THE EFFECT OF TOXINS ON THE THERMAL STABILITY OF ACTIN FILAMENTS BY DIFFERENTIAL SCANNING CALORIMETRY

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In our present study we performed the detailed characterisation of jasplakinolide and phalloidin on the thermal stability of actin filaments. The heat absorption curves were analysed by using the model established by Sanchez-Ruiz *et al*. [1]. The analysis provided the activation energies attributed to the heat denaturation of actin filaments in the absence and in the presence of toxins. The results indicated that there are kinetic differences between the toxin-mediated stabilization of the Ca^{2+}-and Mg^{2+}-actin filaments. The effect of toxins appeared to be cation dependent.

Introduction

Actin is one of the most abundant proteins, which can assemble into many different structures in cells [2–4]. The biological activity of actin is regulated by actin-binding proteins, which form a well-controlled regulatory network. During their interactions these biological partners of actin affect its behaviour and conformational properties. In vitro model systems were often applied to elucidate the properties of actin filaments. Previous studies involved the characterisation of the effect of the polymerisation [5, 6], nucleotides [7, 8] and the interaction of actin with myosin [9–13].

Phalloidin, isolated from *Amanita phalloides* mushroom, and jasplakinolide from the marine sponge *Jaspis johnstoni,* are actin-binding toxins, and used extensively for studying the conformational properties of actin filaments. These drugs competitively bind to filamentous actin with high affinity and causes the stabilization of the filaments [14–16]. Differential scanning calorimetry studies showed that phalloidin and jasplakinolide are also able to stabilize actin filaments at substochiometric concentration [17, 18].

It was in numerous cases that differential scanning calorimetry (DSC) is an effective method to study the thermal properties of actin [19–29]. In this work we used DSC to study the complexes of phalloidin and jasplakinolide with actin filaments (F-actin). Apart from the analyses of the heat absorption *vs.* temperature curves we also took into account the kinetic nature of the irreversible denaturation processes. Using the model developed by Sanchez-Ruiz *et al.* [1] we performed the detailed analysis of the

toxin-induced changes in the thermal stability of actin filaments. This method allowed us to determine the activation energies from the Arrhenius plots of the first-order rate constant of denaturation for both phalloidin and jasplakinolide stabilised actin filaments. The results were in agreement with previous observations that the stabilization of actin by these drugs is different. The differences appear not only in the extent of stabilization, but also in the kinetic properties of the toxin modified processes.

Materials and methods

Materials

KCl, $MgCl_2$, $CaCl_2$, EGTA, Tris and phalloidin were obtained from Sigma Chem Co. (St. Louis, MO, USA). Jasplakinolide was purchased from Molecular Probes (Eugene, OR, USA). ATP and 2-mercaptoethanol were obtained from Merk (Darmstadt, Germany). NaN_3 was purchased from Fluka (Switzerland).

Protein preparation

Aceton-dried powder from rabbit skeletal muscle was obtained as described previously [30]. Actin was prepared according to the method of Spudich and Watt [31] and stored in 2 mM Tris-HCl (pH 8.0), 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM MEA (buffer A). Before the measurements the actin monomer solution was clarified by ultracentrifugation at 100.000 g, 2 h, 4°C. The concentration of G-actin was determined spectro-

photometrically using the absorption coefficient of 0.63 mL mg^{-1} cm^{-1} at 290 nm [32].

Cation exchange in the actin monomer and polymerisation

After the preparation actin bound calcium at the high affinity cation-binding site. Ca^{2+}-bound G-actin was converted to Mg^{2+}-G-actin by adding MgCl$_2$ and EGTA to the protein solution to reach the final concentrations of 100 and 200 μM, respectively. The samples were incubated for 10 min at room temperature [33]. Mg^{2+}-G-actin was polymerised by the addition of 2mM MgCl$_2$, and 100 mM KCl (final concentrations) to the samples. The polymerisation was carried out overnight at 4°C prior to the DSC experiments.

