Eur. J. Biochem. **271**, 4132–4140 (2004) © FEBS 2004 doi:10.1111/j.1432-1033.2004.04351.x

Effects of cardiomyopathic mutations on the biochemical and biophysical properties of the human α-tropomyosin

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Mutations in the protein α-tropomyosin (Tm) can cause a disease known as familial hypertrophic cardiomyopathy. In order to understand how such mutations lead to protein dysfunction, three point mutations were introduced into cDNA encoding the human skeletal tropomyosin, and the recombinant Tms were produced at high levels in the yeast *Pichia pastoris*. Two mutations (A63V and K70T) were located in the N-terminal region of Tm and one (E180G) was located close to the calcium-dependent troponin T binding domain. The functional and structural properties of the mutant Tms were compared to those of the wild type protein. None of the mutations altered the head-to-tail polymerization, although slightly higher actin binding was observed in the mutant Tm K70T, as demonstrated in a cosedimentation assay. The mutations also did not change the cooperativity of the thin filament activation by increasing the concentrations of Ca^{2+}. However, in the absence of troponin, all mutant Tms were less effective than the wild type in regulating the actomyosin subfragment 1 Mg^{2+} ATPase activity. Circular dichroism spectroscopy revealed no differences in the secondary structure of the Tms. However, the thermally induced unfolding, as monitored by circular dichroism or differential scanning calorimetry, demonstrated that the mutants were less stable than the wild type. These results indicate that the main effect of the mutations is related to the overall stability of Tm as a whole, and that the mutations have only minor effects on the cooperative interactions among proteins that constitute the thin filament.

Keywords: circular dichroism; differential scanning calorimetry; *Pichia pastoris*; tropomyosin.

Tropomyosins (Tms) are a family of highly conserved proteins found in most eukaryotic cells. The striated muscle isoform is an α-helical protein, which forms a parallel coiled-coil dimer twisted around the long axis of the actin filament. Each polypeptide chain has 284 amino acid residues, and each dimer binds to seven actin monomers and one troponin (Tn) complex (TnC, TnI and TnT). In striated muscle cells the Tm polymerizes in a head-to-tail fashion, and together with the troponin complex, regulates the Ca^{2+} sensitivity of the actomyosin Mg^{2+} ATPase complex [1]. The Tm amino acid sequence shows a seven-residue pattern (a to g) repeated throughout the entire sequence. Positions a and d, on the same side of the helices, are usually occupied by apolar amino acids that allow hydrophobic interactions between chains. Positions e and g are often occupied by charged residues, and therefore contribute to the stabilization of the parallel coiled-coil structure by ionic interactions with residues at positions e′ and g′ of the other helix. Positions b, c and f are occupied by polar or ionic residues and they interact with solvent or other proteins [1]. In addition to the heptapeptide repeat, there are seven consecutive repetitions of approximately 40 residues each in the entire length of the chain, which correspond to the actin binding sites [2].

Recombinant Tms have been produced in different host cells and the proteins used as tools to obtain information about the relevant regions for functional and structural properties. The recombinant Tm was first produced in *Escherichia coli* but the protein was not N-acetylated [3], and therefore, lacked the functional properties that depended on this modification. Fully functional Tm was produced in *E. coli* by changing the primary structure of the protein with the addition of a dipeptide or a tripeptide at the N-terminal methionine [4]. Our group has successfully shown that *Pichia pastoris* and *Saccharomyces cerevisiae* are capable of producing functional Tms unmodified in their primary structure [5,6]. The proteins are probably N-acetylated, their N-terminal methionine is blocked, and they behave identically to the native Tm in their functional properties, thus making them preferable for structure–function studies to probe amino acid mutations that have been described in cardiomyopathic tropomyosins.

Familial hypertrophic cardiomyopathy (FHC) is a clinically and genetically heterogeneous heart disease characterized by hypertrophy and ventricular dysfunction [7]. The incidence of the disease is high [8], and up to the present date numerous mutations within the genes encoding for the sarcomeric cardiac proteins α-tropomyosin, troponin T, and

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Abbreviations: FHC, familial hypertrophic cardiomyopathy; S1, myosin subfragment 1; T_m, temperature of the midpoint of the thermal unfolding transition; Tm, tropomyosin; Tn, troponin.

(Received 9 July 2004, revised 20 August 2004, accepted 31 August 2004)

myosin heavy-chain have been reported. The frequency of mutation in the α-tropomyosin gene (*TPM1*) is lower, accounting for approximately 5% of FHC, however, different point mutations leading to mutant proteins have been described in the last few years: E62Q [9], A63V [10,11], K70T [10], D175N [12] E180G [12], E180V [13], L185R [14]. Mutations occur mainly in two regions of the protein, one located in the N-terminal domain and the other close to the troponin-binding region of tropomyosin.

Several studies based on the cardiomyopathic mutations D175N and E180G have been reported. *In vivo* studies, using transgenic mice as a model showed an impairment of cardiac function by altering the sensitivity of myofilaments to Ca^{2+} [15]. *In vitro* studies, with recombinant proteins carrying the mutations, demonstrated small effects on the overall stability of the protein as measured by circular dichroism [16], and showed alterations in the kinetics of contractile force generation [17]. Studies with mutations A63V and K70T reported higher muscle Ca^{2+} sensitivity both *in vivo* [18], and, more recently, *in vitro* [19], in addition to prominent effects on the Tm thermal stability as monitored by circular dichroism [19].

