Thermally induced chain exchange of smooth muscle tropomyosin dimers studied by differential scanning calorimetry

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Abstract The thermal unfolding of duck gizzard tropomyosin dimers, αβ, αα, and ββ, and of a 1:1 mixture of αα and ββ homodimers was studied by differential scanning calorimetry (DSC). Both αα and ββ homodimers demonstrated a broad thermal transition with maxima at 37.4°C and 44.6°C, respectively. However, a sharp cooperative thermal transition at 41.5°C characteristic for αβ heterodimer appeared on the thermogram of the mixture of homodimers. The appearance of this transition was prevented by disulfide cross-linking of polypeptide chains in the homodimers. Thus, DSC studies clearly demonstrate formation of tropomyosin heterodimers during heating of the mixture of homodimers and in agreement with earlier published reports indicate thermally induced chain exchange between tropomyosin dimers.

Key words: Tropomyosin; Coiled-coil dimer; Thermal unfolding; Differential scanning calorimetry

1. Introduction

Tropomyosin (TM) is associated with actin filaments in all types of muscles and plays an important role in the regulation of muscle contraction and cytoskeleton organization. TM is a dimer composed of two coiled-coil polypeptide chains with close to 100% α-helical content. Two isoforms (α and β) each containing 284 residues are expressed in smooth and skeletal muscles [1]. Smooth muscle TM consists of a 1:1 mixture of α and β chains (sometimes marked as γ and β, respectively [2–5]) which are predominantly assembled in a αβ heterodimer form [4,6]. It has been shown that the αβ heterodimer of TM can be formed via chain exchange during incubation of a mixture of αα and ββ homodimers at physiological temperature [4,6–8].

Thermal denaturation of TM homodimers from skeletal and smooth muscles has been investigated in detail by differential scanning calorimetry (DSC) [5,9,10]. However, previous DSC studies did not include TM heterodimers or a mixture of TM homodimers. In the present paper we report DSC studies of the thermal unfolding of a mixture of smooth muscle TM homodimers. Our DSC results clearly demonstrate formation of TM heterodimer during the heating of the mixture of TM homodimers and in agreement with earlier published data obtained by circular dichroism spectroscopy [3,4,6–8,11] indicate thermally induced chain exchange between TM dimers.

2. Materials and methods

Smooth muscle TM was obtained as a byproduct of caldesmon purification from frozen duck gizzards [12]. A crude preparation of TM was incubated for 3 h at 25°C with 0.4 M β-mercaptoethanol in the presence of 8 M urea (pH 8.0), dialyzed against 50 mM sodium formate (pH 4.0) containing 20 mM KCl, 1 mM EDTA, and 8 M urea, and subjected to chromatography on carboxy-methylcellulose using the method of Smillie [13]. Fractions containing homogeneous α and β isoforms were pooled, dialyzed against water, and freeze-dried. Unfractionated TM was used as the αβ heterodimer. Protein concentration was determined spectrophotometrically, using $E^{1\%}$ at 277 nm equal to 1.9, 2.2, and 1.7 for αβ heterodimer, αα homodimer, and ββ homodimer, respectively [6].

Disulfide cross-linking of TM homodimers was performed by Cu^{2+}-catalyzed $K_3Fe(CN)_6$ oxidation [3]. TM homodimers (2 mg/ml) were incubated for 2 h at room temperature in the presence of 5 mM $K_3Fe(CN)_6$ and 2 μM $CuSO_4$, and the reaction was stopped by addition of excess EDTA. The content of cross-linked αα and ββ homodimers was estimated by 7.5% polyacrylamide gel electrophoresis in the presence of SDS [14] which was run in the absence of β-mercaptoethanol.