Differential scanning calorimetry (DSC) experiments

The differential scanning calorimetry experiments were performed with a SETARAM Micro DSC-II calorimeter. All measurements were carried out between 0 and 100°C with a scanning rate of 0.3 K min^{-1}. Conventional Hastelloy batch vessels were used with 850 μL sample volume. The actin concentration was 3 mg mL^{-1} (69 μM) in the DSC experiments, and buffer A supplemented with 2 mM MgCl$_2$ and 100 mM KCl was used as a reference. The sample and reference vessels were equilibrated with a precision of ±0.1 mg mL^{-1}. Transition temperatures were determined from the maximum of thermal transition. The DSC curves were further analysed using Microcal Origin 6.0 software in order to obtain the activation energy values. The temperature scales were converted into time scales to get the heat absorption functions in the appropriate unit. The total enthalpy changes of the melting process (ΔH_{cal}) were calculated from the area under the heat absorption curves using baseline correction. From these values the $\ln[\ln(\Delta H_{cal}/(\Delta H_{cal} - \Delta H))]$ values were calculated and plotted as a function of the inverse of the experimental temperature. The linear part of these plots, between –3 and 1 values of the above expression (related to the peaks of the heat capacity curves) was fitted by a linear function to obtain optimal fittings. The activation energy values were determined as the product of the slopes of the Arrhenius plots and the gas constant (R). The

melting temperatures were also calculated from these plots, as a ratio of the y-intercept to the slope.

Results and discussion

The denaturation of a protein, i.e. its transition from a native to a denatured conformational state by either heat or by other means (e.g., chemical denaturants such as GuHCl) is a kinetic process. The kinetic nature of heat induced denaturation often disregarded in calorimetric studies. In these studies it is assumed that there is a thermodynamic equilibrium in the protein solutions at all temperatures during the temperature induced protein unfolding. Although this assumption is not fulfilled in many cases it was shown that the DSC results could be applied to determine the appropriate thermodynamic parameters even if the protein unfolding process is irreversible [34–36]. The model described by Sanchez-Ruiz and his colleagues [1] provided an alternative approach to obtain quantitative information from DSC results regarding protein denaturation. In this work we applied this method to characterise the interaction of actin filaments with different bivalent cations in their high affinity cation-binding sites with toxic peptides, phalloidin and jasplakinolide.

In DSC experiments actin filaments (69 μM) were heated from 0 to 100°C and the heat flow was recorded. Figure 1 shows the thermally induced heat flow changes in samples of actin filaments in the absence (Fig. 1A) and in the presence of equimolar ratio of phalloidin (Fig. 1B) and jasplakinolide (Fig. 1C). The DSC curves indicated a large thermal transition in the samples above ~60°C. In agreement with earlier studies we interpreted these transitions as the result of the heat induced denaturation of actin filaments. The temperatures where the heat flow was the largest (T_m) were determined from Fig. 1 and listed in Table 1.

The comparison of the results obtained with Ca^{2+}-F-actin and Mg^{2+}-F-actin shows that the replacement of the cation at the high affinity binding site introduced only little changes into the thermal denaturation profile of actin filaments. The largest cation effect was observed in the absence of toxins, but even in this case the difference between the T_m values characteristic for Ca^{2+}-F-actin and Mg^{2+}-F-actin was less

Table 1 The melting temperature (T_m) values determined in this study for Ca^{2+}- and Mg^{2+}-F-actin. The values are presented from experiments in the absence of toxins, or in the presence of either phalloidin or jasplakinolide. T_m^1 was determined directly from Fig. 1, while T_m^2 was obtained from the x-axis intercepts of Fig. 2. The x-axis intercept was calculated as the ratio of the y-axis intercept to the slope

