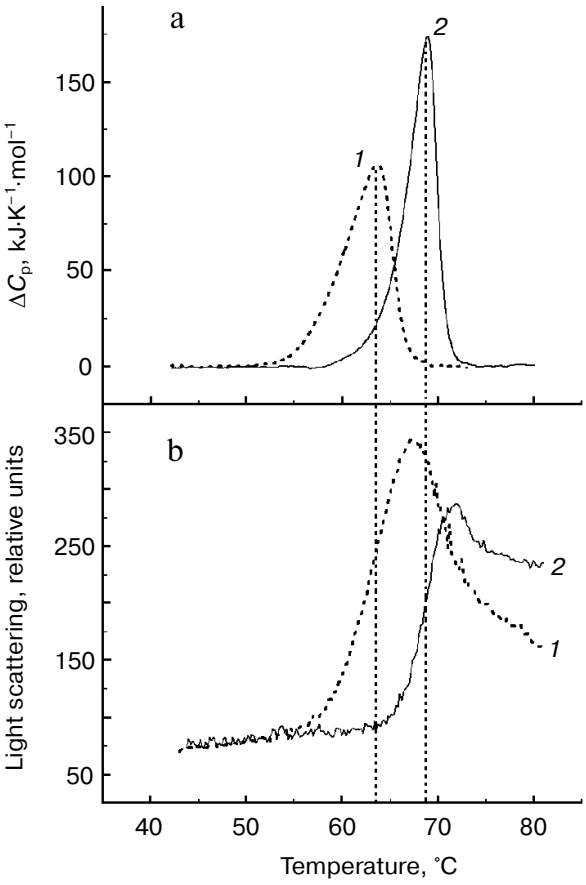


Fig. 7. Effect of ADP on thermal denaturation (a) and aggregation (b) of F-actin. F-Actin (1 mg/ml) in the absence of added nucleotides (curves *1*) or in the presence of 2 mM ADP (curves *2*) was heated with the constant rate of 1°C/min and thermal denaturation or aggregation were measured by DSC (a) or light scattering (b). Dotted lines show the values of the maximum temperature (T_m) of the thermal transition of F-actin in the absence and in the presence of ADP.

DISCUSSION

Our results suggest that at least two reversible stages precede the irreversible thermal denaturation of F-actin. One of them is dissociation of nucleotide (ADP) from nucleotide-binding sites of actin subunits, and the other is fragmentation of actin filaments or dissociation of monomers (or short oligomers) from the filament. The denaturation process is completed by aggregation of unfolded protein molecules. Thus, the proposed mechanism for the thermal denaturation of F-actin filaments can be presented by the Scheme shown below. During heating, destabilization of actin filaments occurs, leading to increased mobility of the filament and to weakening the bonds between subunits. As a result, actin monomers (or short oligomers) dissociate from one of the ends of polar actin filament (most likely, from the pointed (or "–") end where the dissociation rate is much higher than on growing barbed (or "+") end of the filament). These monomers either lose bound ADP and then immediately denature and aggregate, or the ADP-containing monomers may bind again to the actin filament, but most likely to another filament and to the other end ("+"-end). This model (Scheme) explains why thermal unfolding of F-actin depends on both ADP and protein concentration. The presence of excess free ADP in solution impedes the thermally induced reversible dissociation of tightly bound ADP from actin subunits, while the increase in the protein concentration increases the number of actin filaments and, correspondingly, the number of their "+"-ends to which actin monomers can bind.

