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Inter-monomer cross-linking affects the thermal transitions in F-actin

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Abstract Chemical cross-links which covalently connected the Cys-374 and Glu-41 residues of adjacent monomers in the same strand of F-actin were used to follow the consequences of the modification for the motional and structural properties of the actin filaments. DSC measurements reported that the inter-monomer cross-links shifted the thermal transition temperature and affected strongly the cooperativity of the transition in comparison with uncross-linked F-actin. Addition of HMM to F-actin induced significant decrease of the transition temperature to lower value from 69.4 to 67. 5 °C.

Keywords Actin cross-linking · Thermal stability · DSC

Introduction

Actin is one of the main components in the eukaryote cells which plays significant role in many cellular processes, like force-generation, maintenance of the shape of cells, cell-division cycle and transport processes [1, 2]. The filamentous form of actin (F-actin) is the principal component of the contractile and motil systems. Different studies—biochemical and biophysical—indicate that actin filaments are flexible, and the dynamics of the filaments may have significant role in the contractile process [3, 4].

One way to modify the flexibility and perturb inter-monomer motions in F-actin is the chemical cross-linking of adjacent monomers. Site specific inter-monomer cross-links were prepared between Lys-191 and Cys-374 [5], and between Gln-41 and Lys-113 as well as between Gln-41 and Cys 374, respectively [6–8]. It was shown by in vitro motility assay that constrains imposed on actin filaments by cross-linking inhibited the sliding motion of actin filaments on myosin [9, 10]. EPR measurements performed on spin-labelled cross-linked F-actin showed that the rotational dynamics and the interaction with myosin fragments are affected by both interstrand and intrastrand cross-links [11].

In this study, the effect of intrastrand cross-linking between Gln-41 and Cys-374 on the F-actin was studied by means of DSC technique. The main transition of untreated F-actin at 70.2 °C shifted to 69.4 °C after cross-linking and affected the cooperative interaction between monomers. Addition of heavy meromyosin induced further change of the transition temperature and cooperative interaction.

Materials and methods

Chemicals

Synthesis of ANP [*N*-(4-azido-2-nitrophenyl)-putrescine] and ABP [*N*-(4-azidobenzoyl)-putrescine] was described in earlier publications [6] and [7], respectively. Maleimide-TEMPO

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spin label and the other chemicals used in the experiments were purchased from Sigma (Germany).

Protein preparation

Rabbit skeletal actin and heavy meromyosin (HMM) were isolated from the hind leg and back muscle of domestic white rabbits by standard methods [12, 13].

Cross-linking of actin by ABP or ANP

The spin-labelled G-actin (30%) was diluted with unlabelled G-actin (70%) in G-buffer (4 mM Tris/HCl, pH 7.6, 0.2 mM ATP, 0.2 mM $CaCl_2$), and the concentration of G-actin was 2 mg/mL. The "mixed" G-actin was incubated with 8 M excess of ABP or ANP reagent to 1 mol of actin in the presence of transglutaminase enzyme (0.5 unit/mL) in dark for 4 h at room temperature to obtain samples labelled with ABP or ANP on Gln-41. The sample was polymerized with 2 mM $MgCl_2$ and pelleted by ultracentrifugation. The ABP or ANP labelled F-actin pellets were homogenized in F-buffer (G-buffer plus 2 mM $MgCl_2$) and incubated for 1 h on ice under N_2 stream. The photo cross-linking of the double-labelled F-actin samples were carried out as described earlier [6, 7], respectively.

DSC measurement

Thermal unfolding of untreated and cross-linked F-actin was monitored by a SETARAM Micro DSC-II calorimeter. All experiments were conducted between 5 and 80 °C, the heating rate varied between 0.3 and 1.0 K/min in all cases. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 μL sample volume in average. F-actin buffer solution was used as a reference sample. The sample and reference vessels were equilibrated with a precision of ±0.1 mg. There was no need to do any correction between sample and reference vessels. The repeated scan of denatured sample was used as baseline reference, which was subtracted from the original DSC curve.

EPR measurements

Conventional and saturation transfer (ST) EPR spectra were taken with an ESP 300E (Bruker Biospin, Germany) spectrometer. First harmonic in-phase absorption spectra were obtained by using 20-mW microwave power and 100-kHz field modulation with amplitude of 0.1 or 0.2 mT. Second harmonic, 90° out-of-phase absorption spectra were recorded with 63 mW and 50 kHz field modulation of 0.5 mT amplitude detecting the signals at 100 kHz out-of-phase. The 63-mW microwave power corresponds in average microwave field amplitude of 0.025 mT in the centre region of the cell, and the values were obtained by using the standard protocol of Fajer and Marsh [8]. The spectra were usually recorded at 23 ± 0.1 °C. A laboratory developed computer algorithm was used to obtain the spectral parameters of the samples.