In the present study, we combined the biophysical assays – circular dichroism and differential scanning calorimetry – and recombinant human Tm produced in *P. pastoris*, to investigate the effects of cardiomyopathic-related mutations on the human skeletal Tm. Our data indicate that the main effects of mutations A63V, K70T and E180G are mainly related to the overall stability of the protein as a whole, rather than on the position of the mutation in the polypeptide chain, as demonstrated by the biophysical assays. Our studies have provided additional contributions to the understanding of the effects of these mutations on the clinical symptoms of patients carrying cardiomyopathic Tms.

Experimental procedures

Construction of expression plasmids and site-directed mutagenesis

The pPIC9 expression vector and *P. pastoris* strain GS115 (*his*4) (Invitrogen, Life Technologies) were used for Tm production. Oligonucleotides were designed based on the sequence of human skeletal muscle cDNA (skα-TM.1) [20]. The full length coding sequence was amplified by PCR with the oligonucleotides Tm-7F (5′-CGGGATCC**ACC**ATGG ATGCCATCAAG-3′) and Tm-9R (5′-ATAAGAATGCG GCCGCTTATATGGAAGTCAT-3′). The underlined sequences correspond to *Bam*HI and *Not*I sites, respectively. The oligonucleotide Tm-7F contains an ACC sequence (shown in bold) immediately upstream of the start codon [21]. The amplified cDNA was digested with *Bam*HI and *Not*I, and subcloned into the same sites of vector to produce the PIC9-WT expression plasmid.

DNA sequences encoding A63V, K70T and E180G mutant Tms were amplified by PCR in two steps using standard procedures [22]. The oligonucleotides AOX-F (5′-GCGACTGGTTCCAATTGAC-3′), AOX-R (5′-GG TCTTCTCGTAAGTGCCC-3′), SKTM-A63V (5′-GAC AAATACTCTGAAGTACTCAAAGATGCCCAG-3′), SK TM-1R (5′-CTGGGCATCTTTGAGTACTTCAGAGTA TTGTC-3′), SKTM-K70T (5′-AAAGATGCACAGGAG ACGCTGGAGCTGGCAGAG-3′), SKTM-2R (5′-CTCTG CCAGCTCCAGCGTCTCCTGTGCATCTTT-3′), SKTM-E180G (5′-CTGGAACGTGCAGGGGAGCGGGCTGAA CTCTCAGAAGG-3′) and SKTM-4R (5′-CCTTCTGA GAGTTCAGCCCGCTCCCCTGCACGTTCCAG-3′) were used for the amplifications. To perform A63V, K70T and E180G point mutations (underlined in the primer sequences), two DNA fragments of each mutation were initially amplified using, respectively, the primers AOX-F/SKTM-A63V and AOX-R/SKTM-1R, AOX-F/SKTM-K70T and AOX-R/SKTM-2R, and AOX-F/SKTM-E180G and AOX-R/SKTM-4R. The entire cDNA sequences containing the mutations were amplified with the AOX-F and AOX-R primers, digested with *Bam*HI and *Not*I and subcloned into pPIC9 vector leading to PIC9-A63V, PIC9-K70T, and PIC9-E180G expression plasmids.

The *E. coli* strain MC1061 [23] was used for plasmid amplification. The complete cDNA sequences were confirmed by automatic DNA sequencing.

Production and purification of recombinant proteins

Expression plasmids were linearized with *Bgl*II, and used to transform competent GS115 cells by electroporation. Cells were also transformed with linearized pPIC9 plasmid not carrying the cDNA. His^+ transformants were selected on minimal medium agar plates containing 0.4% (w/v) yeast nitrogen base without amino acids, 1% (w/v) ammonium sulfate, 4×10^{-5}% (w/v) biotin and 1% (w/v) glucose. Production and purification of recombinant Tms was performed as described previously [5]. After purification the proteins were analyzed by SDS/PAGE [24], and the purified Tms were lyophilized for future analysis.

Purification of muscle proteins

Muscular actin was purified from acetone powder of chicken pectoralis major and minor muscles [25]. Tn complex was assembled [26] after purification of recombinant TnC [27], TnT [28], and TnI [29] produced in *E. coli* (1 L in 4 L flasks). Proper stoichiometry after assembling was verified by SDS/PAGE. Chicken muscle myosin subunit S1 was prepared from fresh hearts, according to Margossian & Lowey [30]. The myosin (S1) and troponin concentrations were determined using the following extinction coefficients (0.1% solution): $E_{280} = 0.79$ for S1 (115 kDa); $E_{259} = 0.137$ for TnC (18 kDa); $E_{280} = 0.623$ for TnT (31 kDa); $E_{280} = 0.497$ for TnI (21 kDa). The tropomyosin and actin concentrations were determined [31] using bovine serum albumin as a standard.

