Effects of Two Familial Hypertrophic Cardiomyopathy-Causing Mutations on α-Tropomyosin Structure and Function†

Nina Golitsina,§ Yongmi An,‡ Norma J. Greenfield,‡ Ludwig Thierfelder,|| Kenji Iizuka,|| Jonathan G. Seidman,|| Christine E. Seidman,|| Sherwin S. Lehrer,§ and Sarah E. Hitchcock-DeGregori*,‡

Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, Boston Biomedical Research Institute, Boston, Massachusetts 02114, and Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: Missense mutations in α-tropomyosin can cause familial hypertrophic cardiomyopathy. The effects of two of these, Asp175Asn and Glu180Gly, have been tested on the structure and function of recombinant human tropomyosin expressed in *Escherichia coli*. The F-actin affinity (measured by cosedimentation) of Glu180Gly was similar to that of wild-type, but Asp175Asn was more than 2-fold weaker, whether or not troponin was present. The mutations had no apparent effect on the affinity of tropomyosin for troponin. The mutations had a small effect on the overall stability (measured using circular dichroism) but caused increased local flexibility or decreased local stability, as evaluated by the higher excimer/monomer ratios of tropomyosin labeled with pyrene maleimide at Cys 190. The pyrene-labeled tropomyosins differed in their response to myosin S1 binding to the actin−tropomyosin filament. The conformations of the two mutants were different from each other and from wild-type in the myosin S1-induced on-state of the thin filament. Even though both mutant tropomyosins bound cooperatively to actin, they did not respond with the same conformational change as wild-type when myosin S1 switched the thin filament from the off- to the on-state.

Missense mutations in α-tropomyosin (TM)[1] can cause familial hypertrophic cardiomyopathy (FHC, Thierfelder et al., 1994; Nakajima-Taniguchi et al., 1995). FHC is inherited as an autosomal dominant trait characterized by cardiac hypertrophy (thickening of the ventricular walls) with associated myocyte disarray and myocyte hypertrophy (Maron et al., 1987). Affected individuals experience chest pain, dyspnea, and sudden death. Missense mutations in a variety of cardiac sarcomeric proteins, β cardiac myosin heavy chain (Jarcho et al., 1989; Geisterfer-Lowrence et al., 1990; Watkins et al., 1992; Anan et al., 1994; Fananapazir & Epstein, 1994), cardiac troponin T (TnT; Thierfelder et al., 1994), cardiac myosin binding protein C (Bonne et al., 1995; Watkins et al., 1995), and ventricular myosin light chains (essential and regulatory; Poetter et al., 1996), as well as α-TM, can all cause this condition. The mechanism by which these mutations cause cardiac hypertrophy and myocyte disarray remains an important question. Further, we do not understand how some mutations cause more serious disease than others. One TM mutation (D175N) causes cardiac hypertrophy but does not have as dramatic an effect on life expectancy of affected individuals as some other mutations (Coviello et al., 1997). We have begun to address these questions by studying the effects of α-TM missense mutations on its structure and function.

Tropomyosin is a coiled-coil actin binding protein bound along the length of the actin filament in muscle, and most eucaryotic cells. The two polypeptide chains in TM are aligned in parallel and in register (Lehrer, 1975; Stewart, 1975). One TM molecule spans the length of seven actin monomers in the filament. Although analysis of the sequence indicates a weak periodicity that corresponds to the spacing of the actin monomers (McLachlan & Stewart, 1976; Phillips et al., 1986), mutagenic analysis has shown that the sequences of the ends are more critical for cooperative actin binding than the sequences of the intervening periods (Cho et al., 1990; Hitchcock-DeGregori & Varnell, 1990; Cho & Hitchcock-DeGregori, 1991; Urbancikova & Hitchcock-DeGregori, 1994; Hammell & Hitchcock-DeGregori, 1996; Hitchcock-DeGregori & An, 1996).

