Alterations of inter-domain flexibility in actin monomers during cyclophosphamide treatment

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Abstract

The actin is one of the main component of the eukaryotic cytoskeleton. The continuous rearrangement of actin filaments is provided by the different complexes with divalent cations (Ca^{2+} or Mg^{2+}) and nucleotides (ATP, ADP). In the medical routine, cyclophosphamide (CP) is applied as cytostatic and it was shown that in vivo muscle filament system was changed by the CP treatment and it has direct interaction with actin monomers as well. The evolutionary importance of physical links between domains is one of the most interesting question to understand the multi-domain development of protein functions. Here, we analyse the thermal stability modifier act of inter-domain links in proteins, monitored by DSC, with the concept of that how did the nucleotide binding cleft between the two main domains of actin monomers affect the activation energy of domains if it was blocked or released by CP binding or dissociation, respectively. We investigated the importance of inter-domain linkers on the thermodynamic properties of actin. Ca^{2+} and Mg^{2+} bound G-actin can be stabilized by CP binding or polymerization. CP treatment of Ca^{2+} -F actin lacks the structural integrity of the more flexible polymer and shows same stability as CP bound monomers. However, Mg^{2+} -F actin did not show any kinetic response to the CP treatment. We can assume that the inter-domain linker of actin reduces the stability of the domains which leads to a more reactive and variable structure as a thermodynamic advantage for the development of a multi-domain protein can be blocked by CP treatment.

Keywords Inter-domain link · Actin · Cyclophosphamide · DSC · Evolution · Thermodynamics

Introduction

Essential units of cells are cytoskeletal proteins [1]. The actin is one of the main component of the eukaryotic cytoskeleton and muscle sarcomeres with highly conservative sequences. The small variety of proteins is coming from the specialization for intracellular functions [2–4]. The variable forms of actin monomers (G-actin) and filaments (F-actin) are playing important role in motility, division, and transport processes of cells [5–11]. The continuous rearrangement of actin filaments is provided by the different complexes with divalent cations (Ca^{2+} or Mg^{2+}) [12] and nucleotides (ATP, ADP) [13–20]. Cation and nucleotide binding modifies the structural stability of actin monomers but the form that is bound to ATP predominates in cells when actin is present in its monomer state [21, 22]. During

actin polymerization, the ATP is hydrolysed to ADP and P_i [13–20]. The inter-domain link provided benefit is that the cation and nucleotide binding of G-actin initiates the remodelling of binding cleft thus modifies the stability of its two main domains [23, 24]. Presumably, the activation energy as the thermal stability related kinetic property of domains in the structure of a protein can be interpret as separated single proteins independently of their interactions and can be described as small units which linked together in one functional protein. Lower activation energy refers to a more advantageous kinetic case of protein stability [25–28].

In the medical routine, cyclophosphamide (CP) is applied as cytostatic [29] and can be used for its beneficial effects nevertheless with some short- and long-term side effects as well [30–35]. Referring to a forensic medicine indication, we have carried out DSC assay to study its long-term effect in Guinea pig muscle [35]. We are able to distinguish results of drug treatment on Guinea pig muscle actin or myosin applying deconvolution with the collected DSC data [36, 37] as it was carried out in case of different nucleotides containing psoas muscle fibres as well [38, 39].

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The effect of ligand binding to actin filaments is often cooperative and induces allosteric conformational changes in the actin filaments distant far from the binding site [40–44]. Previously, we have studied the effect of different toxins—jasplakinolide and phalloidin—on actin [45, 46]. During the thermal denaturation of the treated actin, we have observed a toxin concentration dependent cooperative binding effect. It was also shown [36, 37, 47] that in vivo muscle filament system was changed by the CP treatment and it has direct interaction with actin monomers as well [48]. The evolutionary importance of physical and structural links between domains is one of the most interesting question to understand the multi-domain development of protein functions [49–53]. If the linker exists, it leads to the benefit of thermal stability and improved kinetics of proteins results decreasing activation energy and earn of function type development. Here, we analyse the thermal stability modifier act of inter-domain links in proteins with the concept of that how did the nucleotide binding cleft between the two main domains of actin monomers affect the activation energy of domains if it was blocked or released by CP binding or dissociation, respectively.