Calorimetric measurements were performed on a DASM-4M differential scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia) with platinum 0.48 ml cells, at a scanning rate of 1000/min. Prior to DSC experiments, the proteins were dialyzed against 15 mM HEPES, pH 7.3, 60 mM KCl, 1 mM $MgCl_2$. In the case of reduced proteins the buffer also contained 1 mM β-mercaptoethanol. All measurements were performed at a protein concentration of 1 mg/ml. The reversibility of the thermal transitions was checked by a second heating of the sample immediately after cooling from the first scan. The calorimetric traces were corrected for the instrumental background by subtracting a scan with buffer in both cells. The temperature dependences of the molar heat capacity were further corrected using a chemical baseline [15], and then analyzed and plotted using the Windows-based software package Origin (Microcal Inc.). A molecular weight of 66 kDa was used for all coiled-coil TM dimers.

3. Results and discussion

3.1. Thermal unfolding of TM dimers

Fig. 1 shows the calorimetric data on the thermally induced denaturation of unfractionated smooth muscle TM, isolated αα and ββ TM homodimers, and their 1:1 mixture. In order to avoid disulfide cross-linking of TM homodimers, these experiments were performed in the presence of 1 mM β-mercaptoethanol. Dotted line curves represent DSC scans of the second heating of the samples immediately after cooling from the first scan, thus showing the reversibility of the thermal transitions. Unfractionated TM, which is predominantly αβ heterodimer [4,6], demonstrates a sharp transition with a maxi-

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Abbreviations: TM, tropomyosin; DSC, differential scanning calorimetry

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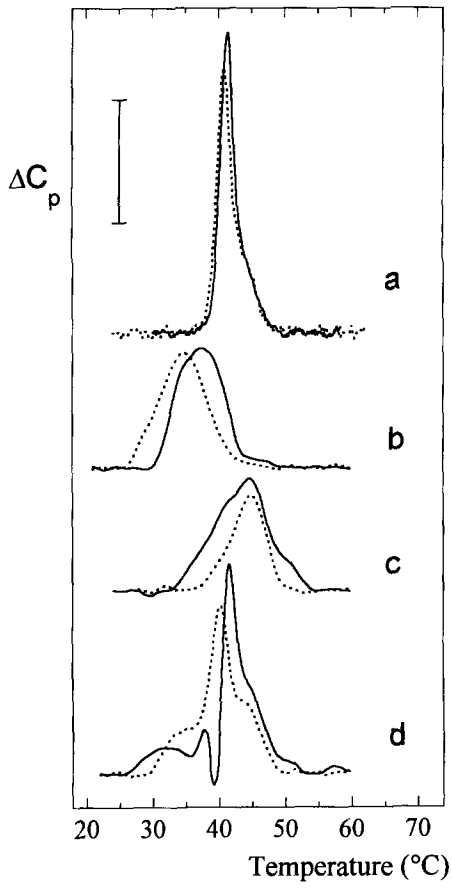

Fig. 1. DSC scans of unfractionated predominantly heterodimeric αβ TM (a), αα homodimer (b), ββ homodimer (c), and 1:1 mixture of αα and ββ homodimers (d). Protein concentration 1 mg/ml. Concentrations of αα and ββ homodimers in their mixture were 0.5 and 0.5 mg/ml, respectively. Conditions: 15 mM HEPES, pH 7.3, 60 mM KCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol. Curves shown by dotted lines were obtained by a second heating of the same samples immediately after cooling from the first scan. Heating rate 1 K/min. The vertical bar corresponds to 100 kJ K^{-1} mol^{-1}.

mum at 41.3°C. This transition was almost completely reversible (Fig. 1a), with calorimetric enthalpy, ΔH_{cal} (the area under the heat sorption curve), equal to 810 ± 40 kJ/mol for both the first and the second heating. In contrast to αβ heterodimer, both αα and ββ homodimers demonstrate a broad thermal transition with maxima at 37.4°C and 44.6°C, respectively (Fig. 1b,c). The width at the half-height of the transition, $\Delta T_{0.5}$, which is a relative measure for the cooperativity of the transition, was 8.8°C for αα and 9°C for ββ, being much larger than the corresponding value for αβ heterodimer (2.5°C). In contrast to αβ heterodimer whose denaturation was almost completely reversible (Fig. 1a), the heat sorption curves of αα and ββ homodimers observed in a second heating were different from those observed in the first heating (Fig. 1b,c). The curve obtained from re-heating of αα-TM was shifted by 2.8°C to the lower temperature (Fig. 1b), without any significant changes of ΔH_{cal} (850 ± 40 kJ/mol) and $\Delta T_{0.5}$ (8.5°C) values. On the other hand, re-heating of ββ-TM led to a significant decrease of the ΔH_{cal} (from 910 ± 50 kJ/mol to 500 ± 30 kJ/mol) and $\Delta T_{0.5}$ (from 9°C to 6°C) without significant changes of the maximum of the transition (Fig. 1c). Thus, αα and ββ homodimers differ from each other, and their thermal unfolding is quite different from that of αβ heterodimer.