In striated muscles, TM functions in concert with Tn to regulate actin−myosin interaction in a Ca^{2+}-dependent fashion (Zot & Potter, 1987; Lehrer, 1994; Tobacman, 1996). Troponin is sufficiently long to span more than one-third the length of TM, and primary binding regions include the Cys 190 region and the C-terminal end, including the overlap region with the N-terminus of the next TM molecule on the actin filament (Flicker et al., 1982; White et al., 1987). Two of the FHC TM mutations (D175N and E180G) are near Cys 190 and occur in residues that have been highly conserved during evolution (Thierfelder et al., 1994; Nakajima-Taniguchi et al., 1995). The third, A63V (Nakajima-Taniguchi et al., 1995), is located far from any known Tn interaction site, and the residue has not been conserved. All three of these sites are *e* or *g* positions in the coiled-coil helix, residues that may be involved in interchain as well as intrachain electrostatic interactions.

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* Author to whom correspondence should be sent: Dr. Sarah E. Hitchcock-DeGregori, Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Tel: (908) 235-5236. Fax: (908) 235-4029. Email: hitchcock@mbcl.rutgers.edu.

§ Boston Biomedical Research Institute.

‡ Robert Wood Johnson Medical School.

|| Harvard Medical School.

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[1] Abbreviations: DTT, dithiothreitol; FHC, familial hypertrophic cardiomyopathy; TM, tropomyosin; Tn, troponin.

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In most instances the effects of the mutations on protein and sarcomere function are not known. To better understand the effects of the FHC-causing mutations on α-TM, we have expressed two (D175N and E180G) of the mutant proteins in *Escherichia coli* and have begun to define the properties of these mutant proteins. The actin binding, thermal stability, and conformation of E180G are not very different from the wild-type protein; however, D175N has significantly different properties. Portions of this work have been reported in a preliminary form (An et al., 1996).

MATERIALS AND METHODS

Construction and Expression of Mutant Tropomyosins

An *Eco*RI-*Bam*HI DNA fragment derived from the normal human striated muscle α-tropomyosin cDNA (MacLeod & Gooding, 1988) was subcloned into *Eco*RI- and *Bam*HI-digested Bluescript vector DNA. Sequences encoding D175N and E180G α-tropomyosin were amplified in two steps by the polymerase chain reaction (PCR) using standard procedures (Ausabel et al., 1994). The nucleotide sequences of the cloned products were redetermined to confirm that only the desired mutations were introduced.

Primers. The following primers were used:

T3:	AATTACCCTCACTAAAGGGAACAAAA
T7:	GTAATACGACTCACTATAGGGCGAAT
175F:	ATCATTGAGAGCAACCTGGAACGTG
175R:	CACGTTCCAGGTTGCTCTCAATGAT
180F:	TGGAACGTGCAGGGGAGCGGGCTGA
180R:	TGAGCCCGCTCCCCTGCACGTTCCA

175Asn. Two fragments were amplified using T7 and 175F primers and T3 and 175R primers. These fragments were gel purified, 20 ng of each fragment were combined and PCR amplified with T3 and T7 primers. The amplified fragment was cleaved with *Eco*RI and *Bam*HI and cloned into Bluescript vector.

180Gly. Two fragments were amplified using T7 and 180F primers and T3 and 180R primers. The fragments were purified, amplified, and cloned as for the 175Asn fragment.

The human cDNAs were cloned in pET11d at the *Nco*I and *Bam*HI sites and expressed in *E. coli* BL21(DE3) cells (Studier et al., 1990). The wild-type and mutant TMs were all expressed at high levels and purified using ammonium sulfate precipitation and chromatography in DE52 cellulose (Whatman) and hydroxylapatite (Bio-Rad) as previously described (Hitchcock-DeGregori & Heald, 1987). The N-terminal Met of the recombinant TMs is unacetylated.

Actin Binding

Actin affinity was measured by cosedimentation of TM with F-actin in the presence and absence of Tn. The pellets and supernatants were analyzed by SDS−PAGE, and the free and bound TM determined by quantitative densitometry using known amounts of TM as a standard (Urbancikova & Hitchcock-DeGregori, 1994). The ionic conditions for each experiment, described in the figure legends, were selected to allow quantitative measurement of the free TM in the range of the binding constant. Therefore, the binding constants can only be compared within an experiment. The data were fit to the Hill equation using SigmaPlot for calculation of K_{app}.