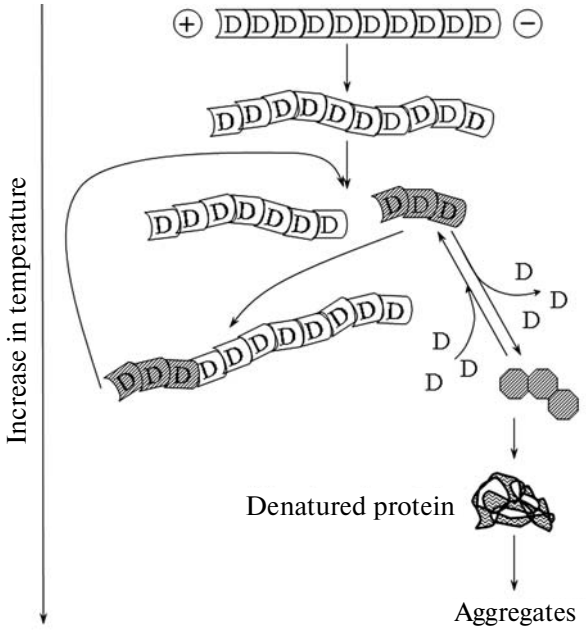
We are aware that the proposed model for the F-actin thermal denaturation (Scheme) is mainly based on indirect data (i.e., on the T_m dependences on actin and ADP concentrations). It is not excluded that these results can be explained by some other way. Let us consider briefly some other interpretations of the dependence of T_m on actin concentration (as to different interpretations of the ADP effect, these were discussed above). For example, it cannot be excluded that the thermal stability of actin filaments may depend on the length of the filaments, which, in turn, may increase with increasing the protein concentration. However, this explanation seems to be very unlikely for the following reason. In all the cases, actin was polymerized at rather high concentration (6-7 mg/ml) and then, before experiments, F-actin was

diluted to final concentrations (from 0.5 to 2.0 mg/ml) using the same yellow tips. Even if mechanical fragmentation of long actin filaments into shorter filaments occurred in this case, it would be the same for all preparations studied. Thus, it seems very unlikely that the length of filaments may depend on final concentration of F-actin.

Another possible interpretation for the dependence of the T_m value on F-actin concentration may be nonspecific lateral sticking of actin filaments (so-called "bundling" effect), which takes place at rather low ionic strength. At high concentration of F-actin such type bundling of actin filaments is more probable that at low actin concentration. It is not excluded that bundling of actin filaments may have some influence on the thermal stability of F-actin. However, this interpretation cannot explain the remarkable effect of AlF_4^- on the dependence of the T_m value on concentration of F-actin (Fig. 6b), since the AlF_4^- anion binds with actin subunits in their specific nucleotide-binding sites, and therefore it seems very unlikely that this anion can have any influence on the bundling of actin filaments. For these reasons, it seems to us that the proposed "dissociative" mechanism of the thermal denaturation of F-actin (Scheme) is the most likely interpretation of the observed dependence of the T_m value on F-actin concentration (Fig. 6b).

It is important to note that two F-actin stabilizers, phalloidin and AlF_4^-, differ from one another in their influence on the T_m dependence on protein concentration (Fig. 6b). Both these stabilizers increase the thermal stability of F-actin substantially (Fig. 6a) although independently from each other [9]. Phalloidin increases the F-actin thermal stability by strengthening the bonds between adjacent subunits in the actin filament, whereas AlF_4^- demonstrates a similar effect by trapping of ADP in the nucleotide-binding site of actin. Thus, the complex ADP–AlF_4^-–actin, which mimics the ADP–P_i–actin intermediate state of actin polymerization, should prevent dissociation of ADP from actin subunits and, on the other hand, it should stimulate the binding of actin monomers to the barbed ("+") end of the filament. This means, according to the model proposed above, that AlF_4^- may influence both reversible stages preceding the irreversible thermal denaturation of F-actin, thus making the T_m dependence on F-actin concentration less pronounced and more similar to those characteristic for monomeric proteins.

