The Effect of Mutations in α-Tropomyosin (E40K and E54K) That Cause Familial Dilated Cardiomyopathy on the Regulatory Mechanism of Cardiac Muscle Thin Filaments

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E40K and E54K mutations in α-tropomyosin cause inherited dilated cardiomyopathy. Previously, we showed, using Ala-Ser tropomyosin (AS-α-Tm) expressed in Escherichia coli, that both mutations decrease Ca$^{2+}$ sensitivity. E40K also reduces $V_{\text{max}}$ of actin-Tm-activated S-1 ATPase by 18%. We investigated cooperative allosteric regulation by native Tm, AS-α-Tm, and the two dilated cardiomyopathy-causing mutants. AS-α-Tm has a lower cooperative unit size (6.5) than native α-tropomyosin (10.0). The E40K mutation reduced the size of the cooperative unit to 3.7, whereas E54K increased it to 8.0. For the equilibrium between On and Off states, the $K_T$ value was the same for all actin-Tm species; however, the $K_T$ value of actin-Tm-troponin at $pCa$ 5 was 50% less for AS-α-Tm E40K than for AS-α-Tm and AS-α-Tm E54K. $K_T$, the “closed” to “blocked” equilibrium constant, was the same for all tropomyosin species. The E40K mutation reduced the affinity of tropomyosin for actin by 1.74-fold, but only when in the On state (in the presence of S-1). In contrast the E54K mutation reduced affinity by 3.5-fold only in the Off state. Differential scanning calorimetry measurements of AS-α-Tm showed that domain 3, assigned to the N terminus of tropomyosin, was strongly destabilized by both mutations. Additionally with AS-α-Tm E54K, we observed a unique new domain at 55 °C accounting for 25% of enthalpy indicating stabilization of part of the tropomyosin. The disease-causing mechanism of the E40K mutation may be accounted for by destabilization of the On state of the thin filaments; however, the E54K mutation has a more complex effect on tropomyosin structure and function.

Inherited cardiomyopathies have been shown to be caused by mutations in proteins associated with contractility. In hypertrophic cardiomyopathy (HCM), 2 the majority of mutations are in proteins of the contractile apparatus (1). In addition, a subclass of inherited dilated cardiomyopathy (DCM), not accompanied by additional symptoms such as conduction disease, is also predominantly associated with mutations in contractile proteins (2, 3).

Cardiomyopathic mutations have been found in all the proteins of the thin filament as follows: actin, tropomyosin, troponin I, troponin C, and troponin T. 11 HCM-causing mutations have been identified in α-tropomyosin, and two mutations, E40K and E54K, have been reported to cause DCM (5).

The effect of HCM-causing α-tropomyosin mutations on thin filament function has been studied by in vitro motility assay and actomyosin ATPase measurements (6–10). In common with mutations in other thin filament proteins, it was found that α-tropomyosin HCM mutations produced an increase in Ca$^{2+}$ sensitivity, and in the majority of cases this was accompanied by an increase in cross-bridge cycling rate or impaired relaxation.

In contrast the two reported DCM-causing mutations, E40K and E54K, cause a decrease in Ca$^{2+}$ sensitivity, and E40K also causes a decrease in cross-bridge cycling rate. In fact, a study of eight DCM mutations in troponin T, troponin C, and α-tropomyosin showed that reduced Ca$^{2+}$ sensitivity and cross-bridge cycling rate are consistent molecular phenotypes in DCM mutations (11).

The question of how DCM-causing mutations produce this molecular phenotype has not been addressed experimentally. The mechanism by which mutations in α-tropomyosin could affect Ca$^{2+}$ sensitivity must be indirect. Tropomyosin has no direct interactions with troponin C or troponin I; however, Ca$^{2+}$ regulation is both allosteric and cooperative, and tropomyosin plays an essential role in transmitting Ca$^{2+}$ activation from troponin C to the whole thin filament (12), and thus it is possible that the mutations interfere with the cooperative mechanism of the thin filament. Steady state and equilibrium measurements of thin filament regulation can be interpreted in...
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terms of the two-state (On and Off) cooperative allosteric model of Monod et al. (13) with tropomyosin acting as the component that confers cooperativity on the system (14). Contraction activity is then determined by the proportion of the thin filament that is in the On state; both the size of the cooperative unit and the On/Off equilibrium constant ($K_I$) vary between different tropomyosin isoforms, and so these parameters may be altered by mutations (15–17). Transient kinetic experiments have indicated that the Off state may in fact consist of two substrates, a “closed” state, where thin filaments can form weak bonds with myosin-ADP-Pi, and a “blocked” state, where myosin-ADP-P cannot bind. The equilibrium between these states ($K_p$) is highly dependent upon $Ca^{2+}$ and also differs between different tropomyosin isoforms (18–20).

