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DIFFERENCES IN THERMAL STABILITY OF FROG AND RABBIT $\alpha\alpha$- AND $\alpha\beta$-TROPOMYOSINS DETERMINED BY OPTICAL ROTATORY DISPERSION

Søren HVIDT

Departments of Chemistry, Risø National Laboratory and Roskilde University, DK-4000 Roskilde, Denmark

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Frog and rabbit $\alpha\alpha$- and $\alpha\beta$-tropomyosins were purified, and their thermal stabilities determined by use of optical rotatory dispersion. The tropomyosins were found to be virtually completely helical at 5°C. Regions of different thermal stabilities were seen for all tropomyosins. Rabbit and frog $\alpha\alpha$-tropomyosin show very similar thermal properties, with main transitions near 47–49°C. The main transition for frog $\alpha\beta$-tropomyosin is at 32°C. The results show that the $\alpha\beta$-tropomyosins are less stable than the $\alpha\alpha$-forms. Only thermal transitions of the $\alpha\beta$-forms appear to be correlated with the body temperatures of the animals.

1. Introduction

Tropomyosin (TM) is an important component of the contractile apparatus of skeletal and cardiac muscles, where it is involved in the calcium-dependent regulation of myosin-actin interactions together with troponin [1]. TM is also found in several nonmuscle cells [2], where its function is at present unclear. Rabbit TM consists of two 284-residue peptide chains, arranged in register and parallel, forming a coiled-coil α-helical structure [1]. Skeletal muscle tissues contain two major forms of TM chains, designated as α- and β-chains. Most tissues contain $\alpha\alpha$- and $\alpha\beta$-TMs, in proportions depending on the type and source of muscle [3]. The physiological significance of the two TM forms remains unknown, and little is known about physicochemical and biochemical differences besides their amino acid sequences [4] and degrees of phosphorylation [5].

The thermal stability of rabbit $\alpha\alpha$-TM has been studied extensively by use of a broad range of techniques including circular dichroism and optical rotatory dispersion (ORD) [6], fluorescence [7], calorimetry [8] and enzyme probes [9]. Most of these studies support the idea that rabbit TM contains regions or domains with different thermal stabilities. Less stable domains could have physiological significance in terms of giving flexibility to the structure or being involved in conformational transitions during regulation [6–10]. Only few studies have been reported on the stabilities of TMs from other species and $\alpha\beta$-forms [10–12]. Therefore, thermal stabilities, as monitored by ORD, were determined of $\alpha\alpha$- and $\alpha\beta$-TMs from frog and rabbit, in order to investigate whether $\alpha\alpha$- and $\alpha\beta$-TM, show different thermal stabilities and to see whether thermal transitions are correlated with the body temperatures of the animals.

2. Materials and methods

Rabbit and frog (*Rana temporaria*) TMs were extracted and purified from ethanol and acetone-

dried powder following standard procedures [13]. Leg muscles were used for frog and rabbit $\alpha\beta$-TM preparations. Back muscles of rabbits were used for $\alpha\alpha$-TM preparations. Crude TM solutions were isoelectrically precipitated twice at pH 4.6, followed by ammonium sulfate fractionation at pH 7.0. Fractions between 48 and 60% saturation were collected. Hydroxyapatite chromatography was used to separate $\alpha\alpha$- and $\alpha\beta$-TMs [14] in 1 M KCl and 15 mM β-mercaptoethanol at pH 7.0. Elutions were performed with linear gradients from 50 to 200 and from 10 to 150 mM phosphate, respectively, for rabbit and frog preparations. Pure fractions were collected, isoelectrically precipitated, and dialyzed against 0.6 M NaCl, 20 mM sodium phosphate (pH 7.0), 0.5 mM EDTA and 0.2 mM NaN_3 (buffer M). Sample purities were determined by SDS-polyacrylamide gel electrophoresis. A Tris-glycine slab gel system was used with 12% acrylamide. The relative content of α- and β-chains was determined from densitometric scans (LKB 2002) of the Coomassie blue-stained protein bands.