Chicken pectoral actin and Tn were purified as previously described (Hitchcock-DeGregori et al., 1982; Potter, 1982). The TM concentration was determined using a microbiuret assay with bovine serum albumin as a standard (Goa, 1953) or by determining the specific tyrosine absorbance (Edelhoch, 1967). Extinction coefficients (1% at 280 nm) were used to calculate the concentration of actin (11.0) and Tn (4.5).

Circular Dichroism Measurements

Data were collected on an Aviv Model 62D spectropolarimeter equipped with a five sample thermal equilibration chamber, as previously described (Greenfield & Hitchcock-DeGregori, 1995). The conditions are described in the figure legends.

Fluorescence Measurements

Recombinant TMs were labeled with pyrene iodoacetamide (PIA) as previously described (Ishii & Lehrer, 1990). The concentration of PIA-TM was determined by the BCA Protein Assay (Pierce) using unlabeled TM as a standard. The concentration of bound pyrene was determined with $\epsilon_{344} = 2.2 \times 10^4\ M^{-1}$. The labeling ratios were 1.65−1.8 mol/mol of TM (82−90%).

For the fluorescence assays, proteins were prepared from rabbit skeletal muscle using previously published methods: actin (Spudich & Watt, 1971), Tn (Ebashi et al., 1971; van Eerd & Kawasaki, 1973), TnT (Greaser & Gergely, 1973), myosin S1 (Weeds & Pope, 1977). Protein concentration, other than labeled TM, was determined by UV spectrophotometry using the following absorbance values ((milligrams/milliliter)$^{-1}$ centimeter^{-1}) and molecular masses kilodaltons: G-actin, 0.63 at 290 nm and 43 000; TM, 0.22 at 277 nm and 66 000; TnT, 0.5 at 280 nm and 30 500. Skeletal rather than cardiac muscle proteins were used for the studies in this paper because they have been extensively characterized in these established assays and because of the possibility to purify them from fresh tissue.

Steady-state fluorescence measurements were obtained with a SPEX Flurolog 2/2/2 photon-counting fluorimeter (Edison, NJ) in the ratio mode with a 2.25-nm band-pass for both excitation and emission with samples in the thermostated housing. Titrations were carried out with excitation at 340 nm and emission at 343 nm to monitor light scattering. A 450 nm cut-on KV Schott filter placed in front of a second photomultiplier at 90° to the excitation was located close to the sample to simultaneously measure fluorescence. Before titration, the sample was clarified by centrifugation at low speed for 10 min.

The binding profiles of TnT added to PIA-TM obtained from the fluorescence change with total added ligand were analyzed by nonlinear least-squares using a computer program to fit fluorescence intensity, F, using $F = (\Delta F)\upsilon$ where ΔF is the fluorescence change at saturation and υ is the fraction of TnT bound. υ is given by the root of

$$nK_bA\upsilon^2 - (1 + nK_bA + K_bB)\upsilon + K_bB = 0$$

where K_b = binding constant, n = stoichiometry, and A = [TnT], as described earlier (Morris & Lehrer, 1984).

RESULTS

To ascertain the effect of FHC mutations D175N and E180G on TM structure and function, recombinant wild-type and mutant TMs were expressed in *E. coli* and purified following published procedures (Hitchcock-DeGregori & Heald, 1987). The N-terminal Met is unacetylated, and the lack of post-translational modification reduces actin affinity but has little effect on stability and other TM functions (Hitchcock-DeGregori & Heald, 1987; Urbancikova & Hitchcock-DeGregori, 1994; Greenfield & Hitchcock-DeGregori, 1994).