To understand the mechanism of mutation-induced change in thin filament function, we have measured cooperative unit size, On/Off equilibrium constants, and $K_p$ for the E40K and E54K DCM mutations in comparison with wild type and native tropomyosin. To understand the structural changes that underlie the functional effects of the mutation, we have made thermostability measurements using CD and differential scanning calorimetry (DSC) (21–23).

We have found that E40K and E54K α-tropomyosin mutations alter thin filament function by different mechanisms. The effect of the E40K mutation can be accounted for by a decreased cooperative unit size and lower $K_I$; however, the E54K mutation does not alter these parameters and appears to alter regulation by a more complex mechanism.

MATERIALS AND METHODS

Protein Preparations—Rabbit skeletal muscle actin, myosin, and myosin subfragment-1 (S-1) were prepared by established methods (24–26) The N-ethylmaleimide derivative (NEM S-1) was prepared as described previously (27). Rabbit skeletal muscle and sheep cardiac muscle tropomyosin were prepared by the original Bailey method (28) and purified by hydroxyapatite chromatography (29). Ala-Ser-α-tropomyosin (AS-α-Tm) and the two mutants were expressed in Escherichia coli and purified as described previously (11). All tropomyosin species were labeled at Cys-190 with N-(1-pyrenyl)iodoacetamide (referred to in the text as pyrene) (Molecular Probes) as described by Lehrer and co-workers (30).

Steady State ATPase Assay—Actin-tropomyosin activation of S-1 ATPase was assayed as described previously (31) in ATPase buffer (10 mM PIPES, 75 mM KCl, 5 mM MgCl$_2$, 0.1 mM DTT, pH 7.2). Phosphate liberated by ATP hydrolysis was measured by the method of Taussky and Schorr (32).

Steady State Fluorescence Titrations—Steady state fluorescence measurements were obtained with a Fluoromax-2 photon counting fluorimeter in the ratio mode with monochromators for both excitation and emission wavelength selection. Titrations were carried out with excitation at 343 nm and emission at 90 °C at 350 nm to monitor light scattering and 485 nm to monitor the excimer fluorescence of pyrene labeled tropomyosin. Before titrations all proteins were clarified by centrifugation in a refrigerated bench top centrifuge for 10 min at 25,000 × g. All buffers used were filtered before use.

Stopped-flow Experiments—All transient kinetic measurements were performed on a Hi-Tech Scientific SF-61 double mixing stopped-flow system using a 100-watt Xe/Hg lamp and a monochromator for excitation wavelength selection. Pyrene fluorescence was excited at 364 nm, and pyrene excimer emission was monitored through a 455 nm cut-off filter. Light scattering was observed at 90° to the incident beam using $\lambda_{ex} = 364$ nm and a UG-5 emission filter (light over 400 nm was cut off). The measurements were carried out in ATPase buffer at 20 °C unless otherwise stated. The temperature of the stopped-flow machine was maintained within 0.1 °C during the course of the experiment. All buffers were filtered, and all proteins were clarified by centrifugation in a refrigerated bench-top centrifuge for 10 min at 25,000 × g just before use. Usually four to nine transients were collected and averaged. The stated concentrations of reactants are those in the stopped-flow observation cell after mixing.

