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# Effects of Actin-Binding Proteins on the Thermal Stability of Monomeric Actin

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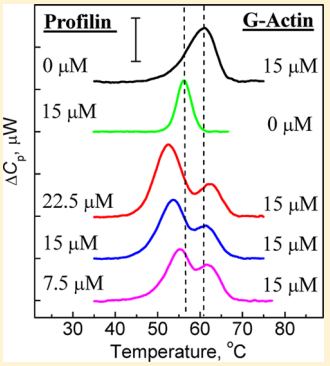
**ABSTRACT:** Differential scanning calorimetry (DSC) was applied to investigate the thermal unfolding of rabbit skeletal muscle G-actin in its complexes with actin-binding proteins, cofilin, twinfilin, and profilin. The results show that the effects of these proteins on the thermal stability of G-actin depend on the nucleotide, ATP or ADP, bound in the nucleotide-binding cleft between actin subdomains 2 and 4. Interestingly, cofilin binding stabilizes both ATP−G-actin and ADP−G-actin, whereas twinfilin increases the thermal stability of the ADP−G-actin but not that of the ATP−G-actin. By contrast, profilin strongly decreases the thermal stability of the ATP−G-actin but has no appreciable effect on the ADP−G-actin. Comparison of these DSC results with literature data reveals a relationship between the effects of actin-binding proteins on the thermal unfolding of G-actin, stabilization or destabilization, and their effects on the rate of nucleotide exchange in the nucleotide-binding cleft, decrease or increase. These results suggest that the thermal stability of G-actin depends, at least partially, on the conformation of the nucleotide-binding cleft: the actin molecule is more stable when the cleft is closed, while an opening of the cleft leads to significant destabilization of G-actin. Thus, DSC studies of the thermal unfolding of G-actin can provide new valuable information about the conformational changes induced by actin-binding proteins in the actin molecule.




Actin is one of the most abundant and highly conserved proteins, and it is essential for numerous cellular functions. Monomeric or G-actin polymerizes in a polar fashion to form actin filaments or F-actin, and this process is accompanied by hydrolysis of tightly bound ATP followed by a slower release of $P_i$. Upon depolymerization of F-actin, ADP bound to actin monomers is replaced by ATP. In vivo, the transition between G- and F-actin is a precisely regulated process controlled not only by nucleotide binding and hydrolysis but also by a large number of actin-binding proteins (ABPs).[1−3] Among the central regulators of this dynamic process are actin depolymerizing factor (ADF)/cofilins, twinfilins, and profilins.

ADF/cofilins are small proteins (molecular masses of 15−19 kDa) that bind to both monomeric and filamentous actin. Their most important physiological function is to increase actin dynamics by promoting the disassembly of actin filaments. ADF/cofilins promote severing of actin filaments and increase the rate of dissociation of actin monomers at filament pointed ends by ∼25-fold.[4−6] They also inhibit spontaneous nucleotide exchange on monomeric actin. Most cofilins bind to the ADP−G-actin with a significantly higher affinity ($K_d$ = 0.02−0.15 $\mu$M) than they bind to the ATP−G-actin ($K_d$ = 0.6−8.0 $\mu$M).[4,7] The only exception identified so far is the mammalian muscle cofilin-2, which binds with high affinity to both ADP− and ATP−G-actin.[8]

Twinfilins are highly conserved ∼40 kDa ABPs containing two ADF/cofilin-like domains (ADF homology or ADF-H domains) that are separated by a short linker region followed by a C-terminal tail region of ∼35 residues. Despite their sequence being homologous to that of ADF/cofilins, twinfilins do not bind to the sides of actin filaments. Instead, twinfilins bind and sequester $\alpha$-G-actin, and at least mammalian twinfilins also cap filament barbed ends.[2,9−11] Like ADF/cofilins, twinfilins bind to the ADP−$\alpha$-G-actin with an ∼10-fold higher affinity ($K_d$ ∼ 0.05 $\mu$M) than they bind to the ATP−$\alpha$-G-actin ($K_d$ = 0.5−2.0 $\mu$M) and inhibit nucleotide exchange on actin monomers.[12] Although twinfilin contains two actin-binding sites (in the ADF-H domains), it forms a 1:1 molar complex with G-actin. The high-affinity binding site is in the C-terminal ADF-H domain, whereas the N-terminal ADF-H domain seems to function as an actin entry domain.[2,12]

Profilins are small ABPs (molecular masses of 12−19 kDa) that are among the most strongly expressed cytoplasmic proteins.[1,2,13] Contrary to proteins with ADF-H domains, profilins bind exclusively to the monomeric actin. The main physiological function of profilins is to increase the dynamics of the pool of monomeric actin in the cytoplasm and to promote

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the incorporation of the ATP−G-actin into the filament barbed ends. Literature data about the binding of profilins to G-actin are somewhat contradictory: they either display a higher affinity for the ATP−G-actin ($K_d$ = 0.1−0.15 $\mu$M for both Ca-bound G-actin and Mg-bound G-actin) than for the ADP−Mg−G-actin ($K_d$ = 0.5−3.3 $\mu$M)[2,14] or bind with a similar affinity to ATP−Mg−G-actin and ADP−Mg−G-actin ($K_d \sim 0.6$ $\mu$M).[15]

