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EFFECT OF PHALLOIDIN ON THE SKELETAL MUSCLE ADP-ACTIN FILAMENTS

Réka Dudás, Tünde Kupi, Andrea Vig, J. Orbán and D. Lőrinczy[*]

University of Pécs, Medical School, Department of Biophysics, Szigeti str. 12, Pécs 7624, Hungary

The effect of phalloidin on the thermal stability of skeletal actin filaments polymerized from ADP-binding monomers was investigated with the method of differential scanning calorimetry. Phalloidin shifted the melting temperature of the ADP-F-actin from 59.1±1.0 to 80.0±1.2°C. The stabilizing effect of phalloidin propagated cooperatively along the filament. The cooperativity factor according to the applied model was 1.07±0.11. With these measurements it was possible to demonstrate that the binding of phalloidin has lower influence on the adjacent protomers in ADP- (k=1) than in ATP-actin filaments (k=3).

Keywords: *ADP-actin filament, calorimetry, phalloidin, stability, thermodynamics*

Introduction

Actin filaments play an important role in the formation of cytoskeleton, and in the muscle contraction as well [1, 2]. Actin filaments (F-actin) are composed from globular monomers (G-actin). One monomer can bind an ATP and a divalent magnesium ion in its central cleft under physiological conditions [3]. The quality of the bound cation can influence the properties of the actin monomer and filament as well [4–8]. The ATP is hydrolyzed during the polymerization of the actin monomers. The final product of this hydrolysation is an inorganic phosphate (P_i) which dissociates from the actin filaments and an ADP molecule remains bound to the protomer. Filaments can be formed from ADP-binding monomers under special, ATP-depleted circumstances (e.g. fatigue, ischemia) [9]. The structure and dynamics of the ADP-actin monomers differs from the ATP-binding ones [10–13]. The filaments formed by the ADP-binding actin monomers are more flexible than the filaments constructed from ATP-binding monomers [11, 14].

Phalloidin is a bicyclic heptapeptide, which is the product of the highly poisonous Amanita phalloides [15, 16]. The cytotoxic drug of this mushroom can bind to F-actin with high affinity (K_d=30–36 nM) [17], while it does not bind to the actin monomers. Phalloidin can stabilise actin filaments even at low concentration [18, 19]. The toxin strongly decreases the actin's critical concentration and accelerates the nucleation and the rate of polymerization as well [20]. Several workgroups used the phalloidin to stabilise the structure of the actin filament [21–26]. Previous calorimetric measurements showed that actin filaments have higher melting temperature when the filaments were prepared from ATP bound monomers (63°C) compared to the ADP-actin filaments (57°C) [12, 13]. These observations indicated that the DSC method can be effectively used to study the thermal properties of actin in details [27–38].

The aim of this study was to investigate the effect of phalloidin on the thermodynamic properties of actin filaments prepared from skeletal ADP-actin monomers by differential scanning calorimetry (DSC). The use of phalloidin made it possible to explore differences between the cooperative properties of ADP- and ATP-actin filaments. The results showed that the cooperativity within the actin filaments prepared from ADP containing monomers is less pronounced compared to the filaments prepared from the ATP saturated monomeric actin molecules.

Experimental

Materials

Chemicals

KCl, $MgCl_2$, $CaCl_2$, *D*-glucose, hexokinase, EGTA, MOPS and phalloidin were purchased from Sigma-Aldrich (Budapest, Hungary). ATP, ADP and β-mercaptoethanol were obtained from Merck (Darmstadt, Germany). NaN_3 was purchased from Fluka (Lausanne, Switzerland).

* Author for correspondence: denes.lorinczy@aok.pte.hu

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Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

Sample preparation

Skeletal actin was prepared from acetone powder of rabbit psoas muscle [39, 40]. The calcium saturated actin monomers were stored in a 2 mM MOPS buffer (pH 8.0) containing 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM β-mercaptoethanol and 0.005% NaN_3. The concentration of the actin monomers was calculated by using the extinction coefficient of 0.63 mL mg^{-1} cm^{-1} at 290 nm [41].

The exchange of the bound ATP on actin monomer for ADP was done by incubating the protein in the presence of 1.65 mg mL^{-1} hexokinase, 0.5 mg mL^{-1} glucose and 1 mM ADP for 1 h at 0°C.

The bound calcium ion (Ca^{2+}) on the actin monomers was changed for magnesium by incubating the samples for 5 min in the presence of 0.2 mM EGTA and 0.1 mM $MgCl_2$ at room temperature [3]. The actin monomers were polymerized by increasing the $MgCl_2$ concentration to 2 mM and the KCl to 100 mM at room temperature for 6 h [42]. After polymerization, the filaments were stored overnight at 0°C.