Functional assays

Viscosity measurements were carried out at room temperature using a Cannon–Manning semimicroviscometer (A50). The affinity of Tm to actin in the presence of Tn was carried out by cosedimentation in a Beckman model LE-80K ultracentrifuge (Beckman), and analyzed by SDS/PAGE. The actomyosin S1 Mg^{2+} ATPase was determined in the absence of troponin as a function of tropomyosin concentration, and in the presence of troponin and Ca^{2+} concentration varying from 10^{-6} to 10^{-3} M. Inorganic

phosphate was determined colorimetrically according to Heinonen & Lahti [32]. All assays were carried out according to Monteiro *et al.* [4], and conditions are described in the figure legends.

Circular dichroism (CD)

CD measurements were recorded on a Jasco J-810 spectropolarimeter with the temperature controlled by a Peltier-type Control System PFD 425S using a 10 mm path length cuvette. The Tm concentration varied from 1 μM to 16 μM in 10 mM sodium phosphate buffer, pH 7.0, containing 200 mM NaCl. The data were collected from 260 nm to 195 nm, and accumulated 10 times, for spectral measurements, and at 222 nm for stability measurements. The average of at least three unfolding experiments was used to construct each curve profile. The value of T_m, which corresponded to the midpoint of the thermal transition unfolding, was determined from the derivative of the transition curve. Curve fitting was performed using ORIGIN (Microcal Software).

Differential scanning calorimetry (DSC)

The microcalorimetric study of Tm denaturation was performed using a scanning microcalorimeter MicroCal Ultrasensitive VP-DSC and standard software for data acquisition and analysis. Tm concentrations were of 15 μM in 10 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl and 1 mM dithiothreitol. Protein samples were dialyzed against the same buffer during 12 h and degassed for 30 min before loading into the calorimeter. Runs were performed with heating/cooling rates of 30, 60 and 90 °C·h^{-1} with no observable change between them, and the process was considered to be in equilibrium. The unfolding was more than 95% reversible and the scan rate independent. The data obtained were subtracted from a baseline of buffer against buffer, corrected for concentration and fitted using ORIGIN DSC ANALYSIS (MicroCal).

Results

α-Tropomyosin production in *Pichia pastoris*

We have previously demonstrated that recombinant chicken muscle Tm produced in the yeast *P. pastoris* had similar functional properties when compared to the native muscle protein [5]. A recombinant human Tm produced in this organism could therefore be a good model for probing amino acid mutations described in cardiomyopathic Tms. The mutations A63V, K70T and E180G were introduced by PCR in the cDNA encoding the human skeletal muscle Tm, skαTM [20], and the mutations were confirmed by DNA sequencing. Expression plasmids carrying mutant (PIC9-A63V, PIC9-K70T, and PIC9-E180G) and nonmutant (PIC9-Tm) cDNAs were used to transform yeast cells, and recombinant clones expressing the proteins were utilized in a large-scale production. Wild type and mutant Tms were produced in yeast at high levels after methanol induction (ranging from 20 to 30 mg·L^{-1}), and the recombinant proteins purified to homogeneity. Figure 1 shows samples of each protein after purification. Recombinant Tms migrated with an apparent molecular mass of 36 kDa and slightly slower migration was observed for the mutant K70T. Mutations A63V and K70T are located at the N-terminal region of the protein and mutation E180G is localized near to the region where troponin interacts with Tm (Cys190, extending to the C-terminal region). Pure recombinant Tms containing point mutations were utilized to evaluate the contribution of the mutant amino acids to the Tm properties.

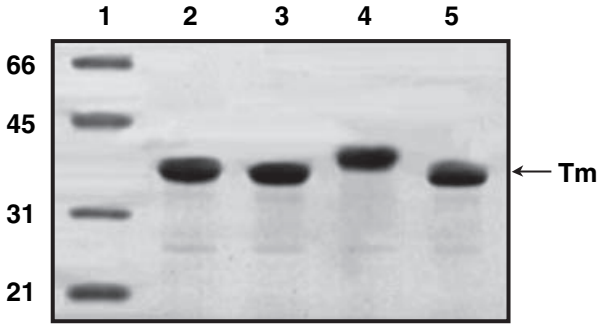

Fig. 1. Gel analysis of Tms. SDS/PAGE (12%) of pure Tms. Ten micrograms of protein were loaded in each well. Lane 1, molecular mass marker (kDa); lane 2, wild type Tm; lane 3, mutant Tm A63V; lane 4, mutant Tm K70T; and lane 5, mutant Tm E180G.

Functional properties of mutant tropomyosins

Recombinant Tms were assayed by structural (head-to-tail polymerization and binding to actin) and regulatory (regulation of myosin S1 Mg^{2+} ATPase activity) properties. Chicken muscle proteins [native actin and myosin (S1), and recombinant troponins] were used in our experiments as they have previously been well characterized in these assays. Polymerization ability of Tms was analyzed by viscosity as a function of the salt concentration. All Tms exhibited maximal viscosity in the absence of salt and lowering viscosity as the salt concentration increased (Fig. 2). No difference in polymerization was observed among the mutant Tms and between mutants and wild type Tm. In the thin filament Tm polymerizes head-to-tail, and polymerization depends on the formation of a complex between amino acid residues (at least nine) at the N-terminal end of one Tm and residues at the C-terminal end of a second molecule. Mutations along the polypeptide chain, far from the complex region involved in the polymerization were not expected to have any influence on the protein polymerization.