A large body of biochemical data shows that most profilins enhance the nucleotide exchange on actin monomers (on both $\alpha$- and $\beta/\gamma$-actin isoforms),[14−19] whereas cofilin and twinfilin inhibit nucleotide exchange.[4,9,12,18−21] The opposite effects of these ABPs on the rate of nucleotide exchange can be explained by their different effects on the G-actin structure. The actin molecule consists of two domains, separated by two diametrically opposed clefts, each domain being subdivided into two subdomains.[22] The larger cleft, between subdomains 2 and 4, is the nucleotide-binding cleft (so named because it constitutes the nucleotide-binding site), whereas the smaller cleft, between subdomains 1 and 3 (also known as the hydrophobic cleft), is a primary target for most ABPs and therefore is also called the "target-binding cleft".[23] The binding of the ABPs to the hydrophobic cleft may have an allosteric effect on the conformation of the nucleotide-binding cleft, making it more open or more closed. The atomic structures of the actin−profilin complexes showed that the binding of profilin to the hydrophobic cleft can lead to the opening of the nucleotide-binding cleft caused by the relative movement of the two main domains.[24−26] In contrast, the crystal structure of the ATP−G-actin complex with the C-terminal ADF-H domain of twinfilin (Twf-C) suggests that binding of Twf-C to the hydrophobic cleft may "lock" the nucleotide-binding cleft in a closed conformation.[27] Thus, the effects of ABPs on the rate of nucleotide exchange on actin monomers, an increase or decrease, seem to be determined by ABP-induced opening or closing of the nucleotide-binding cleft.

There is, however, some discrepancy between the actin crystal structures and the data from solution studies of nucleotide exchange. For example, the solution studies performed on muscle actin are consistent with the opening of the nucleotide-binding cleft upon the transition from the ATP− to ADP−G-actin,[28,29] whereas only the closed conformation of this cleft was observed in the crystal structures of the ADP−$\alpha$-G-actin.[30,31] Another example is $\alpha$-G-actin specifically cleaved within the DNase I-binding loop (D-loop) in subdomain 2, which displayed an increased rate of nucleotide exchange in solution,[32] although in the crystal structure the nucleotide-binding cleft was in a typical closed conformation.[33] Moreover, it should be noted that a wide-open nucleotide-binding cleft was only observed on profilin−$\beta$-actin structures obtained under some special crystallization conditions,[24,26] whereas other structures of profilin complexes with different actin isoforms ($\beta$- or $\alpha$-actin or *Dictyostelium* actin) revealed the nucleotide-binding cleft in a moderately open or almost fully closed state.[26,34−36] It is possible that crystallization favors a closed state for G-actin even though the state of the nucleotide-binding cleft in solution may vary.[33,37] To avoid ambiguity, it seems important to apply additional approaches to examine the nucleotide-binding cleft conformation, more open or more closed. In our opinion, the thermal stability of G-actin can be successfully used for this purpose.

The thermal unfolding of non-muscle $\beta/\gamma$-G-actin in complex with profilin was indirectly studied by the DNase-I inhibition assay, and the results suggested that binding of profilin to G-actin leads to a significant decrease in the thermal stability of the ATP−G-actin but increased the stability of the ADP−G-actin.[38] On the other hand, we have applied differential scanning calorimetry (DSC), which is the most direct and effective method for studying the thermal unfolding of proteins,[39,40] and it can be successfully applied to reveal the changes in the thermal unfolding of muscle $\alpha$-G-actin induced by its interaction with ABPs[41−43] (see ref 44 for a review). In particular, DSC experiments revealed that the binding of cofilin to the ATP−$\alpha$-G-actin significantly increases the thermal stability of actin.[41,42] More recently, it was shown by DSC that both replacement of actin-bound ATP by ADP and specific cleavage of $\alpha$-G-actin within the D-loop (i.e., the changes for which the transition of the nucleotide-binding cleft to its open state was predicted from solution studies[28,29,32]) strongly decrease the thermal stability of G-actin.[45] These studies suggested that the thermal stability of G-actin may depend, at least partially, on the conformational state of the nucleotide-binding cleft: the actin molecule would be stable when the cleft is closed, while an opening of the cleft leads to significant destabilization of G-actin.

Here, we applied DSC to characterize and compare the effects of different ABPs (cofilin, twinfilin, and profilin) on the thermal unfolding of muscle $\alpha$-G-actin in its different states, with either ATP or ADP as a bound nucleotide. Our results show that cofilin binding stabilizes both ATP−G-actin and ADP−G-actin, whereas twinfilin increases the thermal stability of the ADP−G-actin but not that of the ATP−G-actin. By contrast, profilin strongly decreases the thermal stability of the ATP−G-actin but has no appreciable effect on the ADP−G-actin. These results are discussed with regard to effects of ABPs and nucleotides on the conformation of the nucleotide-binding cleft of G-actin.

## EXPERIMENTAL PROCEDURES

**Protein Preparations.** Mouse wild-type twinfilin-1, cofilin-1, and profilin-1 were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* BL21 cells from plasmids pPL78, pPL92, and pPL147, respectively, as described previously for mouse twinfilin and cofilin.[8,46] GST fusion proteins were enriched from the lysis supernatant with glutathione agarose beads (Sigma, St. Louis, MO); proteins were cleaved off the GST by 0.05 mg/mL thrombin and then further purified with Q-Sepharose high-performance anion-exchange and Superdex-75 HiLoad gel filtration columns (Amersham Biosciences AB, Uppsala, Sweden). The peak fractions containing the desired proteins were pooled, concentrated in a Centricon 10 kDa cutoff device, frozen in liquid $N_2$, and stored at −70 °C. Concentrations of these proteins were determined by absorbance at 280 nm using an $E^{1\%}$ equal to 11.4 $cm^{-1}$ for profilin 1, 7.9 $cm^{-1}$ for cofilin 1, and 9.8 $cm^{-1}$ for twinfilin.