Actin was labelled in F-form with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide spin label (MSL) in a molar ratio of 1:1.2 for 12 h at 2 °C. Unreacted label was removed by pelleting the actin by ultracentifugation. The pellet was resuspended, homogenized and dialyzed in G buffer. The samples were filled in WG-808-Q flat cell from Tebu-Bio (France), to obtain optimal sensitivity.

Results and discussion

Evaluation of thermal transitions in actin

The reversibility of denaturation was checked by comparing the first scan of the protein samples with the second one after cooling the sample to room temperature. The DSC transitions were calorimetrically irreversible. Systematic analysis of irreversible denaturation of protein systems started with the model by Lumry and Eyring [14] and Sanchez-Ruiz [15]. After these basic reports, several experimental studies were reported with the conclusion, that, in many cases, the irreversible denaturation of proteins by DSC can be described on the basis of a simple two-state irreversible model [16–19]:

$$\mathrm{N} \xrightarrow{k_{\mathrm{ap}}} \mathrm{F},$$

where N and F are the native and irreversibly denatured proteins, respectively. The k_{ap} reaction rate constant that governs the conversion from N to F is strongly temperature dependent and is a first-order rate constant [20]. The theoretical analysis also suggests that the irreversible step from unfolded state to irreversible state is fast, and the amount of unfolded state is low [20].

The transition temperatures of G-actin depended on scan rate, therefore it could be assumed that the melting of the actin samples was determined by kinetic processes and could be described by the two-state kinetic model (Fig. 1); the transition temperature T_{m} was 60 °C. This result agrees with earlier observations [21]. The simulation of the melting curve supports the two-state model because the calculated transition curve based on this model fits well the experimental DSC trace. The comparison of the main transitions for globular and filamentous actin showed significant increase of T_{m} up to 70 °C, which is due to the specific interaction between the actin monomers (Fig. 2). The remarkable decrease of the line width at half height of the transition trace of F-actin is due to the cooperative

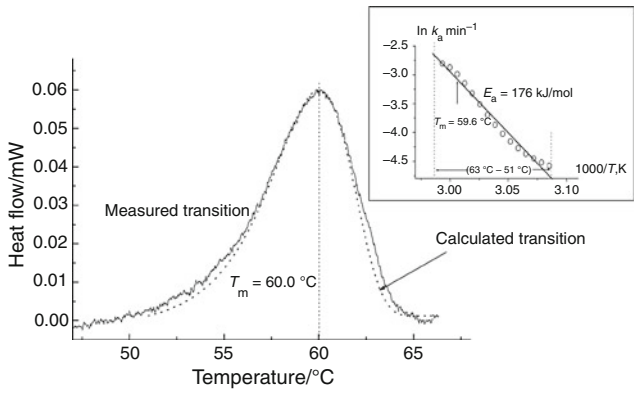

Fig. 1 DSC transition of monomer actin. *Solid line* shows the experimental DSC trace, whereas *dotted line* was obtained after simulation of the transition using the activation energy and transition temperature of experiment. The *inset* shows the dependence of ln k_a as a function of reciprocal absolute temperature

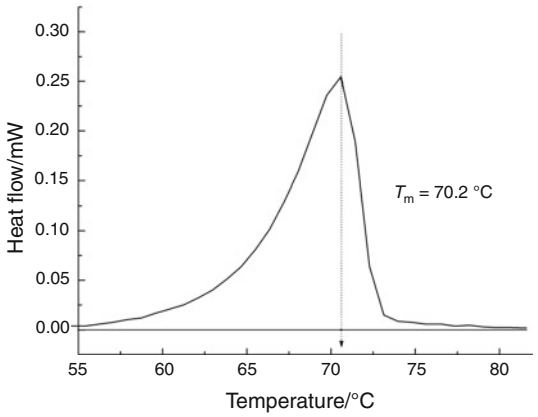

Fig. 2 DSC trace of uncross-linked F-actin. Concentration of actin was 80 μM/L. Polymerization of G-actin was performed in buffer solution containing 100 mM KCl and 2 mM $MgCl_2$ at room temperature

interaction between the protomers in the helical arrangement of the filament.