	Ca-F-actin		Mg-F-actin	
Toxin	$T_m^1/°C$	$T_m^2/°C$	$T_m^1/°C$	$T_m^2/°C$
None	67.3±0.5	66.9	65.6±0.5	65.7
Phalloidin	79.3±0.6	78.2	78.4±0.6	78.5
Jasplakinolide	87.7±0.7	87.4	88.6±0.5	89.4

than 2°C under the applied conditions. In the light of these considerations we concluded that cation exchange had only minor effects on the heat stability of actin filaments. Previously it was shown by using spectroscopic methods that cations have substantial effect on the dynamic behaviour of actin monomers and filaments [37–40]. Our current findings indicate,
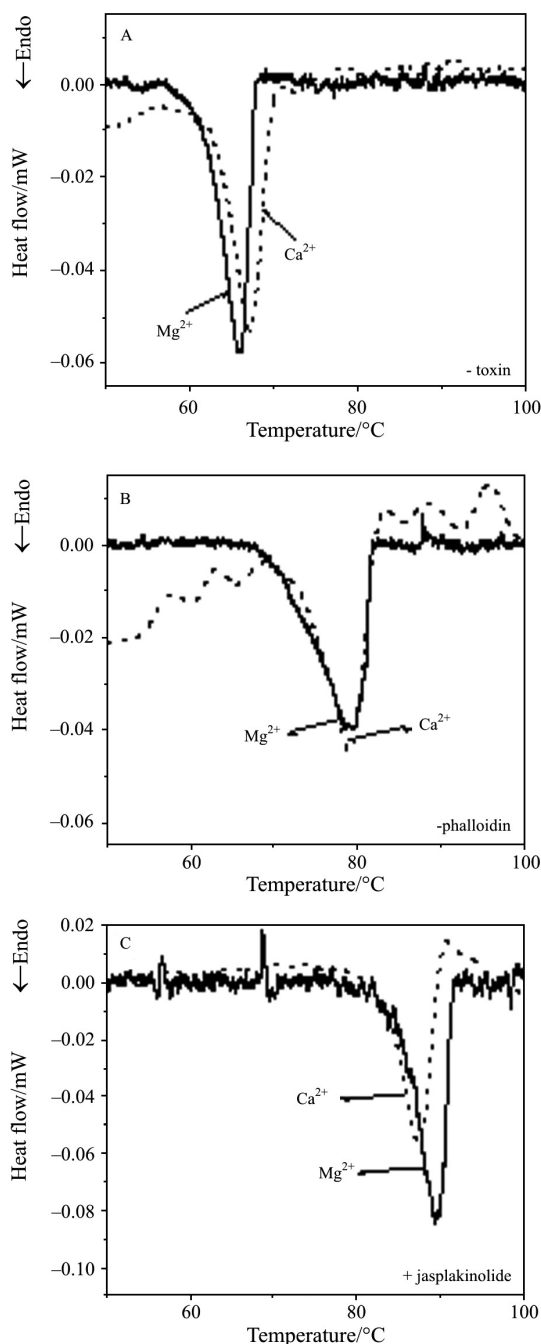

Fig. 1 The denaturation curves for Ca-F-actin and Mg-F-actin. A – in the absence of toxins, B – in the presence of phalloidin or C – jasplakinolide. Solid and dashed lines indicate the data for Mg-F-actin and Ca-F-actin, respectively. The experiments were carried out in buffer A supplemented with 2 mM MgCl$_2$ and 100 mM KCl

that the dynamic properties of filaments characterised by spectroscopic and calorimetric methods do not necessary correlate directly, i.e. the two methods reflect different aspects of the filament dynamics.

The T_m values for the thermal denaturation of actin filaments were shifted to greater values in the presence of either phalloidin or jasplakinolide, reflecting the stabilisation effects of these toxins. The stabilisation by jasplakinolide was more effective than that by phalloidin, in agreement with previous observations [17, 18]. Similar filament stabilising effect was observed with phalloidin using FRET measurements [41].