Rabbit skeletal muscle actin ($\alpha$-actin) was prepared from acetone-dried muscle powder according to the method of Spudich and Watt.[47] G-Actin was stored in buffer containing 2 mM Tris-HCl (pH 8.0), 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM $\beta$-mercaptoethanol, and 0.03% $NaN_3$ (buffer G). The actin molar concentration was determined by absorbance at 290 nm using an $E^{1\%}$ of 6.3 $cm^{-1}$ and a molecular mass of 42.3 kDa. The ATP−Ca−G-actin was transformed into the ATP−Mg−G-actin by a 3−5 min incubation with 0.2 mM EGTA and 0.1 mM $MgCl_2$ at 25 °C. To obtain the ADP−Mg−G-actin, the actin-bound ATP was converted into ADP by incubation of the


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ATP−Mg−G-actin with 0.8 mM ADP, 1 mM glucose, and hexokinase (8 units/mL) for 2 h at 4 °C.[48] It is known that, under similar conditions, only ∼0.4% of ATP was determined in the actin samples after incubation for 1 h with glucose and hexokinase.[49]

**Differential Scanning Calorimetry.** DSC experiments were performed on a DASM-4M differential scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia) as described previously.[41,44,45] All measurements were taken at a scanning rate of 1 K/min in 2 mM Hepes (pH 7.6) containing 0.2 mM $CaCl_2$ or $MgCl_2$ (in the case of Ca-bound G-actin or Mg-bound G-actin, respectively) and 0.2 mM ATP or 0.2 mM ADP (in the case of the ATP−G-actin or ADP−G-actin, respectively). The reversibility of the thermal transitions was assessed when the sample was reheated immediately after being cooled from the previous scan. The thermal denaturation of all protein samples was fully irreversible. Calorimetric traces were corrected for instrumental background and possible aggregation artifacts by subtracting the scans obtained from the reheating of the samples. The temperature dependence of the excess heat capacity was further analyzed and plotted using Origin (MicroCal, Northampton, MA). The thermal stability of actin was described by the $T_m$, and the calorimetric enthalpy was calculated as the area under the excess heat capacity function.

**Analytical Ultracentrifugation.** Sedimentation velocity experiments were conducted at 22 °C in a model E analytical ultracentrifuge (Beckman) equipped with absorbance optics, a photoelectric scanner, a monochromator, and a computer on line. A four-hole An-F Ti rotor and 12 mm double sector cells were used. The rotor speed was 56000 rpm. The sedimentation profiles of G-actin and G-actin−profilin complexes were recorded by measuring the absorbance at 285 nm. All cells were scanned simultaneously. The time interval between scans was 2.5 min. The sedimentation coefficients were estimated from the differential sedimentation coefficient distribution [$c(s)$ vs $s$] using SEDFIT.[50]

## RESULTS

**Stabilization of G-Actin by Cofilin and Twinfilin.** Prior to these experiments, the ATP−Ca−G-actin was first converted into the ATP−Mg−G-actin, and then the bound ATP was replaced with ADP as described in Experimental Procedures. Substitution of $Ca^{2+}$ with $Mg^{2+}$ was used in this case because successful replacement of ATP with ADP is only possible with Mg-bound G-actin.[48] Thus, in all DSC experiments presented in this section, we compared the effects of ABPs on the thermal stability of ATP−G-actin and ADP−G-actin containing $Mg^{2+}$ as a bound cation. Previous studies have shown that the replacement of $Ca^{2+}$ with $Mg^{2+}$ decreases the melting temperature of the ATP−G-actin.[38,45]

Figure 1 summarizes and compares the effects of cofilin (mammalian cofilin-1 isoform) and twinfilin (mammalian twinfilin-1 isoform) on the thermal unfolding of the ATP−Mg−G-actin (Figure 1A) and the ADP−Mg−G-actin (Figure 1B). Cofilin strongly stabilizes ATP−Mg−G-actin and ADP−Mg−G-actin. In good agreement with previous DSC studies of ATP−Ca−G-actin,[41,42] cofilin increases the thermal stability of the ATP−Mg−G-actin by shifting the actin thermal transition by 6 °C (from 56.6 to 62.6 °C) (Figure 1A). An even more pronounced effect of cofilin is observed with the ADP−Mg−G-actin (Figure 1B). In this case, when G-actin is much less thermostable, cofilin binding increases the $T_m$ of its thermal

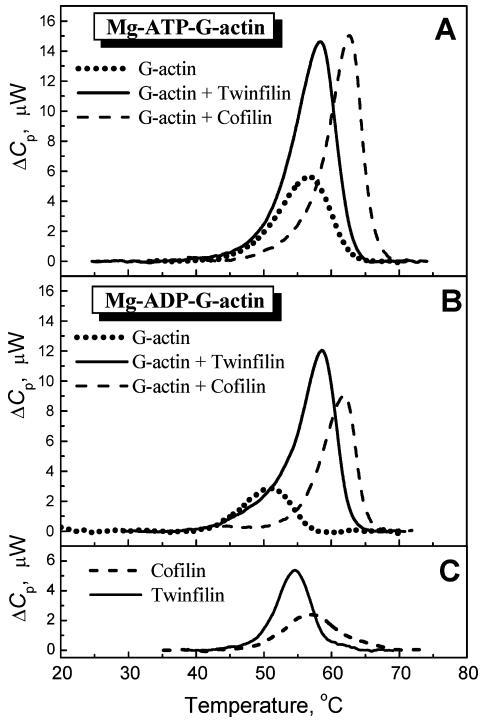


**Figure 1.** Temperature dependencies of the excess heat capacity ($\Delta C_p$) of ATP−Mg−G-actin (A) and ADP−Mg−G-actin (B) in the absence and presence of cofilin-1 or twinfilin-1. The concentration of actin was constant (15 $\mu$M), and the molar concentrations of cofilin and twinfilin in the complexes were 15 $\mu$M. (C) DSC curves of cofilin (15 $\mu$M) and twinfilin (15 $\mu$M) in the absence of actin. The heating rate was 1 K/min.

transition by 11 °C, from 50.8 to 61.8 °C. Importantly, in the complexes with cofilin, the thermal transition of the ADP−G-actin becomes similar to that observed for the ATP−G-actin (Figure 1A,B). In both complexes, actin and cofilin are mutually stabilized and denature as a unit, resulting in a new sharp thermal transition with a maximum at ∼62 °C.