Actin Affinity. One basic TM function is cooperative binding to F-actin, and the ability of Tn to increase the striated TM actin affinity (Yang et al., 1979; Wegner, 1981; Wegner & Walsh, 1981). Actin affinity is sensitive to changes at the N- and C-terminal ends, as well as in the internal sequence of TM [reviewed in Hitchcock-DeGregori 1994)]. Although actin affinity of unacetylated TM is substantially lower than that of acetylated TM isolated from muscle, it can be measured in 0.1 M NaCl. The affinity of E180G was close to that of wild-type TM, but that of D175N was more than 2-fold weaker. Troponin, in the presence of Ca^{2+}, increased the affinity of wild-type TM and both mutants for actin (Figure 1B) and in the absence of Ca^{2+} resulted in a further increase, as expected (Figure 1C). In order to measure the affinity of the TMs for actin with Tn, higher ionic strength was used to weaken the affinity. Therefore, the affinities in the different conditions in Figure 1A−C cannot be compared. The results show that the affinity of E180G was close to that of wild-type TM whereas D175N was reduced 2−3 fold, regardless of the ionic conditions and the presence of Tn with or without Ca^{2+}.

Since the relative differences in affinity of the TMs were the same in all three experimental conditions, we conclude that the fundamental mutant phenotype for D175N is altered actin affinity, rather than interaction with Tn. Even though only one residue has been changed in each mutant, the difference in affinity was similar to that seen when one exon is substituted for another or deleted (Hitchcock-DeGregori & Varnell, 1990; Cho & Hitchcock-DeGregori, 1991; Hammell, R. L. and Hitchcock-DeGregori, S. E., unpublished results).

Thermal Stability and Conformational Analysis. D175 and E180 are in *g* and *e* positions, respectively, in the heptad repeat, sites that allow interchain as well as intrahelical electrostatic interactions (McLachlan & Stewart, 1975). Both mutations are in the least stable region of TM (Ueno, 1984; Ishii et al., 1992). The effect of these mutations on the overall stability of TM was evaluated using circular dichroism. The temperature of unfolding was evaluated by measuring the loss of ellipticity at 222 nm, a measure of α-helix content. Figure 2, panels A and B, shows the thermal denaturations and the first derivatives. The temperature of the major transition of E180G was similar to that of wild-type TM (45.1 vs 44.5 °C) whereas D175N unfolded with two major transitions, one at a lower temperature (40.4 °C) than the major transition. The results were similar for TMs where Cys 190 was modified with pyrene iodoacetamide, though E180G was slightly more stable than wild-type and lacked a pretransition (results not shown).

The TMs were labeled at Cys 190 with pyrene iodoacetamide to provide a conformational probe close to the sites of the mutations (Ishii & Lehrer, 1990). The excimer/monomer ratios of both D175N and E180G were greater than that of the wild-type (Table 1), suggesting that the mutations have increased the flexibility, or decreased the local stability, of TM near Cys 190 allowing the pyrenes to interact more favorably. At lower salt concentrations (30 mM NaCl vs 100 mM), the excimer/monomer ratios of wild-type, and E180G were unchanged, but that of D175N was lower, and closer to wild-type indicating that the conformation of this region in the mutant is sensitive to ionic strength (results not shown).

The excimer fluorescence can be used to monitor unfolding of the TM coiled coil in the region of Cys 190 (Figure 2C). The excimer fluorescence of wild-type and D175N increased significantly between 20 and 40 °C, similar to previous data for striated αα-TM (Ishii et al., 1992), and correlates with