Recombinant Tms were assayed by their ability to bind to actin, in a cosedimentation assay, in the absence and in the presence of troponins. In the absence of troponins, binding of Tms to actin was very weak and only small amounts of Tm were detected in gels after centrifugation (data not shown). The addition of troponins to the reaction mixture increased the capacity of Tms to bind to actin (Fig. 3, lanes 3, 6, 9, and 12), and only minor differences in binding capacity among the Tms were observed. A slightly stronger binding capacity, compared to the wild type Tm was observed in the K70T mutant because no Tm was visualized

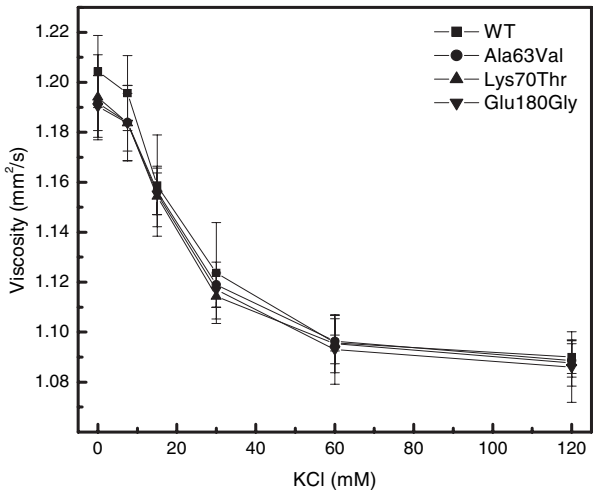

Fig. 2. Effect of ionic strength on Tm polymerization. The determinations were carried out in triplicate, and the data are shown as the average ± standard deviation. Assay conditions: Tm was dialyzed in 10 mM imidazole, pH 7.0, 2 mM dithiothreitol, and 1 mL samples containing 0.5 mg·mL^{-1} were used in the assays. The viscosity measurements were carried out at 25 ± 1 °C using a Cannon–Manning semimicroviscosimeter (A50). (■) Wild type Tm; (●) mutant Tm A63V; (▲) mutant Tm K70T; (▼) mutant Tm E180G.

in the supernatant after centrifugation (Fig. 3, lane 8). The fact that the actin and troponin proteins utilized in this assay were from chicken should be considered. Slight changes in the overall structure of the mutant Tms could not be detected mainly due to the fact that proteins from different organisms were utilized in the assay.

Mutant Tms were compared to the wild type Tm in their ability to regulate the actomyosin S1 Mg^{2+} ATPase activity. ATPase activity was first assayed by varying the concentration of Tm in the presence of constant concentration of F-actin and myosin S1. In this condition, Tm inhibits the ATPase activity as its concentration increases [33]. Figure 4 shows that all mutant Tms were able to inhibit the ATPase activity as the Tm concentration increased, however, they were less effective than the wild type protein. Maximum inhibition (≈ 50%) was observed at the concentration of 1.5 μM (α-Tm/actin ratio of 1 : 5) for the wild type Tm, and 2.0 μM (ratio of 1 : 3.5) for the mutant Tms. In addition, comparison of mutants showed that the E180G mutant was a more effective inhibititor than the K70T mutant. Because the salt concentration used in this assay was very low (40 mM KCl), it is supposed that all Tms were partially polymerized and thus, the differences observed were due to the mutations.

Mutant Tms were also evaluated for alterations in Ca^{2+} sensitive regulation of actomyosin S1 Mg^{2+} ATPase activity in the presence of troponins. In this condition, the tropomyosin–troponin complex inhibits or activates the actomyosin ATPase in the absence and in the presence of calcium, respectively. All mutant Tms were able to regulate the ATPase activity by Ca^{2+}, and the regulation was cooperative for all Tms (Fig. 5). No differences between wild type and mutant Tms were observed. Maximum activation was achieved at pCa = 3.5, and the calcium concentration where the activation was 50%, was close to 10^{-4} M (pCa = 4.0) for all Tms. Both pCa values are higher than those obtained when recombinant chicken Tm was assayed [5,6]. The difference between the present results and those previously reported [5,6] may reflect the different sources of proteins used in the present study to reconstitute the thin filament *in vitro*.

Biophysical properties of mutant tropomyosins

The effect of the mutations on the overall stability of the proteins was evaluated by circular dichroism (CD) and differential scanning calorimetry (DSC). The CD spectra of Tms were typical of folded proteins, with no notable difference among them, and were independent of concentration from 2 μM to 16 μM (data not shown). The ellipticity at 222 nm showed that the mutations did not cause any severe loss of secondary structure (Table 1). The thermal-induced unfolding of wild type Tm monitored by CD is shown in Fig. 6A. The actual melting temperatures were determined from derivative plots of the melting curves of wild type and mutant Tms (Fig. 6B). Two transitions were