Unlike cofilin, twinfilin has no significant effect on the thermal stability of the ATP−G-actin as it increases the $T_m$ of the G-actin thermal transition by only 1.7 °C, from 56.6 to 58.3 °C, although its binding to G-actin strongly increases the enthalpy of the transition (the area under the heat sorption curve) (Figure 1A). This suggests that in the ATP−G-actin−twinfilin complex both proteins may denature together as a single unit, although almost at the same temperature as the ATP−G-actin itself. However, by contrast with the ATP−G-actin, twinfilin is able to stabilize the ADP−G-actin that is much less thermostable than the ATP−G-actin (Figure 1B). In this case, we observed a new calorimetric peak at 58.6 °C (Figure 1B), which is very similar to the peak of the ATP−G-actin in the presence of twinfilin ($T_m$ = 58.3 °C) (Figure 1A). This suggests that twinfilin strongly stabilizes the ADP−G-actin, making its thermal unfolding very similar to that characteristic of the ATP−G-actin.

**Destabilization of the ATP−Ca−G-Actin by Profilin.** The experiments with the ATP−G-actin complexes with profilin were conducted using G-actin containing $Ca^{2+}$ as a bound cation for the following reasons. First, in most cases, solution experiments with actin−profilin complexes were performed with the muscle ATP−Ca−G-actin,[14,16,17,19,51−54] and it was shown that the affinity of profilin for the ATP−Ca−


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G-actin and ATP−Mg−G-actin is very similar.[14,51] Second, previous studies of $\beta/\gamma$-G-actin showed that profilin binding similarly decreases the thermal stability of the ATP−Ca− and ATP−Mg−G-actins.[38]

Figure 2A shows the results from DSC experiments on the ATP−Ca−G-actin complexes with profilin-1 obtained at different profilin:actin molar ratios, from 0.125 to 1.5. At ratios of 1.5:1 and 1:1, we observed on the DSC profiles the appearance of a new peak with a maximum at a temperature lower than those for the isolated ATP−G-actin (61 °C) or profilin (56 °C). The maximum of this peak shifts to a higher temperature when the profilin:actin molar ratio decreases from 1.5 to 0.125. At the highest profilin:actin molar ratio (1.5:1), the maximum of this peak was observed at 52.5 °C; at a 1:1 ratio, the $T_m$ was equal to 53.7 °C, and a further decrease in the profilin:actin molar ratio was accompanied by an increase in the $T_m$ of this peak, up to 58.5 °C at the very low molar ratio of 0.125:1 (Figure 2A). The dependence of the $T_m$ value for the peak of profilin-destabilized G-actin on the profilin:actin molar ratio is shown in Figure 2B. According to predictions,[55] such type dependence suggests that at any time the system can be in rapid dynamic equilibrium between two states, the profilin−actin complex and the mixture of free proteins.

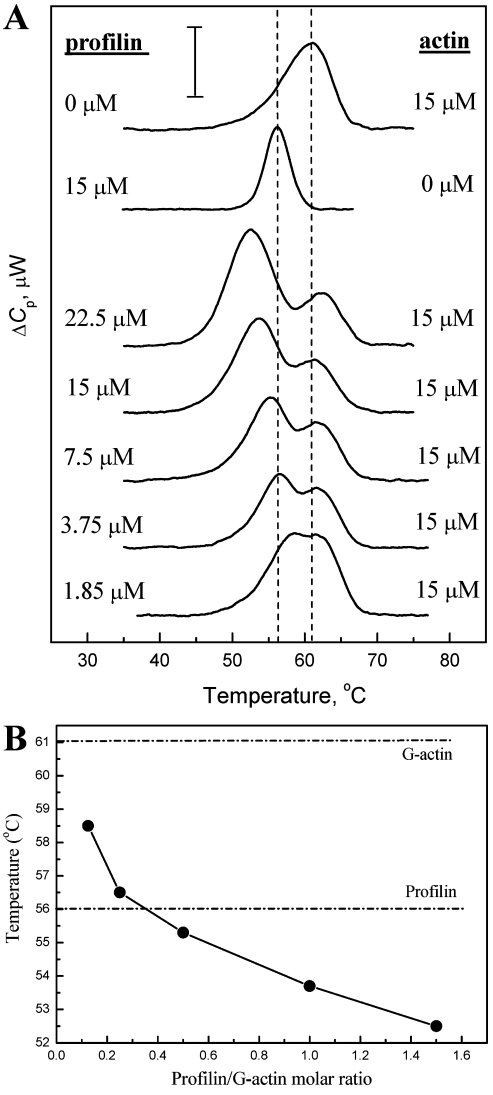


**Figure 2.** Destabilizing effects of profilin on the thermal unfolding of the ATP−Ca−G-actin. (A) DSC curves of the ATP−G-actin, profilin-1, and their mixtures obtained at different profilin:actin molar ratios. The concentration of actin was constant (15 $\mu$M); molar concentrations of profilin varied as indicated on each curve: 22.5 $\mu$M (profilin:actin molar ratio of 1.5:1), 15 $\mu$M (1:1), 7.5 $\mu$M (0.5:1), 3.75 $\mu$M (0.25:1), and 1.85 $\mu$M (0.125:1). Vertical dashed lines indicate transition temperatures for the ATP−G-actin (61 °C) and profilin (56 °C). The heating rate was 1 K/min. The vertical bar corresponds to 5 $\mu$W. (B) Dependence of the maximal temperature ($T_m$) of the peak of the profilin-destabilized ATP−G-actin on the profilin:actin molar ratio. The $T_m$ values were determined from panel A.