Protein concentrations were determined by fringe counting in a model E analytical ultracentrifuge, assuming 40 fringes/1% solution. The ultraviolet absorptions of the same solutions were also measured on a Varian spectrophotometer at 5°C. ORD measurements were made using a Cary model 60 spectropolarimeter equipped with a thermostatted 1 cm cell. The Moffitt-Yang parameter (b_0), the corrected mean residue specific rotation at 231.4 nm ($[m']_{231.4}$) and the ratio of rotational angles at 215 and 231.4 nm ($\alpha_{215}/\alpha_{231.4}$) were determined at 5°C, as described elsewhere [15]. The thermally induced unfolding of TM between 5 and 70°C was monitored by following the temperature dependence of $[m']_{231.4}$ in steps of 1–3°C. Most ORD measurements were made on samples dialyzed extensively against buffer M, to which 0.5 mM dithiothreitol was added as a sulfhydryl-reducing agent, since cysteines are believed to be in the reduced state in muscles. ORD unfolding measurements were also performed on frog $\alpha\beta$-TM samples with methylcarboxyamidated sulfhydryl groups in buffer M. Iodoacetamide was used following the procedure described by Lehrer et al. [7]. No free sulfhydryl groups were detected after the reaction, indicating nearly complete blocking of cysteines [7]. Excess iodoacetamide was removed by isoelectric precipitation followed by dialysis against buffer M without reducing agent. In order to follow the unfolding of TM under more nearly physiological salt concentrations a solution of reduced frog $\alpha\beta$-TM was also dialyzed against 90 mM KCl, 20 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 2 mM $MgCl_2$, and 0.5 mM dithiothreitol prior to the ORD measurements.

3. Results

Frog TM resembles rabbit TM in showing two bands on polyacrylamide gels, as shown in fig. 1. The fastest migrating band for rabbit has been designated the α-band, and the slowest one the β-band. The same notation will also be used for frog TMs. Hydroxyapatite chromatography, which has often been used to separate rabbit $\alpha\alpha$- and $\alpha\beta$-forms, also results in an excellent separation of the two frog forms. An estimate of the purity of the $\alpha\alpha$- and $\alpha\beta$-TM preparations was obtained by use of densitometric scans based on the assumption of similar staining intensity of α- and β-chains. Samples of purified $\alpha\alpha$-TM never contained more than 2% $\alpha\beta$-TM contamination, and purified $\alpha\beta$-TM contained between 48.5 and 50% β-chains, which corresponds to, at most, 3% $\alpha\alpha$-TM contamination. The rabbit $\alpha\alpha$-TM preparations only showed trace amounts of $\alpha\beta$-TM,

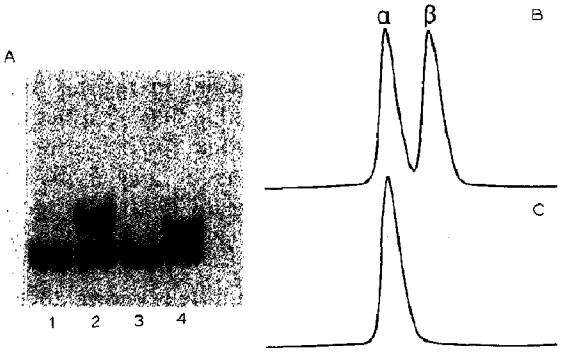

Fig. 1. SDS-polyacrylamide gel electrophoresis of TMs. (A) Lane 1, rabbit $\alpha\alpha$; lane 2, rabbit $\alpha\beta$; lane 3, frog $\alpha\alpha$; lane 4, frog $\alpha\beta$. (B and C) Densitometric scans of frog $\alpha\beta$- and $\alpha\alpha$-TM, respectively.

whereas rabbit $\alpha\beta$-TM preparations often contained between 10 and 15% $\alpha\alpha$ forms.