To create phalloidin bound F-actin we added the toxin to the sample after the nucleotide exchange, at the same time when the polymerization was started. To get a satisfactory polymerization on ADP-actin monomers, the elapsed time of polymerization was always 6 h at room temperature.

DSC measurements and theoretical considerations

The thermodynamic investigation of ADP-bound actin filaments was performed with a Setaram Micro DSC-II calorimeter. The DSC measurements were done in the temperature range of 0–100°C and the heating rate was 0.3 K min^{-1}. For the DSC measurements the actin concentration was adjusted to 69 μM (3 mg mL^{-1}). The experimental buffer with no protein content was used as a reference in the DSC experiments and for baseline correction during the data processing. The sample and the reference solution were heated in the range of 0 to 100°C under isobaric conditions. The difference between the energy uptake of the sample and the reference cell was recorded in the function of temperature.

The data were analyzed with the Microcal™ Origin software (version 7.5). We applied a model described by Visegrády *et al*. [19] to analyze the phalloidin concentration dependence of the actin filament's DSC data to define the degree of cooperativity along the structure of the polymer. The model assumes that phalloidin can stabilise the conformation of the directly bound protomer. In the case of cooperative binding, phalloidin can also stabilise adjacent protomers as the effect of the toxin can propagate along the actin filaments. The number of actin protomers influenced by one phalloidin molecule can be determined with this model. We used the following equation to analyze how the ratio of the actin population that was unaffected by phalloidin (A) changed due to the increasing phalloidin concentration:

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

where p is the probability that an actin protomer in the filament binds phalloidin and k is the cooperativity factor related to the size of the cooperatively influenced actin segment.

The denaturation curves at certain phalloidin concentrations can be divided into 3 different regions. These regions can be related to different protein fractions in the sample solution. The peak with the lowest T_m value belongs to that protein fraction which is not affected by the phalloidin while the peak that is represented by the highest T_m value is belonging to the phalloidin bound part of the actin filament. In the presence of non-saturating phalloidin concentration a third peak can be identified with an in-between T_m value. This fraction represents that part of the protein that is not bound the toxin molecule but indirectly affected by it through long range allosteric interactions. The value of A can be determined by the under-curve area of the denaturation peak of the actin molecules that are not affected by phalloidin relative to the whole under-curve area of the heath transition curve. The peaks were approximated with Gaussians during the data analysis process.

The value of k can be determined by fitting the above equation to the experimental data, so the number of actin protomers affected by one phalloidin molecule can be calculated as $2k+1$ [19].

Results and discussion

In the present study we investigated the effect of phalloidin on the thermodynamic properties of ADP-actin filaments. During the calorimetric experiments the actin was used in 69 μM concentration. The samples were prepared from skeletal ADP-actin monomers in the presence of 2 mM $MgCl_2$ and 100 mM KCl. The contribution of the G-actin content to the results was very small during the measurements as the critical concentration of ADP-actin is around 1 μM [14]. The maximum effect on the DSC curves that was caused by the monomers was less than 2% so the impact of the actin monomers on the heat denaturation curve was considered negligible.

The denaturation curve can be characterized by the melting temperature (T_m), i.e. the temperature where the heat transition curve reaches its maximal amplitude. Comparing the different T_m values it is possible to characterize the thermodynamic stability of the

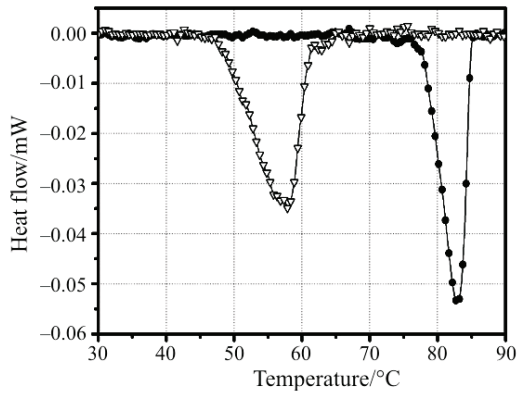

Fig. 1 The recorded DSC denaturation curve of ADP-F-actin (69 μM) ▽ – without phalloidin, and ● – in the presence of 69 μM phalloidin as well (phalloidin:actin molar ratio=1:1, pH 7.3)