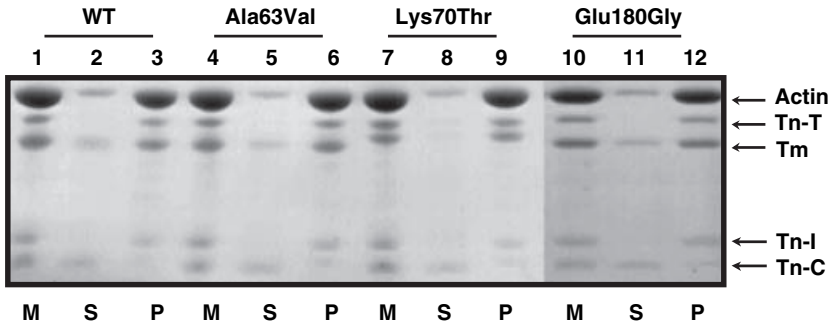

Fig. 3. Actin-binding of wild type and mutant Tms in the presence of troponin complex. Mixtures (M), supernatants (S), and pellets (P) of actin and Tm from actin-binding experiments are shown. Lanes 1–3, wild type Tm; lanes 4–6, mutant Tm A63V; lanes 7–9, mutant Tm K70T; and lanes 10–12, mutant E180G. Assay conditions: 7 μM actin, 1 μM troponin and 1 μM Tm were mixed in 150 mM NaCl, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 mM, EGTA 0.003% (w/v) sodium azide, 10 mM Tris/Cl, pH 7.0 and 1 mM dithiothreitol. The binding of tropomyosin-troponin to F-actin were carried out at 25 °C, for 15 min and ultracentrifuged at 150 000 ***g*** for 30 min, 20 °C, in a Beckman model Optima LE 80K ultracentrifuge, Ti 90 rotor.

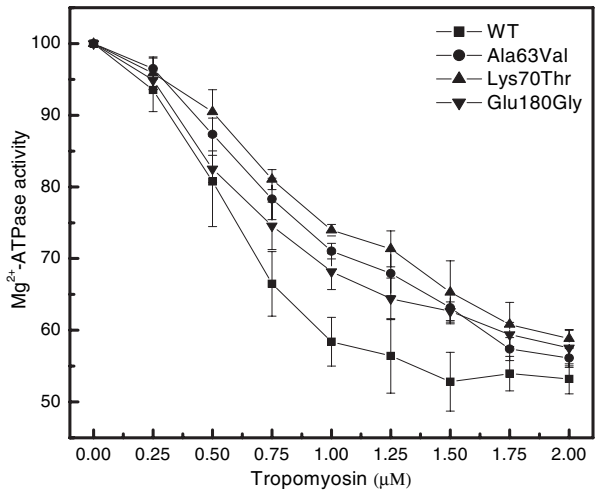

Fig. 4. Inhibition of actomyosin S1 Mg^{2+} ATPase activity by Tm. ATPase activity was measured as a function of Tm concentration. The results are the average of four independent experiments for each protein at each Tm concentration. Assay conditions: 7 μM actin, 0.5 μM myosin (S1), 0–2.0 μM Tm in 5 mM imidazole/HCl, pH 7.0, 40 mM KCl, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM Na_2ATP. (■) Wild type Tm; (●) mutant Tm A63V; (▲) mutant Tm K70T; (▼) mutant Tm E180G.

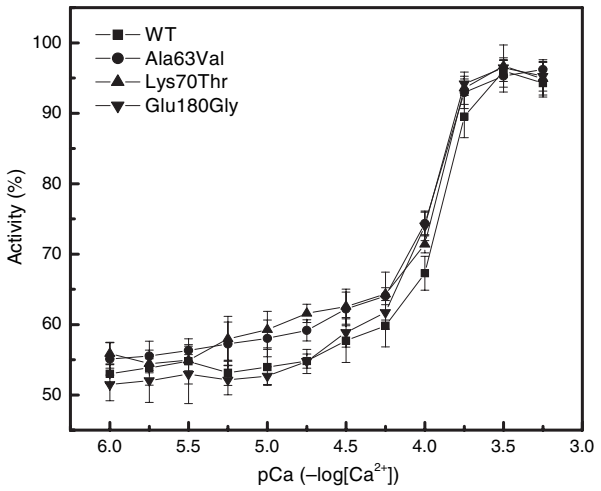

Fig. 5. Calcium regulation of the actomyosin S1 Mg^{2+} ATPase activity by Tm in the presence of troponin. The results are expressed as a percentage of the actin-activated Mg^{2+} ATPase of myosin S1 obtained in the absence of troponin and Tm. The results are the average of four independent determinations at each pCa. Assay conditions: 7 μM actin, 1 μM Tm, 1 μM troponin, 0.5 μM myosin S1 in 20 mM imidazole/HCl, pH 7.0, 6.5 mM KCl, 1 mM dithiothreitol, 3.5 mM $MgCl_2$, 0.5 mM EGTA, 0.01% (w/v) NaN_3, 1 mM Na_2ATP and $CaCl_2$ to give the free Ca^{2+} concentration indicated. (■) Wild type Tm; (●) mutant Tm A63V; (▲) mutant Tm K70T; (▼) mutant Tm E180G.

identified in the thermal-induced unfolding of Tm, and the values for the wild type and mutant Tms are shown in Table 1. The mutants K70T and A63V were less stable than the wild type at T_{m2}.