Materials and methods

Actin preparation from rabbit skeletal muscle

G- and F-actin with Ca^{2+} or Mg^{2+} cations were prepared in the usual way from acetone powder of rabbit skeletal muscle as described earlier by Spudich and Watt [41], and stored in MOPS buffer (2 mM MOPS, 0.2 mM ATP, 0.1 mM CaCl_2 , 0.1 mM β -mercaptoethanol, pH 7.4). Actin concentration was determined from the absorption spectra (Jasco V-550 spectrophotometer, as the average concentration by $\epsilon = 1.11 \text{ mL mg}^{-1} \text{ cm}^{-1}$ at 280 nm and $\epsilon = 0.63 \text{ mL/mg} \cdot \text{cm}$ at 290 nm). We applied 2 mM EGTA then 2 mM MgCl_2 treatment for exchange calcium to magnesium on 2 mg mL^{-1} actin monomers; this way we remained close to the physiological concentration of actin. Actin polymerization process was initialized by addition of 100 mM KCl follow the same protocol as in our previous study [48].

Cyclophosphamide treatment

In our in vitro measurements, the applied dosage of cyclophosphamide (CP) was the same as the human dosage ($150 \text{ mg kg}^{-1} \text{ b.m.}$) during chemotherapeutic treatments [7–10]. The average actin content of skeletal muscle is roughly 10% of the actual muscle mass [41] thus the average mass of Guinea pig gastrocnemius muscle (from our previous study [8]) divided by 10 then by the mass of CP passed in the muscle [$150 \text{ mg kg}^{-1} \times (\text{m}_{\text{gastrocnemius}}/\text{m}_{\text{body}})$] resulted that the actin to

CP ratio has to be 2000/3 (it means 2 mg actin to 3 μg CP) as a single dose. However, as we used actin from rabbit skeletal muscle, we can assume that the distribution of CP in rabbit skeletal muscle should be the same as in Guinea pig skeletal muscle. To achieve a more pronounced effect, we carried out our experiments with 5 times conventional dose of CP to treat actin followed by incubation at room temperature for 1 h (in case of model experiment the animal underwent to a real, long lasting chemotherapeutic protocol as described in [35–37, 47]).

DSC measurements

The actin samples with 2 mg mL^{-1} concentration were freshly prepared before all measurements. The analysis was made by a SETARAM Micro-DSCII calorimeter between 0 and 100°C with heating rate of 0.3 K min^{-1} . Conventional Hastelloy batch vessels ($V_{\text{max}} = 1 \text{ mL}$) were used for the experiment to investigate denaturation with 950 μL sample volume in average. Samples' masses were between 920 and 970 mgs. MOPS buffer was used as a reference. The reference and sample vessels were equilibrated with a precision of $\pm 0.1 \text{ mg}$; this way we did not need to do any correction between vessels' heat capacity. With the help of a two-point SETARAM peak integration setting, calorimetric enthalpy was calculated from the area under the heat absorption curve, and then, the results [denaturation or melting temperature (T_m) and calorimetric enthalpy (ΔH_{cal}) data of samples] were compared. This method is identical with the protocol as we applied in our previous study [48].

Calculation of activation energy

With the exception of low molecular mass globular proteins, denaturation is irreversible in biological systems. The first model of irreversible denaturation kinetics of protein systems is named after Lumry and Eyring [54], which was further developed by Sanchez-Ruiz et al. [25, 55, 56] as well as by Vogl et al. [57]. We used the model of Sanchez-Ruiz. The collected infinitesimal DSC enthalpy change data (dH , average of three independent measurements) were integrated (dH_{cal}) in the function of time related change of the temperature. Then, $\ln(\ln(dH_{\text{cal}} / dH_{\text{cal}} - dH))$ vs. $1/T$ function was fitted with a linear [55]. The slope of the line was divided by of its intercept results the melting temperature (T_m). The slope multiplied with the Regnault number is equal to the activation energy (E_a).