A 1:1 mixture of the two TM homodimers shows a complicated DSC profile (Fig. 1d) which cannot be obtained by simple summation of the αα and ββ profiles (Fig. 1b,c). Up to 32°C it follows the unfolding of αα homodimer; however, above 33°C, when ββ homodimer begins to denature (Fig. 1c), an exothermic peak appears on the thermogram (Fig. 1d). This exothermic peak is superimposed with thermal transition of αα homodimer at 37.5°C, and above 39°C it turns into a sharp endothermic peak with a maximum at 41.5°C (Fig. 1d). The exotherm probably reflects chain exchange between αα and ββ homodimers leading to formation of αβ heterodimer. This newly formed heterodimer unfolds at 41.5°C. The data presented agree with earlier published results obtained with circular dichroism spectroscopy [3,4,6–8]. Very similar results were obtained for the mixture of the two TM homodimers at high ionic strength, in the presence of 0.5 M NaCl (data not shown). This means that the end-to-end interaction of TM molecules which is strongly diminished at high ionic strength [3] does not contribute to the sharp thermal transition at 41.5°C. Thus, the appearance of this transition characteristic for αβ heterodimer during the heating of the homodimers mixture is caused only by chain exchange during thermal unfolding.

During the second heating of the αα+ββ mixture the exotherm completely disappeared (Fig. 1d, dotted line curve), thus indicating that the chain exchange process was already completed. The curve obtained after the second heating was

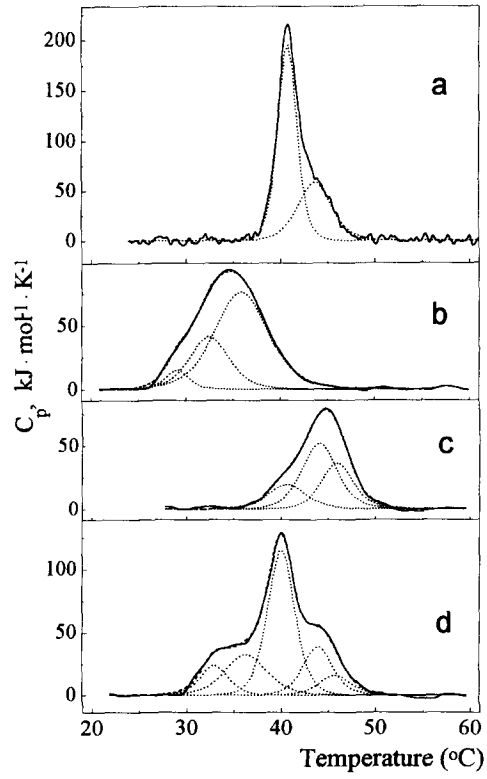

Fig. 2. Deconvolution of endotherms obtained from the second heating (dotted lines in Fig. 1) for αβ heterodimer (a), αα homodimer (b), ββ homodimer (c), and the mixture αα+ββ (d). Solid lines represent the experimental curves, dashed lines are results of fitting of experimental data to the non-two-state model, and dotted lines represent the individual components (calorimetric domains).

shifted by 1.3°C to a lower temperature without any significant changes of the ΔH_{cal} value (995 ± 50 kJ/mol). The curves obtained from the third heatings of all samples of TM were almost fully identical to the curves obtained from the second heatings (data not shown). This means that thermal transitions resulted from the second heating are reversible and therefore these curves can be used for deconvolution into individual components.