Table 1. Circular dichroism parameters for the thermal-induced unfolding of wild type (WT) and mutant Tms. The values are the mean ± standard deviation of at least three experiments. T_{m1} is the midpoint of the thermal transition unfolding calculated from the derivative. T_{m2} is the main transition.

Tm	$[\Theta]_{222}$ at 37 °C (deg·cm^{-2}·dmol^{-1})	T_{m1} (°C)	T_{m2} (°C)
WT	−30500 ± 800	39.9 ± 1	43.3 ± 1
A63V	−29000 ± 1200	40.3 ± 1	41.6 ± 1
K70T	−28500 ± 1000	38.3 ± 1	39.6 ± 1
E180G	−30500 ± 1000	40.5 ± 1	42.2 ± 1

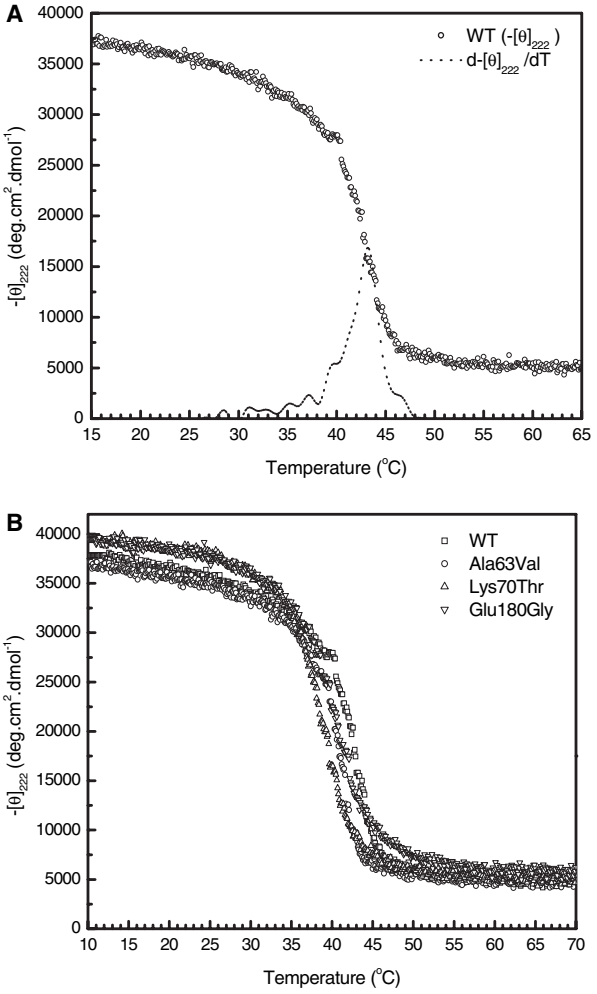

Fig. 6. Change in ellipticity at 222 nm as a function of temperature. (A) The change in ellipticity of wild type (WT) Tm at 222 nm as a function of temperature (○) and its derivative curve (····). (B) Thermal-induced unfolding of WT and mutant Tms monitored by the changes in ellipticity at 222 nm. The unfolding was more than 95% reversible for all proteins. Experimental conditions: the CD measurements were recorded on a Jasco J-810 spectropolarimeter with the temperature controlled by Peltier-type control system PFD 425S using a 10 mm path length cuvette and a scan rate of 60 °C·h^{-1}. The protein concentration was 15 μM in 10 mM sodium phosphate buffer, pH 7.0, containing 200 mM NaCl and 1 mM dithiothreitol.