Also, another interesting feature of the actin−profilin complexes was observed from their DSC curves, the presence of a pronounced peak at 61−62 °C (Figure 2A). This peak can be clearly and separately observed by using the previously described "successive annealing" procedure.[56] After irreversible denaturation of profilin-destabilized G-actin by heat treatment of the equimolar actin−profilin complex to 52.5 °C and following cooling, only a thermal transition with a $T_m$ of 61.7 °C was observed on the DSC profile during reheating (Figure 3). This transition corresponds to thermal denaturation of actin that is free of profilin (see Figure 2A), and its calorimetric enthalpy (200 ± 20 kJ/mol) represents more than 40% of that for initially added G-actin (460 ± 40 kJ/mol). This suggests that, under the conditions used, ~60% of G-actin molecules are destabilized by profilin in the actin−profilin complexes, whereas other actin molecules (~40%) remain free of profilin and denature independently of the presence or absence of profilin. Several possible reasons can be proposed to explain this surprising effect, including the fact that the complex of actin with profilin cannot be fully formed even at room temperature (e.g., because of rapid dynamic equilibrium or the presence of very short actin oligomers in the G-actin preparation). Such short oligomers can be formed from actin dimers with nucleation activity, which have been identified in solutions of

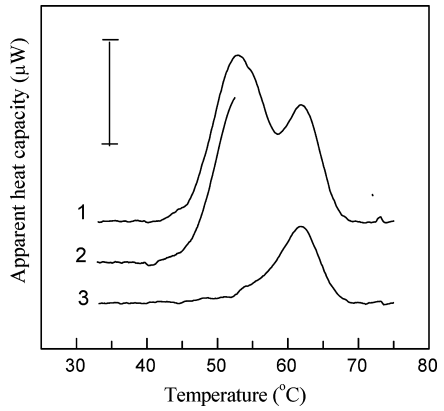


**Figure 3.** Separation of the calorimetric peak of free actin from profilin-destabilized G-actin by the successive annealing procedure. First, the DSC curve of the equimolar actin−profilin complex (20 $\mu$M actin and 20 $\mu$M profilin) was measured (curve 1). Then, a separate sample of the complex was heated to 52.5 °C (curve 2), cooled, and again heated to 75 °C. Only the thermal transition of free G-actin ($T_m$ = 61.7 °C) was observed on the DSC profile during reheating (curve 3). This approach allows estimation, at least approximately, of the content of free actin in the actin−profilin complex. The vertical bar corresponds to 5 $\mu$W. Other conditions were as described for Figure 2A.


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G-actin prepared from muscle acetone powder.[57] Another possible explanation is that the actin−profilin complexes dissociate upon being heated.

We performed additional experiments to examine possible reasons for the presence of the free actin peak on the DSC profiles of actin−profilin complexes. First, we applied analytical ultracentrifugation to analyze G-actin preparation for the presence of short actin oligomers, which cannot bind profilin. Careful analysis of differential sedimentation coefficient distribution $c(s)$ (Figure 4) revealed, besides the main peak

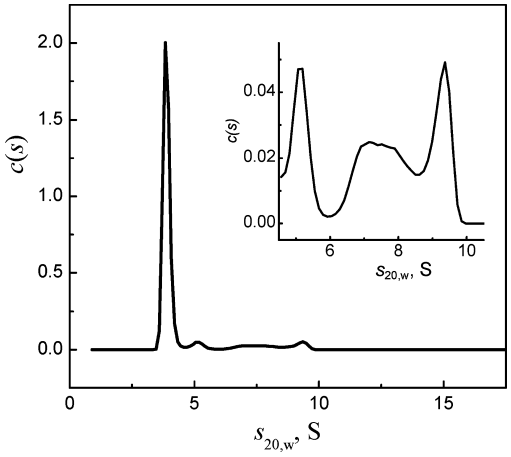


**Figure 4.** Sedimentation velocity analysis of G-actin (20 $\mu$M). Differential sedimentation coefficient distribution $c(s)$ was obtained at 22 °C and converted to standard conditions ($s_{20,w}$). The rotor speed was 56000 rpm. Other conditions were as follows: 2 mM Hepes (pH 7.6) containing 0.2 mM $CaCl_2$ and 0.2 mM ATP. The inset shows an expanded view of the region from 4.5 to 10.5 S.

of monomeric G-actin with an $s_{20,w}$ of 3.9 S, several small actin oligomers with $s_{20,w}$ values of 5, 7−8, and 9.3 S (Figure 4, inset). These $s_{20,w}$ values roughly correspond to apparent molecular masses from ∼70 to ∼170 kDa. However, the total content of these short actin oligomers (probably no longer than tetramers) did not exceed 10% of actin molecules in the G-actin preparation, and therefore, they by no means can account for a large content of free actin molecules in the profilin−actin complexes. Thus, it seems more likely that this effect observed by DSC (Figures 2A and 3) results from the rather low affinity of profilin for G-actin.