The ultraviolet absorption spectra of the four TM forms are very similar. The extinction coefficient at 277 nm was found to be 0.290 ± 0.006 ml/mg per cm for all four samples, after subtraction of the absorption at 320 nm. The ratios of absorptions at 277 and 260 nm were between 2.3 and 2.6, indicating little or no contamination with nucleotides. ORD properties of the four TMs are also very similar at 5°C, as shown in table 1. Three indicators of helical content were used, and the calculated helical contents, based on assumed reference ORD values for completely helical and random states [15], are shown in parentheses. Fig. 2 illustrates the unfoldings of reduced frog $\alpha\beta$-TM samples in buffer M and in the solvent with more nearly physiological ionic strength. Unfolding occurs at 2–3°C higher temperatures in buffer M than in the lower ionic strength solvent. The unfolding of methylcarboxyamidated frog $\alpha\beta$-TM is also shown in fig. 2, and it is seen to be virtually identical to the unfolding of the reduced form. The reversibility of the unfolding was followed by cooling the TM solutions back to 5°C from the highest temperature. Typically 90–95% of the loss in $[m']_{231.4}$ during the thermal unfolding was recovered after cooling. One way of characterizing the thermal transitions is to find the temperature, $T_{1/2}$, where the $[m']_{231.4}$ value is the average of its values at 5 and 70°C. Values of $T_{1/2}$ for all four TMs are summarized in table 2. The solid lines in Fig. 2 correspond to the temperature-dependent helical and random coil reference states [15]. The difference quotients were then calculated as $\Delta f_h/\Delta T$, where ΔT is the temperature increment between two measurements, and then plotted against the average temperature before and after the temperature increase. The unfolding process of frog $\alpha\beta$-TM is seen to result in two well-resolved major transitions, and the three other systems show a minor transition between 33 and 38°C together with a major transition between 47 and 50°C. The temperatures corresponding to the first and second peak are denoted T_1 and T_2, respectively, and were reproducible to within ± 1°C. Values of T_1 and T_2 are summarized in table 2. The approximate fractions of the structures involved in the low-temperature transitions were estimated from the areas under the peaks, based on a somewhat arbitrary separation of the transition curve into two components. Only the frog $\alpha\beta$-TM transition curve is resolved well enough for accurate estimates of the fractions.

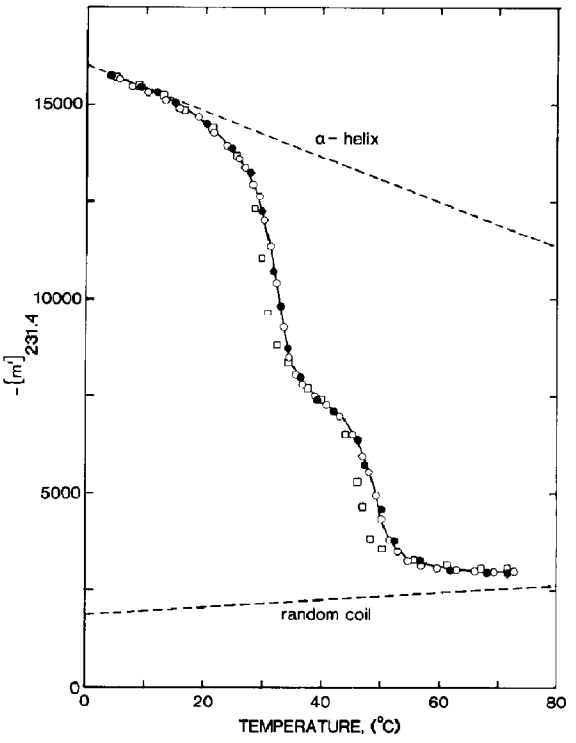

Fig. 2. Thermal unfolding of frog $\alpha\beta$-TM, followed by ORD. (○) In buffer M, (●) iodoacetamide-treated TM in buffer M without dithiothreitol, (□) in physiological solvent. A smooth curve is drawn through the data in buffer M. Broken lines refer to the temperature-dependent helical and random coil reference states [15].