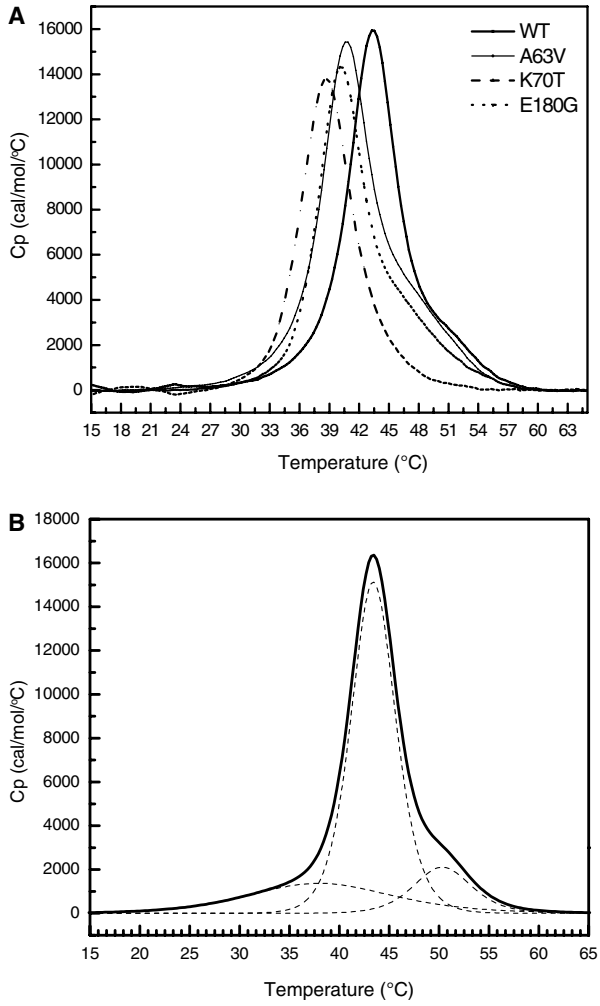

Fig. 7. DSC scans. (A) Typical DSC curves for wild type (WT) and mutant Tms after subtraction of the buffer baseline and removal of the heat capacity increment of unfolding followed by normalization of the concentration. (B) Typical DSC curve for WT. The solid curve represents the observed data and the dashed curves represent the deconvolution of the individual transition into three independent transitions. See Table 2 for the thermodynamic parameters of the individual transitions. Experimental conditions: 15.15 μM of protein in 10 mM sodium phosphate buffer, pH 7.0 containing 100 mM NaCl and 1 mM dithiothreitol.

Figure 7A shows the heat capacity profile for wild type and mutant Tms measured by DSC at a scan rate of 60 °C·h^{-1}. In the experimental conditions of assay the Cys190 residue was in the reduced state (data not shown). The heat capacity profile of the proteins showed a very broad transition, which suggested that they unfolded in a multistep process. The thermal-induced unfolding was highly reversible (> 95%), as shown by the repeatability of the DSC endotherms upon rescanning and the recovery of the native far-UV CD spectra upon cooling (data not shown). The T_m of each Tm transition is shown in Table 2, and they were used to rank the proteins in order of stability: wild type > A63V = E180G > K70T. The maxima of the transitions were not dependent on scan rate and the spectra

Table 2. Summary of the thermodynamic parameters determined by DSC for the wild type (WT) and mutant Tms. The uncertainties listed are the standard errors of the mean and included the uncertainty in the determination of protein concentrations. The values are the mean ± standard deviation of at least three experiments. T_m is the midpoint of the thermal transition unfolding; ΔH_{cal} is the calorimetric enthalpy of the whole transition. T_{m2} is the main transition.

Tm	T_m at the maximum of the transition (°C)	ΔH_{cal} (kcal·mol^{-1}·°C^{-1})	T_{m1} (°C)	T_{m2} (°C)	T_{m3} (°C)
WT	43.5 ± 1	130 ± 10	39.0 ± 1	43.4 ± 1	50.1 ± 1
A63V	40.8 ± 1	135 ± 10	39.4 ± 1	41.0 ± 1	47.1 ± 1
K70T	38.7 ± 1	110 ± 10	38.0 ± 1	39.6 ± 1	42.4 ± 1
E180G	40.4 ± 1	120 ± 10	40.1 ± 1	42.2 ± 1	47.3 ± 1

were essentially the same for scan rates of 30, 60 and 90 °C·h^{-1} (data not shown). Figure 7B shows the fitting of the DSC scan for wild type Tm obtained using three endotherms. The T_ms of the wild type and mutant endotherms are shown in Table 2. It is evident from the data that the unfolding of the wild type and mutant Tms involved more than a single two-state transition. There was a good agreement between the T_{m1} and T_{m2} calculated using CD and the corresponding values calculated using DSC (Tables 1 and 2).

Discussion

In individuals with FHC, mutations in Tm are thought to affect the surface of the protein, which may compromise the integrity of the thin filament, resulting in defects in force transmission. In order to understand the functional consequences of the mutations at a molecular level, recombinant human Tms were produced, and used as model proteins to study the interactions that govern the stability of the thin filament. Three mutations described as causing cardiomyopathy were introduced in the cDNA encoding the human skeletal muscle tropomyosin. One mutation (E180G) is located near to the troponin binding site, and occurs in a Tm region highly conserved during evolution. This mutation occurs at the e position of the heptad repeat, and introduces changes in the surface charge of Tm. The two other mutations (A63V and K70T) are located at the N-terminal region, far from the troponin binding region and occur at the g position of the repeat. The K70T mutation also introduces changes in the surface charge of Tm. All the mutant amino acids are involved in interchain and intrachain interactions and therefore are important for the stabilization of the parallel coiled-coil structure.

A number of studies on the effects of cardiomyopathy mutations in Tm are available, the D175N and E180G being the best characterized so far. However, in all of them, the N-terminal methionine was either unacetylated or modified by the addition of an Ala-Ser extension in order to compensate for the inability of *E. coli* to N-acetylate recombinant Tm. Amino and carboxy terminal ends of Tm are critical for polymerization and binding to actin. Because Tm binds cooperatively in a head-to-tail fashion, modification of the amino terminus can alter the function of the

protein, even though the rest of the polypeptide chain is identical to the wild type protein. The capacity of *P. pastoris* to produce functionally active Tm, without modifications of its primary sequence, provides, for the first time, a suitable protein to be used in this type of study. Recombinant human wild type and mutant Tms were produced in the yeast *P. pastoris*, and were properly N-acetylated as they were able to polymerize and to bind to actin.