3.2. Deconvolution of endotherms

Fig. 2 shows the results of deconvolution analysis of the curves obtained from the second heating of native TM, αα and ββ homodimers, and their 1:1 mixture. The main calorimetric parameters for individual thermal transitions (transition temperature, T_m, and calorimetric enthalpy, ΔH_{cal}) are summarized in Table 1. The curve for native TM, which is predominantly αβ heterodimer, was decomposed into two individual transitions: the main sharp transition 1 with a maximum at 40.8°C, and a smaller transition 2 with a maximum at 43.7°C (Fig. 2a). The thermal transitions of αα and ββ homodimers are multistate, requiring at least three individual transitions (or calorimetric domains) to fit the DSC data (Fig. 2b,c, Table 1), in good agreement with earlier DSC studies on these proteins [5]. The DSC curve for αα+ββ mixture has been decomposed into five individual transitions (Fig. 2d). Among them, transitions 1 and 2 correspond, respectively, to transitions 2 and 3 of αα homodimer, and the sharpest and most intense transition 3 corresponds to the main transition 1 of αβ heterodimer (Fig. 2, Table 1). As to transitions 4 and 5, they can be assigned both to transition 2 of αβ heterodimer and to transitions 2 and 3 of ββ homodimer.

Thus, the results presented above clearly demonstrate the formation of αβ heterodimer from the mixture of homodimers due to thermally induced chain exchange. On the other hand, the presence of individual transitions characteristic for αα homodimer on Fig. 2d indicates that some chains remain assembled into homodimers even after second heating of the homodimer mixture. Chain exchange can be prevented by interchain disulfide cross-links in the homodimers.

Table 1
Calorimetric parameters of individual thermal transitions shown by dotted lines in Fig. 2 for unfractionated TM (αβ heterodimer), αα and ββ homodimers, and their 1:1 mixture (αα+ββ)

TM dimer	T_m (°C)	ΔH_{cal} (kJ/mol)
αβ		
Transition 1	40.8	525
Transition 2	43.7	275
αα		
Transition 1	29.1	48
Transition 2	32.5	228
Transition 3	35.9	570
ββ		
Transition 1	40.7	102
Transition 2	44.1	257
Transition 3	46.0	162
αα+ββ		
Transition 1	33.0	94
Transition 2	36.4	192
Transition 3	40.1	441
Transition 4	44.0	162
Transition 5	45.8	71

The absolute error of the given T_m values did not exceed ±0.3°C; the relative error of the given ΔH_{cal} values did not exceed 15%.

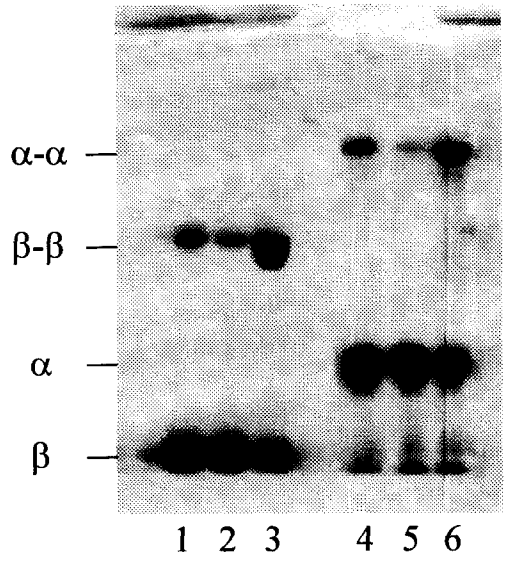

Fig. 3. SDS-gel electrophoresis pattern of TM ββ (1–3) and αα (4–6) homodimers. Lanes 1 and 4 represent the samples reduced in the presence of 1 mM β-mercaptoethanol prior to DSC experiments; lanes 2 and 5 represent the same samples extracted from calorimetric cells after the second heating; lanes 3 and 6 represent the homodimers oxidized by $K_4Fe(CN)_6$. The monomer bands are labeled α and β, the corresponding disulfide cross-linked homodimers are labeled α-α and β-β.