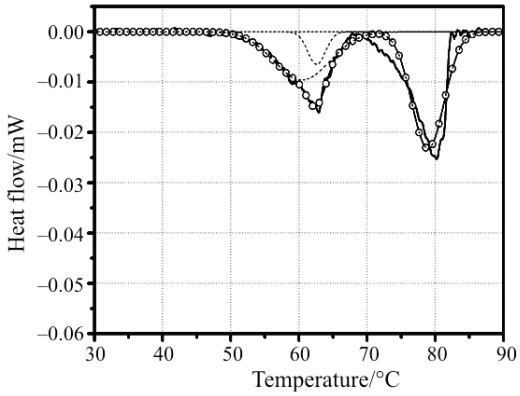

Fig. 2 DSC curve — – in the presence of 13.8 μM phalloidin (phalloidin:actin molar ratio=0.2:1), and ○ – Gaussian fit of the curve. --- – Gaussian fits of the different protein fractions

different forms of the proteins [43, 44]. Generally, high T_m values are related to thermodynamically stable protein conformations [43]. The position of the T_m values is basically independent from the applied thermodynamic model describing the denaturation process.

The DSC curve of the phalloidin-free sample of the ADP-actin filaments showed one peak with a T_m value of 58°C. This result is in good agreement with previous studies when the same shift to a smaller T_m value was observed compared to the ATP-actin filaments.

The effect of phalloidin was tested at different actin:phalloidin concentration ratios (0.2:1, 0.3:1, 0.5:1, 1:1). In the presence of phalloidin an extra peak appeared on the transition curves at 82.9°C (Fig. 1). This peak can represent the actin population that is directly stabilised by the toxin molecules. At the lower ratios of the applied toxin the peak that belongs to the lower T_m value became distorted due to the presence of protomers that were not binding phalloidin but were affected indirectly by the drug through allosteric interactions. To evaluate this situation, a previously successfully applied model was used. The model described by Visegrády and his colleagues used Eq. (1) to reveal the connection between the stabilizing effect of phalloidin and the change that was induced on the DSC curves. In this equation k is the size of the cooperatively influenced unit in the actin filament. A higher k value can be related to the higher degree of cooperativity. When k equals 0 then no cooperativity can be identified within the filament.

In our case Gaussian curves were assigned to the different populations during the fitting procedure. The three different clusters that were identified during the fitting procedure are the toxin bound protomers, the toxin free protomers not influenced by phalloidin and the toxin free protomers that are to some extent influenced by the phalloidin molecules (Fig. 2). The under-curve area of the toxin free protomers not influenced by phalloidin divided by the total under-curve area of the heat transition curve can give us A, that is needed for the fitting procedure (Fig. 3). The fitting of Eq. (1) to the data points could reveal that the value of k was 1.07±0.11 for the actin filaments prepared from ADP saturated actin monomers. This k value shows that the effect of one toxin molecule can be transferred to 3 protomers ($2k$+1) including the directly bound actin protomer as well. These results can clearly show that the cooperativity of the ADP-actin filaments is lower compared to ATP-actin.

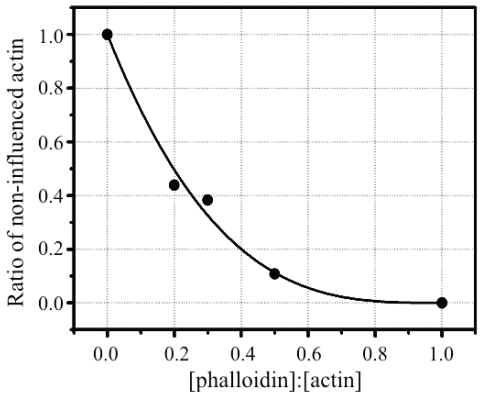

Fig. 3 The ratio of non-influenced actin protomers (A) in terms of the phalloidin:actin concentration ratio (k=1.07±0.11)

Conclusions

Our present results could confirm the previously shown fact that skeletal ADP-actin filaments are thermodynamically less stable than ATP-filaments. We also found that phalloidin could effectively stabilize the ADP-actin filaments as well. The binding of phalloidin to ADP-actin filaments induced cooperative

stabilization in the neighbourhood of the directly affected protomers. Our results showed that the size of the cooperatively affected region spreads over 3 protomers ($2k$+1) in ADP-filaments, which is smaller than 7 that was identified in the case of ATP-filaments.

The decreased cooperativity might indicate the decreased effectivity of the interprotomer interactions within the ADP-actin filaments. The decreased cooperativity of these filaments might be involved in the reduced function of the ATP depleted region of the muscle tissues.

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