The stability of human Tm

CD and DSC experiments were used as methods for evaluating the effect of mutations on the stability of Tms [34]. The thermal-induced unfolding of the rabbit [35–37], rat, and chicken [38,39] skeletal Tms have been characterized as a multistep process with at least two melting transitions. Human Tm shows two melting transitions (T_ms), one at about 40 °C and the other at about 43 °C during the thermal-induced unfolding monitored by CD. Previous investigations using CD of the thermal-induced unfolding of skeletal Tm from other organisms also identified two melting transitions: rabbit Tm has T_ms at 43 and 51 °C [35], and rat Tm has T_ms at 30 and 44 °C [38]. Chicken smooth Tm has T_ms at 32 and 44 °C as determined by DSC [39]. The T_ms reported above were different from those calculated for human Tm. The smallest difference between the first and second temperature of melting above described is 8 °C (rabbit), which is much greater than the difference between the two melting temperatures for human Tm, only 3 °C.

The heat capacity profile of human Tm shows a broad transition that is better fitted with three endotherms. This finding agrees with the DSC results for chicken skeletal muscle [40] and duck smooth muscle [41] Tms, which have at least three melting transitions. The first two T_ms measured by DSC were similar to the two T_ms identified by CD during thermal-induced unfolding. The third T_m measured by DSC occurred at 50 °C, whereas the CD signal at 222 nm showed no further change at temperatures > 46 °C. Although the CD signal at these temperatures was low, it was greater than the signal from a random coil structure. The CD signal at 222 nm was unable to monitor the third transition, either because of lack of resolution or because the transition was invisible to this probe. Thus, the analysis of the melting profile of human Tm was enhanced by the use of different probes.

The mutations affect the stability of the protein

Heller *et al.* ([19] and references therein) identified two T_ms in the unfolding of chicken Tm monitored by CD and suggested that the lower T_m (T_{m1}) reflected the stability of the C-terminus and the higher T_m (T_{m2}) reflected that of the N-terminus. These authors showed that the mutations A63V and K70V affected only T_{m2} in the chicken Tm. In good agreement with these data, our results showed that none of the mutations studied here affected T_{m1}, but that the mutations on residues A63 and K70 decreased the T_{m2}. The mutation on residue E180 did not decrease T_{m1} or T_{m2} but, like the other mutants, it reduced T_{m3}. These results agree with the general view that FHC pathology results from low stability of the mutant Tms.

The mutations did not affect the structure of the protein as there was no significant alteration in the function or in the amount of the secondary structure. However, the mutations did affect the stability of the protein, and the most destabilizing mutation was K70V, which is the most deleterious mutation in FHC. Individuals carrying these mutations have a high incidence of sudden death [11]. The global T_m for the wild type Tm is well above the normal human body temperature (43 vs. 37 °C), which makes this protein very stable under physiological conditions. However, the T_m of the mutant Tms, especially K70V, were closer to the human body temperature, making them more susceptible to partial unfolding under physiological conditions and thus, affecting their normal function. These conclusions could only be reached because we worked with the human Tm instead of Tms from other organisms with different T_ms (see above).

Although all mutations caused destabilization of the coiled-coil, the effect of each mutation, individually, might be due to different effects. Based on previous studies it is known that Tm contains stable coiled-coil regions interrupted by domains without stable secondary structure [42–44]. For example, Hitchcock-DeGregori *et al.* [45] identified a region, from residues 166–188, that is the most important for both function and stability of the rat Tm. This region contains the mutation E180G, which was shown in our results to be the least deleterious mutation in the human Tm. On the other hand, Tm function was insensitive to a deletion of a region from residues 47–88 [45], which contains the destabilizing mutations A63V and K70T observed in our results. Why are the A63V and K70T the most destabilizing mutations? Both mutations are located in exon 2, a highly conserved region in striated Tms from different organisms. In addition, mutation A63V is close to one of the seven alanine clusters that occur periodically along tropomyosin [46]. The alanine residues have been implicated in the wrap-around bending of Tm on the actin helix [47], and the mutation A63V probably allows local unfolding. The mutation K70T changes a long charged side chain to a noncharged side chain at position g of the heptad repeat, a position involved in the stabilization between the helices of the coiled-coil. The substitution could cause a local change in Tm conformation and therefore in stability.

Because the mutations did not affect the normal function of the thin filament and the mutant Tms did not aggregate at the high protein concentrations tested here, it could be argued that the cause of FHC is something other than low stability. However, this pathology is not detected in patients until they reach a certain age [48]. The low stability of the mutants may cause a very slow loss of functionality that accumulates over time. This hypothesis supports the fact that the mutation that causes the greatest loss in stability also causes FHC pathology at the youngest age [11].

Acknowledgements

We thank Dr C. Gooding, University of Cambridge, UK, for the gift of human Tm cDNA; Dr S. C. Farah, Instituto de Química, USP, São Paulo, for helpful discussions and for providing the *E. coli* clones carrying the plasmids pET3a-TnT, pET3a-TnC and pET3a-TnI; Dr J. A. Ferro, Faculdade de Ciências Agrárias e Veterinárias, UNESP,

Jaboticabal, for discussions; Dr A. Nhani Jr. for help in the myosin S1 preparations, and Dr R. E. Larson, Faculdade de Medicina de Ribeirão Preto, USP, for careful reading of the manuscript. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). E. H. was a graduate fellow from FAPESP.

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