It should be noted that literature data on the affinity of mammalian profilins for the muscle ATP−Ca−G-actin are contradictory, and the equilibrium dissociation constants for profilin−actin complexes measured by different methods vary within a wide range, from 0.1−0.2 $\mu$M[2,14,51−53] to 0.6 $\mu$M[54] or even 3.0−6.0 $\mu$M.[16,58,59] If the $K_d$ of this interaction is ≥3 $\mu$M, the fraction of the profilin-free actin in the equimolar mixture of these proteins should exceed 30% even at room temperature. To examine this assumption, we applied analytical ultracentrifugation to study sedimentation properties of actin−profilin complexes in comparison with those of free actin and profilin (Figure 5). Consistent with a previous report,[51] the complex of G-actin with profilin displayed a peak with an $s_{20,w}$ of 4.4 ± 0.3 S that was independent of the actin:profilin molar ratio (Figure 5C−E), which could not be separated from the peak of free G-actin with an $s_{20,w}$ of 3.9 ± 0.1 S (Figure 5A). However, we also observed a peak corresponding to free profilin with an $s_{20,w}$ of 2.5 ± 0.2 S (Figure 5B−E), and the area

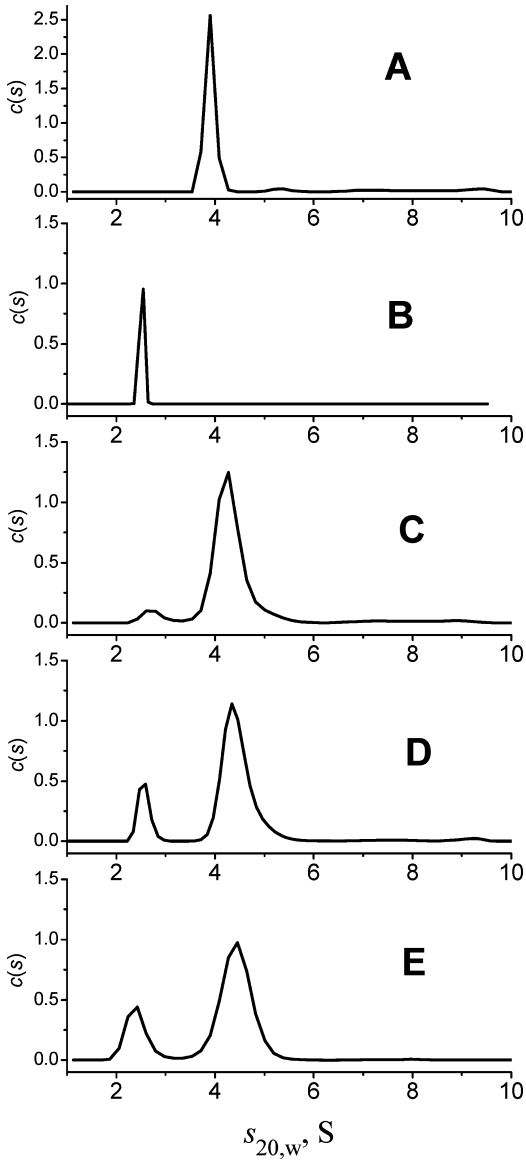


**Figure 5.** Sedimentation velocity analysis of the ATP−Ca−G-actin (20 $\mu$M) (A), profilin-1 (20 $\mu$M) (B), and their mixtures obtained at different profilin:actin molar ratios. The concentration of actin was constant (20 $\mu$M); molar concentrations of profilin in the complexes were 10 (C), 20 (D), and 30 $\mu$M (E). Differential sedimentation coefficient distribution $c(s)$ was obtained at 22 °C and converted to standard conditions ($s_{20,w}$). Other conditions were as described in the legend of Figure 4.

under this peak increased with an increase in the profilin concentration (Figure 5C−E). According to simple calculations, almost half of profilin remained unbound to actin even in the equimolar mixture of the proteins, thus indicating the presence of profilin-free actin in the samples. This may explain the presence of the peak of free G-actin on the DSC profiles of actin−profilin complexes (Figures 2A and 3). Overall, these results confirm that mouse profilin-1 has a micromolar affinity for the muscle ATP−Ca−G-actin,[16,58,59] which is much lower than that often reported in the literature (0.1−0.2 $\mu$M).[2,14,51−53] Most importantly, however, the DSC results (Figure 2) clearly demonstrate the destabilizing effect of profilin on the ATP−Ca−G-actin.


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**Interaction of Profilin with the ADP−Mg−G-Actin.** DSC experiments with profilin complexes with the ADP−G-actin were performed with actin containing $Mg^{2+}$ as a bound cation (as mentioned above, successful replacement of ATP with ADP is only possible with the Mg−G-actin[48]). In contrast to the ATP−Ca−G-actin that was destabilized by profilin (Figure 2), the binding of profilin to the ADP−Mg−G-actin did not have an appreciable effect on its thermal stability (Figure 6A). In the presence of profilin, the thermal transition of the