Results

Figure 1 shows the activation energies for all investigated actin conditions in the absence of CP. On the basis of accurate plots, two distinct activation energies appear (blue and

red lines) where the E_a values representing two different denaturation kinetics as components of the result of linear fit on the whole denaturation range (black lines on Figs. 3 and 4). It is highly probable that the contribution of the inter-domain linker can be explicit because it refers to an intermediate kinetics of the two large structural domains "scissor-like" motion during denaturation of the whole protein. Figure 2 represents the effect of CP treatment on activation energies in case of same set of experiments with actin. CP binding increased the activation energy of all kind of monomeric actin but decreased in case of Ca^{2+} -F actin and did not show any effect on Mg^{2+} -F actin polymers. To surprise, except of Mg^{2+} -F actin, in all cases, we could fit three straight lines—exhibiting the effect of CP binding—as an additional intermediate kinetic component of the denaturation in the whole temperature range.

The most characteristic thermal parameters determined by the Sanchez's method (T_m ($^{\circ}\text{C}$): denaturation temperature and E_a (kJ mol^{-1} : activation energy) are summarized in Tables 1 and 2.

We have seen on the basis of plotting raw data and using the literature [58–60] that we can decompose the heat flow curves into cooperating thermal domains. It can be seen from Fig. 3 and 4 that the thermal parameters of deconvolved curves (R^2 stands for the goodness of fit) are in a good agreement with data given by the Sanchez's method (see Tables 1 and 2).

Accepting that, the different actin forms are consisted on the variety of conserved domain dimers, and this way corresponding to the thermal domains, we examined the applicability of the Sanchez method to each of the deconvolved components (see Figs. 5 and 6 and Table 3).

Fig. 1 The black values refer on the whole temperature range (fitting cannot be seen), blue lines on the higher and red linear on the lower one. Analysis of thermal denaturation curves of different G- and

F-actin by the Sanchez model (closed squares) as the composition of two different kinetic tendencies (low: blue, high: red)

Fig. 2 Except of Mg²⁺-F actin three linear can be fitted. Analysis of thermal denaturation curves of different G- and F-actin in the presence of CP by the Sanchez model (closed squares) as the composi-

tion of three different kinetic tendencies if the CP had direct effect on actin (low: blue, moderate: red, high: green)

Table 1 Thermal data achieved by Sanchez's fit on the whole denaturation range. Data are averages of three independent measurements

	Ca ²⁺		Mg ²⁺	
	T_m /°C	E_a kJ mol ⁻¹	T_m /°C	E_a kJ mol ⁻¹
<i>G-actin</i>				
Native	59.1	437.08	53.2	851.9
CP treated	59.1	654.74	55.9	1019.41
<i>F-actin</i>				
Native	67.2	848.28	66.6	780.4
CP treated	67.1	651.21	67.8	817.44

Discussion and conclusions

The first thermal denaturation of actin was performed on G-actin (Tatunashvili et al. [58]), during which they obtained a DSC signal indicating the existence of at least two interacting thermal domains. Structural confirmation was obtained by determining the atomic structure of G-actin (Kabsch et al. [24]), which consists two main units with a deep cleft and each of them can be divided into two additional subunits. The study of F-actin is another example of how DSC, as a method, is suitable for the study of the consequence of polymerization, which manifests itself in a significant increase in denaturation temperature and calorimetric enthalpy in F-actin and a decrease in the half-width of the DSC signal (Bertazzon et al. [59] and Lőrinczy et al. [60]).

Table 2 Thermal data after fitting with multiple lines

	Ca ²⁺		Mg ²⁺	
	$T_m/^\circ\text{C}$	$E_a \text{ kJ mol}^{-1}$	$T_m/^\circ\text{C}$	$E_a \text{ kJ mol}^{-1}$
<i>G-actin</i>				
Native	56.8	704.28	52	1350.44
	59.1	331.29	53.2	674.74
CP treated	57.5	1054.21	54.6	1912.66
	58.9	604.28	55.8	832.18
	59.1	461.74	55.9	745.08
<i>F-actin</i>				
Native	66	1403.47	65.5	1139.2
	67	615.81	66.6	622.2
CP treated	64.5	1185.44	66.3	1288.69
	66.9	636.52	67.8	617.32
	67.1	578.96	—	—