Effect of cross-linking on thermal transitions

It is known from earlier experiments that the nitroxide riporter molecules were rigidly attached to actin monomers and indicated the motion of a larger domain in the monomer. Only a small fraction of the labels (smaller than 5% of the total absorption) exhibited fast, sub-microsecond rotational motion in the conventional EPR time domain, which might be due to the motion of the labels relative to protein, or the motion of the label-binding region of actin. At increasing temperature, the transition of slower motion to faster one increased significantly, and at about the denaturation temperature (~60 °C) of G-actin only the component of faster motion was detected. Modification of the

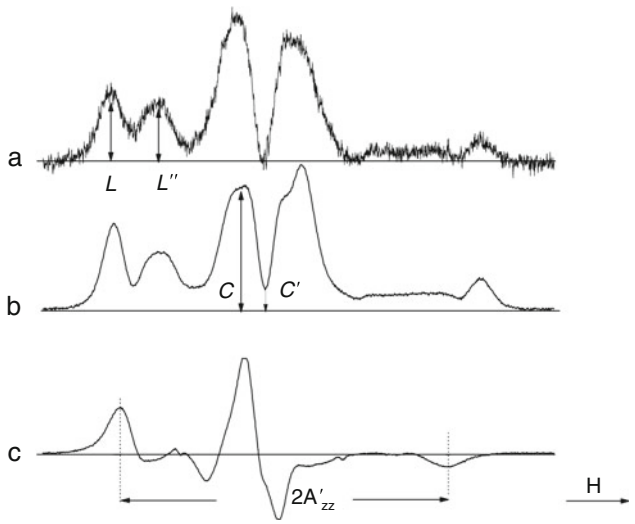

Fig. 3 Conventional and saturation transfer (ST) EPR spectra of spin-labelled uncross-linked and cross-linked (Cys-374 × Gln-41) F-actin. Maleimide nitroxide probe molecules (MSL) were used to label F-actin on the Cys-374 site. **a** ST-EPR spectrum of uncross-linked MSL-F-actin. **b** ST-EPR spectra of intrastrand cross-linked MSL-F-actin. The increase of L''/L and C'/C parameters indicates the slower molecular motion of F-actin. **c** Conventional EPR spectrum of cross-linked MSL-F-actin

filament structure by intrastrand cross-linking between the amino acid residues Cys-374 and Glu-41 in F-actin produced significant changes in both local and global conformations. According to ST-EPR experiments, the motional dynamics of F-actin modified by intrastrand cross-linking was much slower in the sub-millisecond time domain in comparison with control samples and interstrand cross-linking (Fig. 3).

The thermal transition of cross-linked F-actin showed two smaller additional transitions as well, one of them at about 50 °C, whereas the transition at 75 °C seems to be an exotherm one (Fig. 4). The shift of the temperature (<1 °C) at the main transition for cross-linked F-actin is in the usual range of the experimental error for that of F-actin. Using Gaussian functions for preliminary deconvolution, it was possible to resolve the contributions of different domains in the complex DSC curve (dotted lines in Figs. 4, 5). The transition temperature of the main transition almost coincides with the transition temperature of untreated F-actin, but the larger width at half height refers to a decrease of cooperativity between subunits. The transition at 50 °C arises probably from small broken filaments of F-actin remained in the sample during the process of preparation. The reason of occurence for the exotherm transition might be the partial aggregation of manipulated filaments.

It is known that the EPR spectra of macroscopically oriented F-actin labelled with maleimide riporter molecules at the Cys-374 site exhibit orientation dependence with respect of the laboratory magnetic field. The narrow

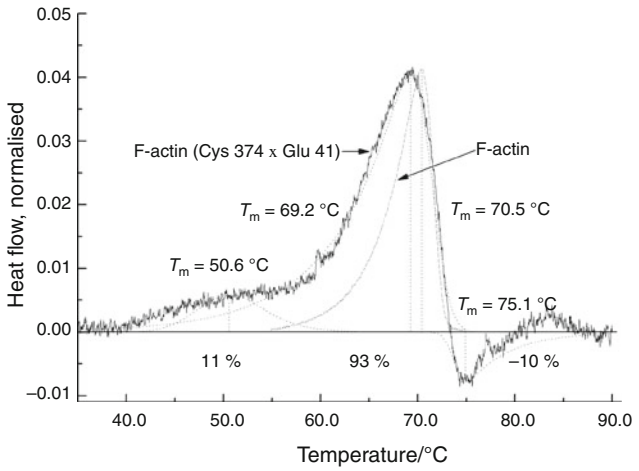

Fig. 4 DSC trace of cross-linked (Cys-374 × Gln-41) F-actin. Concentration of actin was 40 μM/L. *Thin line* shows the DSC trace of an untreated F-actin sample with transition temperature of 70.5 °C. *Dotted lines* are the result of deconvolution with Gaussian functions. The numbers in percent characterize the area of components obtained during deconvolution process