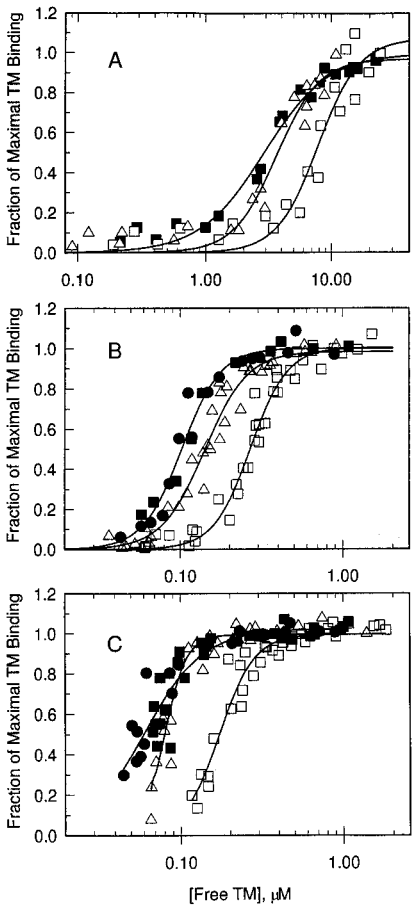

FIGURE 1: (A) Binding of tropomyosins to F-actin. Symbols: ■, human wild-type; □, D175N; △ , E180G. Data are from two independent experiments. The K_{app} (±SE) values calculated from analysis of the data using the Hill equation were wild-type, (0.34 ± 0.03) × 10^6 M^{-1}; D175N, (0.13 ± 0.02) × 10^6 M^{-1}; E180G, (0.27 ± 0.04) × 10^6 M^{-1}. Conditions: 100 mM NaCl, 2 mM $MgCl_2$, 20 mM imidazole, pH 7.5, 0.5 mM DTT, 5 μM actin, 0.5−20 μM TM. (B) Binding of tropomyosins to F-actin, with troponin, +Ca^{2+}. Symbols: same as in Figure 1A, also ●, rat wild-type TM. Data are from two or three independent experiments. K_{app} (±SE) values are wild-type, (10.1 ± 0.3) × 10^6 M^{-1}; D175N, (3.7 ± 0.1) × 10^6 M^{-1}; E180G, (7.1 ± 0.3) × 10^6 M^{-1}. Conditions: 200 mM NaCl, 2 mM $MgCl_2$, 20 mM imidazole, pH 7.5; 0.5 mM DTT, 0.2 mM $CaCl_2$, 2.5 μM actin, 0.3−2.5 μM TM, 1:1.2 TM:Tn (molar ratio). (C) Binding of tropomyosins to F-actin, with troponin, +EGTA. Symbols: same as in parts A and B. Data are from two or three independent experiments. Removal of Ca^{2+} increased the affinity of all TMs. For all except D175N, the affinity was too tight to obtain a valid K_{app}. Conditions: same as for Figure 1B except with 250 mM NaCl and 0.2 mM EGTA (no $CaCl_2$).

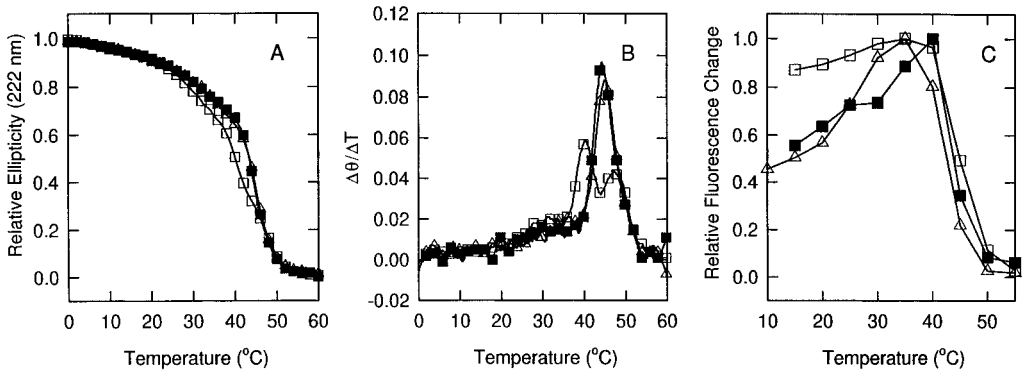

FIGURE 2: (A and B) Circular dichroism of unlabeled tropomyosins. (A) Isotherm; (B) first derivative of the data in Figure 1A. Symbols: ■, human wild-type; □, D175N; △, E180G. The temperature dependence of unfolding was evaluated by measuring the loss of ellipticity at 222 nm, a measure of the α-helix content. Conditions: 1.5 μM TM in 500 mM NaCl, 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT. (C) Temperature dependence of the relative excimer fluorescence of PIA-tropomyosins. Symbols: same as in Figure 2A. Conditions: 0.5 μM TM in 0.1 M NaCl, 5 mM $MgCl_2$, 5 mM DTT, 20 mM HEPES, pH 7.5.