Let us consider in detail the main statements of the proposed mechanism (Scheme). One of them is that the actin filament denatures not as a whole, but as separate monomers or short oligomers dissociated from the filament during heating. In favor of this assumption are the results of recent studies on the thermal denaturation of actin using the hydrophobic fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (ANS). It has been shown that both G-actin and F-actin demonstrate a very



A scheme illustrating the dissociative mechanism for the thermal denaturation of F-actin filaments. The temperature increase is shown from top to bottom. ADP molecules, both free in solution and bound to actin monomers, are marked as D. The shaded actin monomers (or short oligomers) are those that dissociate upon heating from the "–"-end of actin filament and can bind to the "+"-end of the same or other filament. See text for details

Scheme

similar increase in ANS fluorescence during thermal denaturation of the protein, and they both show very similar fluorescence spectra of the dye bound to denatured actin (A. V. Pivovarova and D. I. Levitsky, unpublished results). Strong increase in ANS fluorescence is known to be caused by interaction of the dye with hydrophobic clusters that appear on the protein surface upon denaturation [37-39]. Hence we may conclude that similar increase in ANS fluorescence for G-actin and F-actin reflects similarity between these proteins in hydrophobic properties of the surface of denatured protein, and this is in favor of our assumption that denaturation of F-actin monomers occurs after their dissociation from actin filaments.

Another confirmation of the "dissociative" mechanism of the F-actin thermal denaturation has been recently obtained from studies on the interaction of F-actin with small heat shock proteins [40]. It has been found that these proteins do not interact with intact F-actin and do not protect F-actin from thermal denaturation, but they effectively prevent aggregation of heat-denatured actin by forming small, stable, and highly soluble complexes with denatured actin. The size and sedimentation coefficients of these complexes were much less than that of intact F-actin. The results were interpreted as follows: small heat shock proteins bind to denatured actin

monomers or short oligomers obtained during heating of F-actin and protect them from aggregation by forming small and highly soluble complexes [40].

The other important statement of the proposed dissociative mechanism of the F-actin thermal denaturation is that the ADP-containing actin monomers or short oligomers dissociated from the "–"-end of actin filament may bind again to the "+"-ends of the filaments (Scheme). Let as consider briefly the literature data in favor of this assumption. An "annealing" phenomenon has been found for F-actin, i.e., formation of long polar actin filaments from very short filaments due to interaction between "+"- and "–"-ends of the filaments [41-43]. The annealing process proceeded spontaneously, and, unlike actin polymerization, the presence of ATP-containing G-actin monomers and ATP hydrolysis was not required [41].

Furthermore, there are many reports in the literature that monomeric ADP–G-actin, in which ATP is replaced by ADP, is able to polymerize with formation of F-actin filaments, although more slowly than ATP–G-actin [44-46]. Filaments of F-actin obtained from ADP–G-actin and ATP–G-actin did not differ from one another in their length, viscosity, and mechanical properties [47], although some difference was found in their dynamic properties (in mutual flexibility of subunits in the filament) [48]. It has been also demonstrated that ADP–G-actin is able to bind to the barbed ("+") ends of preformed actin filaments with a rather high rate (only three times less than for ATP–G-actin) [49].

All these data mean that the binding of ADP-containing actin monomers or short oligomers to the barbed ("+") ends of actin filaments, which is postulated in our model (Scheme), seems to be quite possible. In favor of this are also the DSC results on thermal denaturation of F-actin in its complexes with the actin-binding protein gelsolin. In these complexes the maximum temperature of the F-actin thermal transition was very low (T_m ~ 61.5°C), and its dependence on protein concentration was much less pronounced than for F-actin: the T_m value increased by only 0.7°C when concentration of F-actin was increased from 0.4 to 2.1 mg/ml (D. A. Pavlov, personal communication). It is known that gelsolin binds to the growing "+"-end of actin filament thus preventing the binding of actin monomers to this end and further growth of the filament. In the light of the proposed model (Scheme), this should lead to a strong decrease in the F-actin thermal stability making it similar to that of the monomeric protein, and have a significant influence on the dependence of T_m value on actin concentration. All these predictions have been fully corroborated in DSC experiments performed by Dr. D. A. Pavlov.

Thus, the results presented here, together with the data of other authors, testify in favor of the proposed dissociative mechanism of the thermal denaturation of F-actin filaments.

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