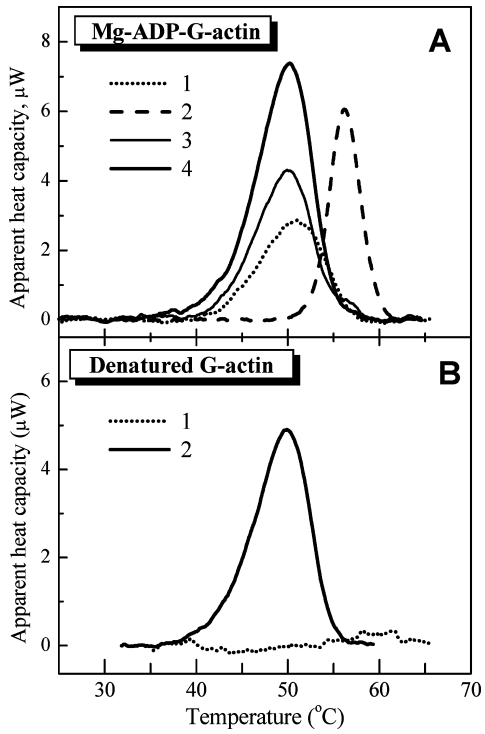


**Figure 6.** DSC curves of the ADP−Mg−G-actin (A) and denatured G-actin (B) in the absence (curves 1 shown by dotted lines) and presence of profilin. (A) The concentration of the ADP−Mg−G-actin was constant (15 $\mu$M); molar concentrations of profilin were 15 (curve 3) or 30 $\mu$M (curve 4). Curve 2 corresponds to profilin (15 $\mu$M) in the absence of actin. Other conditions: 2 mM Hepes (pH 7.6), 0.2 mM $MgCl_2$, and 0.2 mM ADP. (B) G-Actin (15 $\mu$M) was denatured by being preheated at 60 °C for 40 min, and its DSC curves were obtained in the absence of profilin (curve 1) and in the presence of 15 $\mu$M profilin (curve 2).

ADP−G-actin shifts by only 1 °C toward a lower temperature, from 51 to 50 °C (in Figure 6A, compare curves 1 and 3). It should be noted that the peak at 56 °C corresponding to free profilin (Figure 6A, curve 2) fully disappears in the presence of the ADP−G-actin. This suggests that upon interaction of profilin with the ADP−G-actin both proteins may destabilize each other and melt together, thus giving the thermal transition at 50 °C. Surprisingly, the same transition at 50 °C was observed in the presence of a 2-fold molar excess of profilin over actin (Figure 6A, curve 4) without the appearance of the peak at 56 °C corresponding to free profilin. In this case, an increase in the profilin concentration only elevates the area of the thermal transition at 50 °C by ~60% (in Figure 6A, compare curves 3 and 4). These results suggest that profilin can interact with denatured actin and this interaction leads to its destabilization. To examine this assumption, we analyzed by DSC the equimolar mixture of profilin with preliminary denatured G-actin obtained after a 40 min incubation at 60 °C. It was shown that such type-denatured G-actin (so-called "inactivated" G-actin) is represented by stable homogeneous aggregates (or "monodisperse associates") consisting of a limited number of unfolded protein molecules (up to 15−17 molecules).[60,61] The results showed that denatured (inactivated) G-actin does not display any cooperative thermal transition on the DSC profile (Figure 6B, curve 1), whereas in the presence of profilin, the transition at 50 °C is clearly observed (Figure 6B, curve 2), very similar to those observed for profilin in the presence of the ADP−G-actin (Figure 6A, curves 3 and 4). These results confirm our assumption that upon heating nonspecific interaction of profilin with fully or partially denatured actin occurs, which significantly decreases the thermal stability of profilin. Thus, profilin, when bound to the ADP−Mg−G-actin, has no significant effect on its thermal stability; on the other hand, the thermal stability of profilin strongly decreases when it is nonspecifically and nonstoichiometrically bound to fully or partially denatured actin during the heating.

## DISCUSSION

The data presented here show that cofilin, when bound to muscle $\alpha$-G-actin, increases the thermal stability of both ATP−G-actin and ADP−G-actin, whereas twinfilin stabilizes the ADP−G-actin but not the ATP−G-actin. By contrast, profilin binding decreases the thermal stability of the ATP−G-actin but has no appreciable effect on the ADP−G-actin. Comparing these DSC results with literature data on the rate of nucleotide exchange on G-actin, we assume that the effects of different ABPs on the thermal stability of muscle actin, increase or decrease, are associated with conformational changes in the actin molecule leading to closing or opening of the nucleotide-binding cleft.

Cofilin and twinfilin effectively inhibit the nucleotide exchange on the ATP−$\alpha$-G-actin.[9,12,18−20] Furthermore, the crystal structure of the ATP−$\alpha$-G-actin complexes with the C-terminal ADF-H domain of twinfilin suggests that binding of the ADF-H domain of either ADF/cofilin or twinfilin to the hydrophobic cleft between actin subdomains 1 and 3 may "lock" the nucleotide-binding cleft in a closed conformation.[27] However, we observed some difference between cofilin and twinfilin in their effects on the thermal stability of the ATP−$\alpha$-G-actin (Figure 1A). Unlike cofilin, which significantly increases the ATP−G-actin thermal stability, twinfilin has no appreciable stabilizing effect when bound to the ATP−G-actin. It seems likely that twinfilin only locks the nucleotide-binding cleft in its closed conformation, whereas cofilin may induce further closure of the cleft (e.g., it may promote the transition of the cleft into its "superclosed" state recently suggested in the ATP−G-actin by molecular dynamics simulations[62]). The difference in the effects of cofilin and twinfilin on the thermal stability of the ATP−G-actin as revealed by DSC (Figure 1A) suggests that these proteins may have slightly different binding sites on G-actin. On the basis of the structure of the ATP−$\alpha$-G-actin complex with the C-terminal ADF-H domain of twinfilin,[27] the structural homology between cofilin and twinfilin,[63] and the competitive actin binding of twinfilin against cofilin,[12] both these proteins were suggested to bind to G-actin at the cleft between subdomains 1 and 3.[63] Nevertheless, the DSC data presented here (Figure 1A) indicate that there is some difference between cofilin and twinfilin in their interaction with the hydrophobic cleft between subdomains 1


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and 3, which may lead to different conformational changes in the nucleotide-binding cleft between subdomains 2 and 4.