Table 1

ORD properties of tropomyosins at 5°C

Values in parentheses are helical contents, calculated as described in ref. 15. Units of [m'] and b_0 are deg/cm per dmol.

Sample	$-[m']_{231.4}$	$-b_0$	$-\alpha_{215}/\alpha_{231.4}$
Rabbit $\alpha\alpha$	15600 (0.99)	625 (0.98)	1.181 (0.93)
Rabbit $\alpha\beta$	15400 (0.98)	623 (0.97)	1.190 (0.95)
Frog $\alpha\alpha$	15800 (1.01)	623 (0.97)	1.187 (0.94)
Frog $\alpha\beta$	15600 (0.99)	627 (0.98)	1.195 (0.96)

Table 2

Transition temperatures (°C) of tropomyosins

Sample	$T_{1/2}$	T_1 [a]	T_2
Rabbit $\alpha\alpha$	46	34 (11)	47
Rabbit $\alpha\beta$	44	32 (21)	47
Frog $\alpha\alpha$	47	38 (16)	50
Frog $\alpha\beta$	33	32 (59)	49
Frog $\alpha\beta$ [b]	33	32 (59)	49
Frog $\alpha\beta$ [c]	31	30 (57)	46

[a] Values in parentheses denote percentage of total transition involved in T_1 transition.

[b] Iodoacetamide-treated TM in buffer M without dithiothreitol.

[c] Sample in physiological solvent (see text).

4. Discussion

The results summarized in table 1 indicate that the four TM forms are nearly completely helical at low temperatures, in agreement with findings for rabbit $\alpha\alpha$-TM of many previous investigations. Several studies have also indicated that rabbit $\alpha\alpha$-TM contains regions or domains with different thermal stabilities [6–10]. Evidence for less stable regions near the middle [7–9] or in the C-terminal half [6] of rabbit $\alpha\alpha$-TM has been accumulated. The presence of such regions has been inferred from studies of fragments [6,8] or from the existence of pre-transitions during the thermally induced unfolding process [7,9]. The derivative plots in fig 3 show such low-temperature transitions between 30 and 35°C for all the TMs. The high-temperature transitions occur near 47°C, and the fact that they are not smooth and symmetrical indicate that even these transitions are not simple two-state transitions, but probably involve unfolding of several domains with similar transition temperatures in agreement with earlier studies on rabbit $\alpha\alpha$-TM [6,8]. The transition temperatures depend somewhat on solvent composition, as seen in fig. 1 and table 2. In the solvent with lower salt concentrations the transition temperatures are about two degrees lower than in buffer M for both transitions, and the shapes of the derivative curves are also very similar in the two solvents. The transitions are also seen to be independent of whether the sulfhydryl groups are blocked or reduced, making it unlikely that the two-state unfolding is due to disulfide formation.