3.3. Disulfide cross-linking of homodimers

Each homodimer of smooth muscle TM can be disulfide cross-linked at a single site (Cys-190 in αα and Cys-36 in ββ species), whereas αβ heterodimer cannot form disulfide cross-links since cysteines on α and β chains are far separated from each other [3,16]. Even reduced homodimers in the presence of 1 mM β-mercaptoethanol contain small quantities of cross-linked species (Fig. 3, lanes 1 and 4). After the heating the proportion of cross-linked dimer is significantly reduced (Fig. 3, lanes 2 and 5). This change in the oxidation state of TM homodimers may be the reason for the differences observed between DSC curves obtained from the first and the second heating of the homodimers (Fig. 1b,c). Cross-linked homodimers cannot undergo chain exchange and this will prevent heterodimer formation during the heating of an equimolar mixture of two homodimers. To check this assumption we oxidized two homodimers and studied their mixture by DSC.

We were unable to achieve complete cross-linking of TM monomers; however, after the oxidation procedure the content of cross-linked homodimers was significantly increased, up to ~35% for αα and up to ~70% for ββ (lanes 3 and 6 in Fig. 3). DSC profiles of these cross-linked homodimers and their mixture are shown in Fig. 4. Since the proportion of cross-linked αα homodimer was rather low (about 35%), the curve obtained for oxidized αα homodimer (dotted line curve on Fig. 4) was similar to that of reduced αα homodimer (Fig. 1b). On the other hand, cross-linking of ββ homodimer resulted in a pronounced change of its thermal unfolding. In this case two well separated peaks were observed on the thermogram (dashed line curve on Fig. 4): the smaller peak with maximum at 44.5°C characteristic for reduced ββ homodimer (Fig. 1c), and the main peak with maximum at 50.1°C corresponding to cross-linked ββ homodimer. These results agree with earlier DSC studies showing that intramolecular cross-

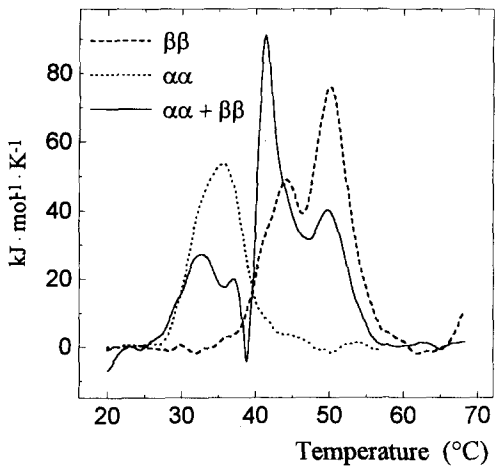

Fig. 4. DSC scans of oxidized TM homodimers αα (dotted line) and ββ (dashed line) (lanes 3 and 6 in Fig. 3) and of their equimolar mixture (solid line). Conditions were the same as in Fig. 1 except that β-mercaptoethanol was omitted.

linking of TM dimers strongly increases their thermal stability [5,10,17]. The DSC profile for the mixture of two oxidized homodimers shows the exotherm reflecting the chain exchange and a sharp thermal transition with a maximum at 41.4°C corresponding to the melting of αβ heterodimer (solid line curve on Fig. 4). However, the peak at 50.1°C corresponding to the melting of cross-linked ββ homodimer is also observed on this curve. Taking into account that the concentration of each homodimer in the mixture (0.5 mg/ml) was half of that for separately measured homodimers (1 mg/ml), and the size of the peak at 50.1°C for homodimers mixture was about half of that for cross-linked ββ homodimer, one can conclude that cross-linked ββ homodimers remain unchanged during the heating of the homodimer mixture. This means that the cross-linked dimer does not participate in the thermally induced chain exchange leading to heterodimer formation.

In conclusion, our present DSC results agree with the data obtained by other methods [3,4,6–8,11] and indicate that heating may induce chain exchange between TM dimers and that this process is prevented by disulfide cross-linking of polypeptide chains in the homodimers.

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