The aim of our present work is to interpret the evolutionary and thermodynamic benefit of inter-domain linker in actin monomers. Both domains of actin can be modelled as separated conservative globular protein units whose stability is based on the energy which explains their unfolding kinetics of structural transition between their native to intermediate and denatured form [25]. All variable sites and linkers out of the domain can turn out novelties for the functional and structural development and results high plasticity and upregulated thermal stability of the multi-domain proteins [61]. The physical link between the domains of actin can be developed by its advantageous thermodynamic properties because formed a structural setup for a nucleotide binding cleft. The structural dynamics of the binding cleft can be a sensitive response to the type of bound nucleotides and cations. Therefore, the more adaptive structural dynamics of domains possibly established new structural advantages for the novel functions of actin and actin-related cytoskeletal

Fig. 3 Deconvoluted thermal domains in thermal denaturation curves of different untreated G- and F-actin (endotherm effect is deflected downwards)

Fig. 4 Deconvoluted thermal domains in thermal denaturation curves of different G- and F-actin in the presence of CP (endotherm effect is deflected downwards)

processes to make sense in the divergent development from anchor prokaryotic and archaeal actin like proteins [62, 63].

It was shown that F-actin can be further stabilized by toxins (e.g. phalloidin, jasplakinolide), which shift the denaturation temperature by $\sim 14^\circ\text{C}$ to a higher range proving the cooperativity of the toxin-binding into the cleft [45, 46, 64]; therefore, phalloidin-stabilized F-actin is used for better measurement of thermal transitions of filamentous actin and actin-binding proteins (e.g. S1, tropomyosin, etc.) to isolate them. Similar to myosin, when G-actin polymerizes to F-actin by hydrolysis of bound ATP, an intermediate state is formed in the actin subunits during the formation of the actin-ADP-Pi complex. This condition can be studied with complexes of F-actin with ADP and Pi analogues ($\text{BeF}_x/\text{AlF}_4^-$) and resulted in the shift of T_m into higher temperature range (in case of muscle fibres too) [38, 39, 65–69]. The reason—of the minor difference between our recent denaturation temperatures compared to the literature data—can be the difference in the concentration of samples, in the heating

rate as well as the different measuring principle of our system (most of the devices use capillary sample holders why our is a big [$V = 1\text{ mL}$] stainless steel cylinder. The instrument is a heat-flux calorimeter.).

As previously shown, the CP treatment modifies the thermodynamic stability of actin subunits and possibly affects the nucleotide binding cleft [48]. Here, we observed that the denaturation of Ca^{2+} bound actin monomers show higher T_m with less stable and more reactive denaturation kinetics (lower E_a) than in case of Mg^{2+} . Ca^{2+} -ATP possibly binds stronger between the domains as a core in a highly reactive structure than in case of Mg^{2+} -ATP in which case domains were taken apart a bit by its size in a more tensed structure. Nevertheless, in both cases, all calculated activation energy values were increased by CP binding which refers to a slower denaturation kinetics. However, the denaturation of Ca^{2+} -F actin shows a more stable and less reactive kinetics than monomers what explains a well-ordered structure in a polymer form. To surprise, the T_m of Mg^{2+} bound

Fig. 5 Analysis of deconvolved thermal domains from Fig. 3, by the Sanchez model as we presume that thermal domains are related to the kinetics of separated domains in the structure of actin

filamentous actin was increased a bit but E_a was almost the same as monomer form refers to the identical denaturation kinetics of monomer and polymer forms of Mg^{2+} actin. The CP treatment modified the structural integrity of the Ca^{2+} bound polymers because the activation energy of Ca^{2+} -F actin without any change of T_m was decreased to the same level as E_a of CP treated monomers. The CP treatment did not have any effect on the stability of filamentous Mg^{2+} actin only the T_m was increased seems like CP can decorate only the sides of filament cannot solve the structural integrity of it. (Table 1) (Fig. 7.)

If we use multiple fitting of curves, the calculated T_m values seem like the components of values are generated by single fitting and follow the same tendency. Multiple fitting of curves measured with filamentous actin resulted E_a values are identical before and after CP treatment in contrast to single fitting but with monomeric actin single

and multiple fitting resulted E_a values show the same tendencies (Table 2). We could wait on the basis of Figs. 2 and 3 that the CP treatment by the deconvolution of heat flow curves give possibility to look into finer details. On the basis of Sanchez's fitting of deconvolved curves (Figs. 5 and 6), we got similar tendencies but all E_a s were definitely bigger than in case of multiple fitting (Table 3, compared to Table 2) during the refining of fitting on the whole denaturation temperature range. According to the application of the Sanchez model directly on measured curves by single and multiple fitting, we have got lower E_a values then did it with deconvolved data what we can assume that the thermal domains are related to the kinetics of separated domains in the structure of actin. What we can observe more that multiple line fitting of basic thermal denaturation curves of CP treated actin resulted E_a values

Fig. 6 Analysis of deconvoluted thermal domains from Fig. 4. of CP treated G- and F-actin forms by the Sanchez model as we presume that thermal domains are related to the kinetics of separated domains

in the structure of actin in which a new component appears by the effect of CP as an isolated domain

lay into the range of E_a values generated by analysis of thermal domains from deconvoluted curves.