Table 1: Fluorescence Properties of Pyrene-Labeled Tropomyosins with Actin, Actin-Myosin S1, Troponin, or Troponin T[a]

	tropomyosin		
	wild-type	D175N	E180G
TM	**1.0** = 100%	**2.5** = 100%	**5.0** = 100%
TM, F-actin (30 mM NaCl)	99%	95%	94%
TM, F-actin, S1 (30 mM NaCl)	155%	58%	89%
TM, Tn (30 mM NaCl)	46%	52%	56%
TM, Tn (100 mM NaCl)	58%	65%	63%
TM, TnT (100 mM NaCl)	54%	40%	62%

[a] Values in bold are pyrene excimer/monomer ratios at 480/405 nm. Percentages are normalized values. All TMs were labeled at Cys 190 with pyrene iodoacetamide. Conditions: NaCl, as indicated; 5 mM Mg^{2+}, 5 mM DTT, 20 mM HEPES, pH 7.5.

the pretransitions of helix unfolding (Ishii & Lehrer, 1990). In contrast, the excimer fluorescence of E180G was high and remained constant in this region, consistent with the lack of a helix pretransition, suggesting that the Cys 190 region is less stable at lower temperature than in wild-type or D175N, allowing for a more favorable interaction between pyrenes. Substitution of Gly for Glu should make the region less favorable for helix formation. The decrease of excimer fluorescence in the main transition correlates reasonably well with the main helix transitions (Figure 2A and B; note different experimental conditions).

Interaction with Myosin S1 on the Actin Filament. The pyrene probe at Cys 190 can also be used to monitor changes in interactions with actin due to myosin S1 binding to actin filaments and also complex formation between TM and Tn. There is a small decrease in fluorescence when TM binds to actin (Ishii & Lehrer, 1990). When myosin S1 binds to actin-TM, the TM excimer fluorescence increases, indicating an increase in flexibility in the molecule near Cys 190 (Ishii & Lehrer, 1990). The same effect was observed with unacetylated, recombinant TM, whereas the excimer fluorescence of D175N decreased, and there was little effect on E180G (Table 1 and inserts of Figure 3, panels A and B), even though all three TMs bound F-actin stoichiometrically in the presence of saturating S1 (from cosedimentation experiments, results not shown). The results suggest that myosin S1 binding to actin filaments had no effect on the flexibility of E180G on actin whereas it decreased that of D175N.

Myosin S1 induces the binding of TM to actin in the "on-state", under conditions where TM alone binds poorly (Eaton, 1976; Golitsina et al., 1995). When TM binding to F-actin

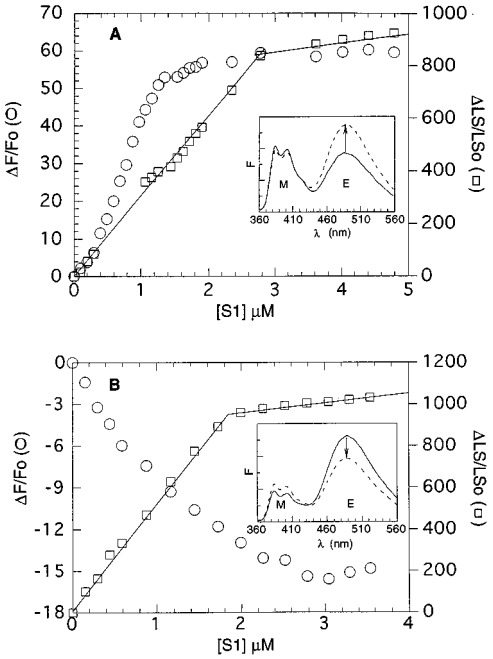

FIGURE 3: (A) wild-type; (B) D175N. Cooperative binding of tropomyosins induced by myosin S1. Excimer fluorescence of TM (○) is monitored with increasing S1 concentration. Light scattering (□) monitors the binding of S1 to actin. Conditions: 50 mM NaCl, 5 mM Mg^{2+}, 5 mM DTT, 20 mM HEPES, pH 7.5. (A) 0.5 μM wild-type TM, 3 μM actin; (B) 0.3 μM D175N TM, 2 μM actin. The inserts show the fluorescence spectra with and without saturating myosin S1. M, monomer fluorescence; E, excimer fluorescence.