After the first heating process the samples were cooled back to 0°C and heated again to 100°C in a second process. During the second heating of the samples no actin denaturation curves were observed, clearly indicating that the thermal denaturation of actin filaments was an irreversible process. A mechanism proposed by Mikhailova and colleagues suggested that the final irreversible part of this process was preceded by at least two reversible stages, one of this is the dissociation of the bound nucleotide, and the another one is the dissociation of subunits from actin filaments [42]. Furthermore the heat flow *vs.* temperature profiles were found to be dependent on the scan rate of the heating process. These observations suggest that the melting process, at least partially, is kinetically controlled. Taking this conclusion into account we applied an alternative way to analyse the DSC data to shed light on the kinetic nature of the denaturation process.

The application of the method described by Sanchez-Ruiz and his colleagues [1] uses the reaction scheme (Eq. (1)), which forms the basis of the treatments of irreversible DSC measurements [43]. This theory assumes that the native protein (*N*) undergoes two transitions: a reversible equilibrium transition to an unfolded state (*D*), followed by a first-order kinetic transition to an irreversibly denatured state (*I*).

$$N \xleftrightarrow{k_1, k_{-1}} D \xrightarrow{k_2} I \qquad (1)$$
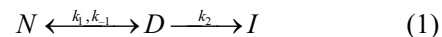
Based on the scheme one considers that $k_2 < k_1, k_{-1}$, then k_2 is the rate limiting step and determines the $N \leftrightarrow D$ extent of the $D \rightarrow I$ irreversibility of the process. If either the $N \leftrightarrow D$ or the $D \rightarrow I$ kinetic step is rate limiting, then the obtained activation energy is most correlated with this step. In our analyses the first-order denaturation rate constant (k_2) was obtained at each temperature according to the following equation:

$$k_2 = \exp\left[-\frac{E_A}{R}\left(\frac{1}{T} - \frac{1}{T^*}\right)\right] \qquad (2)$$

where E_A is the activation energy, T is the experimental temperature, T^* is the temperature at which the rate constant is unity (i.e., $k_2(T^*) = 1$ s^{-1}) and R is the gas

constant. The apparent value for k_2 is k_{app} which can be determined from the experimental results by an alternative way using the enthalpy changes determined from the DSC curves (left side of Eq. (3)). Then the activation energies (E_A) for the irreversible unfolding reactions can be estimated from the Arrhenius plot of the dependence of the k_{app} on the inverse temperature as follows:

$$\ln\left[\ln\left(\frac{\Delta H_{cal}}{\Delta H_{cal} - \Delta H}\right)\right] = \frac{E_A}{R}\left(\frac{1}{T_{1/2}} - \frac{1}{T}\right) \quad (3)$$

where ΔH is the excess enthalpy change, ΔH_{cal} is the total enthalpy change of the melting process and $T_{1/2}$ is the transition temperature.

The dependence of the value of k_{app} on the reciprocal temperature is presented in Fig. 2 for Ca^{2+}-F-actin (Fig. 2A) and Mg^{2+}-F-actin (Fig. 2B) in the absence of toxins, or in the presence of equimolar concentrations of phalloidin or jasplakinolide. Due to their nature these plots can give information regarding the quality of the presentation if one investigates the estimates for T_m from the Arrhenius plots and compares them to the values obtained from Fig. 1. Table 1 contains the T_m values obtained by determining the x-axis intercepts of the Arrhenius plots. The comparison of T_m values from Figs 1 and 2 indicates that the differences are small and the values from the Arrhenius plots adequately reflected the maximum places of the heat absorption *vs.* temperatures curves.

We found that the value of the activation energy from independent experiments and from different analyses showed larger variation than the value of T_m. We estimate the error attributed to the determined activation energies to be ~10–15%. The activation energies (E_A) of actin filaments in the absence of toxins

were 533.4 and 556.8 kJ mol^{-1} for the Ca^{2+}-, and Mg^{2+}-actin filaments, respectively. These values are similar to the results obtained with the majority of the proteins previously examined. The small difference between the two E_A values suggested that the replacement of the bound cation had little effect on the kinetic characteristic of the heat-induced denaturation of actin filaments in the absence of toxin. This conclusion is in agreement with that we drawn from the analyses of the T_m values determined from Fig. 1.