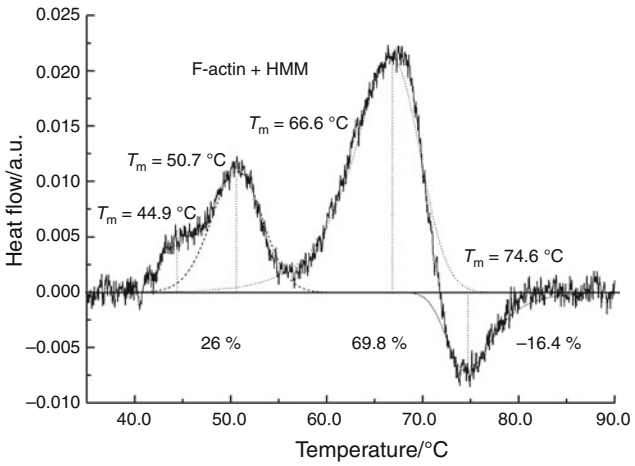

Fig. 5 DSC trace of intrastrand cross-linked (Cys-374 × Gln-41) F-actin in combination with HMM. The molar ratio of HMM to actin was 1 mol of HMM to 5 mol of actin. Concentration of actin was 40 μM/L. A remarkable shift of the transition temperature was induced by binding of HMM to F-actin

angular distribution of spin labels have a center of 36° between the long axis of the filament and the principal axis of spin label, the angular spread at half height 24°. A second, smaller population of spin labels could be detected as well with significant disorder [22, 23]. The disorder may arise from improperly oriented filaments. The cross-links produced a marked change in the orientation dependence of the riporter molecules with respect to the long axis of the filaments, supporting the perturbation produced by the artificially generated binding between the neighbouring monomers.

Addition of myosin and/or HMM affected the structure and the dynamic properties of cross-linked F-actin. When HMM was added to cross-linked F-actin in a molar ratio of 5 mol of actin to 1 mol of HMM, the DSC trace showed three meltings with transition temperatures of 44.9, 51.2 and 67.5 °C (Fig. 5). The first two transitions could be assigned to the S-2 and S-1 fragments of HMM, and the highest transition reflected the melting of cross-linked F-actin. The data for HMM agree well with the data of earlier experiments [24]. It is interesting to note that the transition temperature of cross-linked F-actin shifted to lower temperature with about 2 °C, but the full line width at half height remained the same. The deconvolution of the samples with Gaussian functions resulted almost the same pattern as in the case of F-actin, the area of the broad transition at 50 °C did not increase significantly, but the exotherm transition increased a little due to the altered interactions.

The decrease of T_m in the main transition is probably due to a small destabilization of actin structure induced by myosin fragments bound to cross-linked actin. It is possible that the binding of HMM induces a weakening of the contact between the longitudinal protomers, and this effect is amplified by the distorsion evoked by the intrastrand cross-linking. However, the frequency of rotational motion of the riporter molecules decreased at the same time in the environment of the labelled Cys 374 sites; significant increase in spectral parameter C'/C could be detected in the ST-EPR time domain. It was reported earlier that cross-links impair the motion of actin filaments in vitro motility assay [10, 25]. The domain–domain interaction between the head of myosin and its binding domain on actin provides an interprotein communication that is necessary for a coordinated function. It is possible that this destabilization contributes to the inhibition of the motility.

The actin filaments are internally mobile, and therefore they have different mode of motions. These are required for the proper interaction with myosin and for the necessary conformational changes during ATP hydrolysis and force generation in muscle fibres [26]. The cross-links may perturb the significantly important motions causing alterations in the local and global conformations of actin. The data achived by cross-linking can help in deeper understanding of the thermal behaviour of actin [27] and the effects of toxic agents and nucleotides on the cooperativity of monomers in F-actin [28, 29].

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