was measured by cosedimentation as a function of myosin S1 concentration (in the absence of nucleotide), all three TMs saturated the actin filament when two or more myosin S1 molecules bound per six or seven actins, as has been previously reported for acetylated TM isolated from muscle (Golitsina et al., 1995). The changes in excimer fluorescence, however, were different for the three TMs. The excimer fluorescence of wild-type increased with titration of S1, with a maximal effect when TM saturated the actin at only two to three S1 molecules bound per seven actins (Figure 3A), in agreement with the sedimentation data. The results suggest that the binding of two or more S1 molecules to actin promotes the binding of TM to actin and has a long-range effect on the TM conformation. In contrast, with D175N, the decrease in excimer fluorescence did not reflect TM binding. Whereas the TM saturated the F-actin at two

myosin S1/six to seven actins (measured by cosedimentation, results not shown), the excimer fluorescence saturated at about one S1 per actin monomer (Figure 3B) and monitored S1 binding, rather than TM binding. This suggests that the decrease in excimer fluorescence, indicative of reduced flexibility of the TM in the Cys 190 region, is sensitive only to local S1 binding, near the probe on the TM-actin filament. The effect of S1 on the excimer fluorescence of E180G was too small to measure, even though S1 induced E180G binding to actin. In these experiments, there was little effect on the pyrene fluorescence in the absence of S1, because they were carried out in conditions where the unacetylated TMs bind poorly to actin.

The results suggest that the FHC mutations affect the conformation of TM in the myosin S1-induced on-state of actin-TM. The conformations of the two mutants are different from each other and from wild-type TM.

Interaction between FHC Tropomyosins and Troponin. Both FHC mutations are close to the region of TM of Ca^{2+}-sensitive Tn interaction (Mak & Smillie, 1981; Tao et al., 1990). The pyrene at Cys 190 provides a probe for this interaction. The binding of Tn or TnT to wild-type TM caused an increase in monomer fluorescence, as has been previously reported (Ishii & Lehrer, 1991). With D175N and E180G, there was a similar increase in fluorescence with Tn, and Tn bound with similar but slightly weaker affinity [$(1.0 \pm 0.5) \times 10^7$ M^{-1} vs $(2.0 \pm 0.5) \times 10^7$ M^{-1}]. With TnT, the fluorescence change was less with D175N than wild-type or E180G, but the affinities were similar [$1-2.6 \pm 0.5) \times 10^7$ M^{-1}]. The stoichiometry was close to 1 Tn or TnT:TM in all cases. The binding of Tn or TnT reduced the excimer/monomer ratio in all three TMs, indicating that the mutation does not alter the interaction of TM with Tn in this region (Table 1). The results are consistent with the similar enhancement by Tn of the actin affinity of the three TMs (Figure 1) as well as with observations that major changes in the proximal exon 6-encoded sequence (amino acids 189−213), or deletion of exon 6, do not alter Tn affinity for TM (Hammell & Hitchcock-DeGregori, unpublished results). The Ca^{2+}-dependence of the regulated actomyosin ATPase was not investigated as we have found that all TMs that bind to actin with Tn function similarly in this assay.

DISCUSSION

In vitro analysis of recombinant TMs carrying FHC mutations, D175N and E180G, has shown that the main effects are reduced actin affinity (D175N) and altered conformation when bound to actin in the S1-induced on-state of the thin filament. The mutations have small effects on the stability of TM in the regions of the mutations. However, Tn interaction is not notably affected, either with TnT or the ternary Tn complex.