The activation energy values obtained in the presence of toxins are also summarised in Table 2. The comparison of these values indicates that the effect of toxins on this parameter was cation dependent. The effect of phalloidin on E_A for Ca^{2+}-actin filaments was negligible, while jasplakinolide decreased the value of E_A by 20%. The experiments with Mg^{2+}-actin filaments resulted in opposite tendencies with these toxins, as the value of E_A was unaltered by jasplakinolide, while phalloidin decreased the E_A by more than 30%.

These results indicate that there are kinetic differences between the toxin-mediated stabilization of

Table 2 The activation energy (E_A) values in the absence and in the presence of equimolar (toxin to actin protomer) concentrations of phalloidin and jasplakinolide. The value of E_A was calculated from the slopes obtained in Fig. 2 as the product of the slopes and the gas constant (R)

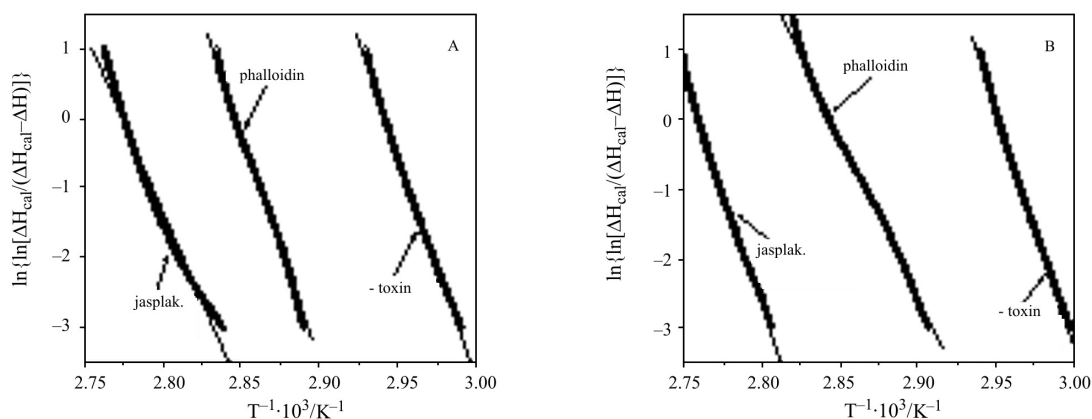| Toxin | E_A/kJ mol^{-1} | |
	Ca-F-actin	Mg-F-actin
None	533.4	556.8
Phalloidin	546.6	380.4
Jasplakinolide	429.2	544.1

Fig. 2 The analyses of the denaturation curves. The figure shows the determined values for $\ln[\ln(\Delta H_{cal}/(\Delta H_{cal} - \Delta H))]$ as the function of the inverse of the absolute temperature. The results in panel A are from experiments with Ca^{2+}-F-actin, while panel B shows the data for Mg^{2+}-F-actin. Linear fit to the $\ln\{\ln[\Delta H_{cal}/(\Delta H_{cal} - \Delta H)]\}$ *vs.* T^{-1} curves are presented as solid lines. The slopes in panel A were -64.1 ± 0.2 K, -65.7 ± 0.2 K and -51.6 ± 0.3 K with intercepts of 188.7 ± 0.5, 187.1 ± 0.5 and 143.2 ± 0.9 in the absence of toxins, in the presence of phalloidin and in the presence of jasplakinolide, respectively. In panel B the corresponding slopes were -66.9 ± 0.1 K, -45.7 ± 0.1 K and -65.4 ± 0.2 K with intercepts of 197.7 ± 0.5, 130.1 ± 0.2 and 180.5 ± 0.7

the Ca^{2+}- and Mg^{2+}-actin filaments. The effect of toxins appeared to be cation dependent. The observations suggest that the melting temperature (T_m) and the activation energy (E_A) are different measures of the thermal stability of actin filaments. To estimate the biological importance of our findings, and to test in more detail the limitations and applicability of the method based on the Arrhenius analyses of DSC curves further experiments will be required.

Acknowledgements

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