The biological significance of actin filament cooperativity is still unclear; however, some findings can be made to the best of our present knowledge. The actin filament system is under the complex influence of actin-binding protein molecules in vivo. Actin-binding protein molecules alter the conformation and dynamic properties of the filament. If a local interaction causes a change in more distant molecular moieties, it is conceivable that effect has a role of transmitting information. In the development of multi-domain

proteins, all linkers were turned to be implicated in the selection forward-looking structural and functional advantages.

Here, we highlighted the importance of inter-domain linkers on the thermodynamic properties of actin. The dynamics and stability of nucleotide binding cleft of monomers are well regulated by binding nucleotides and divalent cations. As we suppose in our model on Fig. 7. Ca²⁺ and Mg²⁺ bound G-actin can be thermodynamically stabilized by CP binding or by polymerization. CP treatment of Ca²⁺-F actin lacks the structural integrity of the more flexible polymer (it can be described with higher

Table 3 The T_m and E_a parameters determined from Sanchez plots (see Figs. 5 and 6) are in a good agreement with the deconvolved parameters (see Figs. 3 and 4)

Calculated parameters				
	Ca^{2+}		Mg^{2+}	
	$T_m/^\circ\text{C}$	$E_a \text{ kJ mol}^{-1}$	$T_m/^\circ\text{C}$	$E_a \text{ kJ mol}^{-1}$
<i>G-actin</i>				
Native	56.3	579.67	50.8	1390.9
	60.4	625.21	53.5	1089.23
CP treated	55.5	1153.62	53	2919.44
	57.9	886.76	54.4	1828.52
	60.6	1082.35	56.4	1359.43
<i>F-actin</i>				
Native	64.9	1567.17	64	1289.65
	67.5	997.17	67	1051.35
CP treated	62.5	1253.68	65.7	1146.97
	65.8	1136.53	68.28	1010.58
	68	1288.9	—	—

inter-monomer flexibility than Mg^{2+} -F actin [45]) and shows same stability as CP bound monomers. However, the Mg^{2+} -F actin did not show any kinetic response to the CP treatment. Alkylation of residues with N atom by CP (as previously show [70–72]) can affect the whole structure of actin. Based on our data, actin filaments are

less sensitive to the CP treatment than monomers can be interpreted as CP can reach the nucleotide binding cleft only in monomers and react with the residues which are implicated in ATP/ADP binding can change the thermal stability of actin. Modification of long well charged residues in the binding cleft can change the open/tight structure of actin monomers. Typically the methylation of H73 can protect and shield ATP binding or mutation at H73A, R177D, S14C or S14C/D157A can reduce ATP binding and leads to a quick nucleotide exchange [73]. The physiologically more relevant Mg^{2+} actin is more reactive to CP treatment and probably getting a tight structure by the alkylation of residues in the nucleotide binding cleft which seems thermodynamically the most important structure of actin as an inter-domain linker.

According to the application of the Sanchez model directly on measured curves by single and multiple fitting, we have got more sensitive thermal stability than did it with deconvolved curves and we presume that thermal domains are related to the kinetics of separated domains in the structure of actin. These results we can interpret as the inter-domain linker of actin reduces the thermal stability of the domains in the structure of actin monomers which leads to a more reactive and variable structure as a thermodynamic advantage for the development of a multi-domain protein and this elementary effect can be blocked by CP.

Fig. 7 Cartoon explains our model of cyclophosphamide treatment modified stability and structural dynamics of actin monomers and polymers

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Data availability There are no additional available data to upload.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval All procedures followed were approved and in accordance with the ethical standards of the responsible committee on animal experimentation (institutional and national) and with the revised Helsinki Declaration of 1975.

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