The mutations are in one of the most highly conserved regions of TM and are included in the region of the fifth actin binding site (McLachlan & Stewart, 1976; Phillips et al., 1986; Hitchcock-DeGregori, 1994). Neither D175 nor E180 is one of the proposed consensus actin binding residues (Phillips et al., 1986; personal communication). However, D175 would be able to form an ion pair with R178 in the same chain and be repulsive to E173 in the *e* position in the other chain, both conserved residues and part of the consensus site. E180 could form an ion pair with R182 in the opposite chain, another conserved residue, but not part of the Phillips consensus site. While we have no detailed understanding of the structural requirements of internal periods for actin affinity, here it is notable that the D175N mutation involves a side chain that is proximal to residues possibly involved in actin binding (Phillips et al., 1986). D175N changes the charge but not the size of the side chain whereas E180G is more dramatic, changing both the charge and size of the side chain.

One role of TM is its involvement with myosin to activate cooperatively the thin filament (Bremel et al., 1973) and switch it from the "off" to the "on" binding state (Lehrer, 1994). Fluorescent probes at Cys 190 of TM have been used to investigate the S1-induced change from the off to the on-state (Lehrer & Ishii, 1988; Ishii & Lehrer, 1990; Geeves & Lehrer, 1994). There are two fluorescence effects of different magnitude associated with S1 binding to actin, both indicative of changes in the local environment of TM on the actin filament (Lehrer & Ishii, 1988). For pyrene-TM, these are manifested as a long-range effect associated with localized unfolding (increased excimer fluorescence) that is complete when only two to three actins bind per TM or per seven actin monomers. The second appears to be a small decrease in excimer fluorescence associated with saturation of the actin filament with myosin S1 and consequent binding in the region of Cys 190. This is a short-range effect, apparently associated with stabilization of the Cys 190 region.

The conformation of the Cys 190 region of both FHC TM mutants in the myosin S1-induced on-state differs from that of wild-type TM. Neither exhibited the increase in excimer fluorescence at low saturation of actin with myosin S1. Since D175N and E180G had high excimer fluorescence in the absence of actin and myosin S1 (Table 1), the long-range effect would not be apparent if the pyrenes are close to the optimum arrangement. The small decrease in excimer fluorescence in E180G upon myosin S1 binding may be due to the short-range effect. What is interesting is that D175N showed a much larger decrease in excimer fluorescence than wild-type or E180G when the thin filament was saturated with myosin S1, that is, when a myosin head bound close to the probe on TM bound to the actin filament, indicative of a short-range effect. Therefore, even though the mutants bind cooperatively to actin, they do not respond with the same conformational change when myosin S1 switches the thin filament from the off to the on-state. Unfortunately, it was not possible to correlate the fluorescence changes with activation of the actomyosin ATPase; the unacetylated recombinant TMs do not show this activation (Bartegi et al., 1990; Cho & Hitchcock-DeGregori, 1991).

The TMs used present studies differed in two respects from those found in the diseased state. First, they were αα-TMs, the major isoform in most skeletal muscles and in the hearts of small mammals. In large mammals, such as man, the major TM is αβ-TM. While there are sequence differences between the two isoforms throughout the molecule, the residues in the β-chain proximal to D175 and E180 are the same as in α-TM (E173, R178, and R182) (MacLeod et al., 1985; MacLeod & Gooding, 1988; Widada et al., 1988). Therefore, one might anticipate the mutations to have the generally same local consequences in αβ-TM as in αα-TM. The second difference is that the recombinant TMs were homodimers; both α-chains carried the mutation. The mutation in humans is dominant where, if the mutant TM is

expressed, in general one polypeptide chain would be wild-type and the other mutant. The interchain ionic interactions involving D175 and E180 would be disrupted in either homodimers or heterodimers. In addition, as previously mentioned, the recombinant TMs are unacetylated.

It is also important to note that D175 and E180 are encoded by exon 5, a constitutively expressed exon found in TMs expressed in virtually every cell and tissue, and in all the TM in certain muscles (Pittinger & Helfman, 1991). Yet, the only reported phenotype so far is the cardiomyopathy. One may speculate that the cardiomyocytes are compromised in the long term because they continuously work under a load. While the mutations have grave consequences for the patients and their families, the phenotype cannot be considered severe as it is not usually lethal until adulthood. Unfortunately, there is no information about the ultrastructure or physiology of the muscles or other cells of individuals carrying these mutations.

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