The effects of cofilin and twinfilin on the nucleotide exchange on the ATP−G-actin have been investigated by many authors, and cofilin has been shown to inhibit the nucleotide exchange also on the ADP−G-actin.[64] However, similar data with respect to the ADP−G-actin are lacking for twinfilin, as well as atomic-resolution structural data for the complexes of ADP−G-actin with these proteins. In this case, valuable information can be provided by DSC results showing that both cofilin and twinfilin strongly increase the thermal stability of the ADP−α-G-actin, which is much less thermostable than the ATP−α-G-actin (Figure 1B). It is important to note that in the complexes with cofilin or twinfilin the thermal transition of the ADP−G-actin becomes similar to that observed for the ATP−G-actin in the complexes with these proteins (compare panels A and B of Figure 1). These results suggest that both proteins induce the closure of the nucleotide-binding cleft, which is initially open in the ADP−G-actin. In a manner independent of the bound nucleotide, ATP or ADP, twinfilin locks the cleft in its closed conformation characteristic of the ATP−G-actin, whereas cofilin induces further closure of the cleft.

In contrast to cofilin and twinfilin that inhibit nucleotide exchange on the ATP−G-actin, most profilins enhance nucleotide exchange on both α- and β/γ-actin isoforms, with either $Ca^{2+}$ or $Mg^{2+}$ as a bound cation.[14−19] According to recent views, binding of profilin to the hydrophobic cleft produces a 4.7° rotation of the two major domains of actin relative to each other, and this rotation is independent of the source of the actin.[36] As a result, the nucleotide-binding cleft becomes moderately more open in the profilin−actin complex,[26,36] and in some cases, under specific conditions, even a wide-open cleft was observed on profilin−β-actin structures.[24,26] In good agreement with these literature data, DSC results show that profilin decreases, in a concentration-dependent manner, the thermal stability of the ATP−α-G-actin (Figure 2). On the other hand, binding of profilin to the ADP−α-G-actin has no significant effect on its thermal stability (Figure 6A). This suggests that profilin is unable to produce further opening of the nucleotide-binding cleft, which is already open in G-actin with ADP as a bound nucleotide. It is noteworthy that destabilization of the ADP−G-actin by profilin was also not shown by the DNase-I inhibition assay, the method that is based on the ability of native G-actin to inhibit the activity of DNase-I.[38] Moreover, unlike our DSC results (Figure 6A), some profilin-induced increase in the thermal stability of the ADP−Mg−G-actin was observed by this method. This disagreement might be explained by our finding that profilin can be significantly destabilized by its nonspecific binding to fully or partially denatured actin (Figure 6B). As a result, the ADP−Mg−G-actin (including those actin molecules that may be stabilized by profilin) and profilin destabilized by denatured actin melt within the same temperature region (from 45 to 55 °C) and their thermal transitions cannot be separated on the DSC profile. Besides this, it should be noted that the experiments with the DNase-I inhibition assay were performed on non-muscle β/γ-actin,[38] whereas our DSC results were obtained with muscle α-actin.

The mechanism of thermal unfolding of profilin−G-actin complexes is very complicated and therefore deserves special attention. First, the results of sedimentation experiments (Figure 5) corroborate the data that show that the affinity of mammalian profilin for the muscle ATP−Ca−G-actin is rather low,[16,58,59] much lower than often reported in the literature.[2,14,51−53] A low affinity of profilin for the ATP−α-G-actin can explain both the presence of the free actin peak on the DSC profiles of actin−profilin complexes and the dependence of the thermal unfolding of profilin-destabilized G-actin on the profilin:actin molar ratio (Figure 2). (Interestingly, in the case of cofilin, any dependence of the thermal stability of the α-G-actin−cofilin complexes on the cofilin:actin molar ratio was not observed in DSC experiments.[42]) Second, the results of molecular dynamics simulations showed that a wide-open nucleotide-binding cleft is structurally and thermodynamically unstable, and the cleft immediately (within 200 ps) closes when profilin dissociates.[65] Thus, in the presence of profilin, the actin molecules seem to be in dynamic equilibrium between two conformational states, with an open or closed nucleotide-binding cleft. It seems most likely that this equilibrium is temperature-dependent, and it may be significantly changed upon heating. Finally, the finding that profilin can nonspecifically interact with denatured actin (Figure 6B) makes the understanding of the thermal unfolding process for the profilin−actin complexes even more complicated. In any case, some intriguing aspects of the events occurring in the actin−profilin complexes upon their heating (such as the dependence of the thermal unfolding of these complexes on profilin concentration, etc.) remain unclear and deserve additional research.

In conclusion, the results presented here support the assumption that the thermal stability of G-actin depends on the conformational state of the nucleotide-binding cleft, more open or more closed. The actin molecule is stable when the cleft is closed, while an opening of the cleft leads to significant destabilization of G-actin. Thus, DSC studies of the thermal unfolding of G-actin can provide new valuable information about the conformational changes induced by actin-binding proteins in the actin molecule.

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### Notes

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## ABBREVIATIONS

ABP, actin-binding protein; ADF, actin depolymerizing factor; ADF-H, ADF homology; DSC, differential scanning calorimetry; $T_m$, thermal transition temperature; Twf-C, C-terminal ADF-H domain of twinfilin.

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