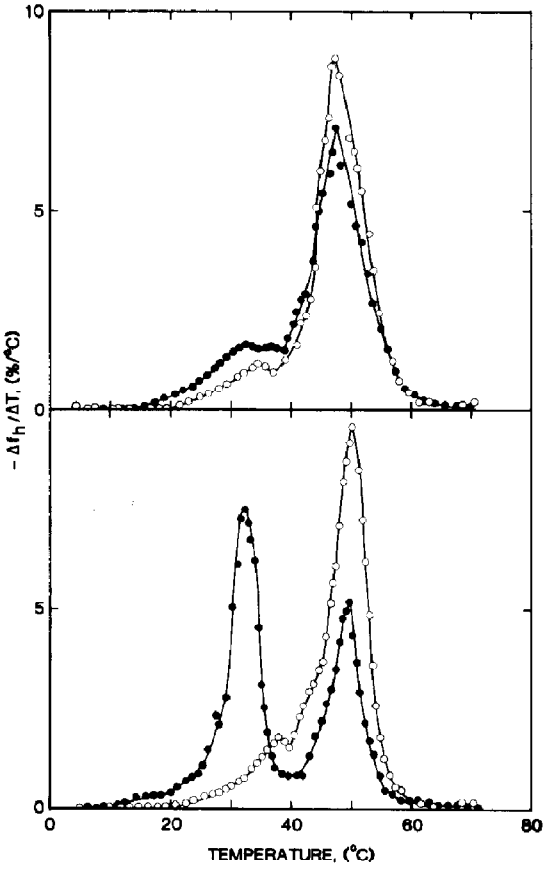

Fig. 3. Thermally induced helix-coil transitions of TMs in buffer M. The difference quotients of loss in helicity to temperature increment are plotted against temperature. Top panel shows results for rabbit; bottom for frog: (○) $\alpha\alpha$-TM, (●) $\alpha\beta$-TM.

The physiological significance of the low-temperature transition and the existence of both $\alpha\alpha$- and $\alpha\beta$-forms remains unclear. Rabbit $\alpha\beta$-TM is seen to be about 2°C less stable than the rabbit $\alpha\alpha$-form, when $T_{1/2}$ is used as an indicator of thermal stability. The frog $\alpha\beta$-TM unfolding demonstrates a very clear example of a low-temperature transition which involves a very significant fraction of the structure (59%). The results also show a clear $T_{1/2}$ difference of 14°C between the frog $\alpha\alpha$- and $\alpha\beta$-TM.

Although the physiological implications are un-

clear, our ORD results for rabbit and frog TM indicate that there are physicochemical differences between the $\alpha\alpha$- and $\alpha\beta$-forms, and that $\alpha\beta$-forms are less stable and therefore perhaps more flexible than $\alpha\alpha$-forms. The body temperature of rabbit is near 37°C, and 13–26°C for frog [16]. This physiological difference does not give rise to differences in thermal stability of the two $\alpha\alpha$-TMs. Frog $\alpha\alpha$-TM is even seen to be slightly more stable than rabbit $\alpha\alpha$-TM. So the thermal transitions in the $\alpha\alpha$-forms do not show any correlation with the body temperatures of the muscle. The two $\alpha\beta$-forms exhibit clear differences in thermal stabilities. The difference in $T_{1/2}$ is 11°C as compared with a body temperature difference of 11–24°C between the two species. The stability of the $\alpha\beta$-form appears therefore to be more nearly correlated with the physiological temperatures. Whether this is also true for $\alpha\beta$-TMs from other species remains to be seen from further investigations.

Conformational transitions in tropomyosin and myosin rod, another coiled-coil α-helical structure from muscles, have been studied extensively in recent years both experimentally and theoretically [17]. Various factors appear to be important for the stability of domains in coiled-coil structures. Such structures contain a high percentage of helix-forming amino acid residues. An amino acid composition analysis on frog $\alpha\alpha$- and $\alpha\beta$-TM (unpublished result) show that the latter contains lower percentages of alanine, leucine and isoleucine, which are among the helix-forming amino acids [18]. Frog $\alpha\beta$-TM is at the same time richer in threonine, valine, and methionine, which are not especially favourable for helix formation. The amino acid composition differences of frog $\alpha\alpha$- and $\alpha\beta$-TM may therefore explain part of the difference in thermal stability of the coiled-coil structures. Another important factor for the stability of the coiled-coil structure is the hydrophobic interactions between the two chains [1,4], but investigations of such effects necessitate knowledge about the amino acid sequences. Thermal stabilities of domains in TM are important in order to understand its physiological role during contraction and to separate localized flexibility in the structure from the general flexibility of coiled-coil proteins [19]. Frog TMs appear to be promising systems for further investigations of the role of $\alpha\alpha$- and $\alpha\beta$-TMs during muscle contraction and of the factors which in general regulate the stability of coiled-coil proteins.

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