Tropomyosin Binding Measurements—The binding of actin to tropomyosin was measured by co-sedimentation. 0.25–12 μM of AS-α-Tm and 21 μM of native rabbit skeletal actin were co-sedimented in the presence or absence of 3 μM NEM myosin S-1 by centrifugation at 313,000 × g in a Beckman TLA100 rotor at 20 °C in 200 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3.87 mM MgCl$_2$, and 0.5 mM dithiothreitol. Tropomyosin in pellet and supernatant fractions was separated by 10.5% SDS-PAGE, stained with Coomassie Blue, and quantified by densitometry. All calculated tropomyosin concentrations were adjusted by percentage pellet recovery, which was independently calculated using the same densitometry system. Apparent binding constants ($K_{app}$) determined by fitting the data to the Hill equation using Kaleidograph (Synergy Software).

DSC Measurements—DSC and light scattering experiments on recombinant AS-α-Tm and its DCM mutants, E40K and E54K, were performed as described earlier for AS-α-Tm and its HCM mutants, D175N and E180G (23). All tropomyosins were fully reduced before experiments by heating in the presence of $β$-mercaptoethanol followed by pre-heating of the samples in the calorimeter cell. The state of Cys-190 (reduced or cross-linked) was checked by SDS-PAGE in the absence of $β$-mercaptoethanol; all tropomyosins studied were in the fully reduced state.

CD Analysis—Thermal stability measurements were made following the molar ellipticity of tropomyosin at 222 nm as a function of temperature in buffer containing 0.5 M NaCl, 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, and 0.5 mM dithiothreitol using a Jasco J720 spectropolarimeter equipped with a variable temperature bath (cell path length 1 cm). Data were obtained at 1.0 °C intervals from 10 to 90 °C using a protein concentration of 0.1 mg/ml. The molar ellipticity was normalized (10 °C = 1, 90 °C = 0) to give fraction folded.

RESULTS

Effect of Tropomyosin Mutations on Acto-S-1 ATPase—Previously we found that the sliding speed of Ca$^{2+}$-activated thin filaments containing the AS-α-Tm E40K mutation was lower than wild type or filaments with the E54K mutation (11). We investigated the activation of actin-tropomyosin ATPase over a range of actin-tropomyosin concentrations. The activation
curves were fitted to a simple saturation equation; this showed that the AS-α-Tm E40K had an 18% lower $V_{\text{max}}$ than wild type AS-α-Tm (37 s$^{-1}$ versus 45 s$^{-1}$), whereas AS-α-Tm E54K had a $V_{\text{max}}$ that was slightly higher than wild type (Fig. 1A). In paired experiments wild type and mutant tropomyosin gave the same $K_m$ value. A similar pattern was observed with fully reconstituted thin filaments at pCa 5. Thus, one effect of the E40K mutation in tropomyosin is to reduce the cross-bridge turnover rate.

Because these actin-tropomyosin activation measurements were made with a large excess of actin over S-1, cooperative effects were not observed. It is possible that mutations in tropomyosin could affect the cooperativity of the thin filament. To test for this, we measured ATPase activity as a function of increasing S-1 concentration with a constant 2 μM actin. Strongly bound cross-bridges switch actin-tropomyosin filaments to the On state, and in these measurements this is manifested as an increase in the slope of the ATPase hydrolysis rate as S-1 concentration increases (33). With pure actin filaments we observed a constant slope indicating no cooperativity. In the presence of tropomyosin or troponin-tropomyosin, the slopes increased indicating cooperative activation of the thin filament. The different tropomyosin species produced different curves, and we observed the same pattern in the absence (Fig. 1B) and presence of troponin (Fig. 1C). When we compared native α-Tm with AS-α-Tm, we observed that filaments containing native tropomyosin consistently produced more activation than those composed of AS-α-Tm. When we compared wild type AS-α-Tm with the two mutants, we observed that for AS-α-Tm E40K the curve was equal to or below the WT curve. In contrast, filaments containing AS-α-Tm E54K tropomyosin gave higher ATPase activation than wild type at all S-1 concentrations, and in the presence of troponin the curve was very similar to that found using native cardiac tropomyosin.

Although these experiments demonstrate differences in cooperative properties in the mutant tropomyosin species, a detailed explanation is not possible because the shape of the curve depends upon several independent parameters, including $V_{\text{max}}$, cooperative unit size, and $K_m$, the equilibrium between the On and Off states. To understand the change in function caused by the mutations, we therefore need to make direct measurements of these parameters.

**Determination of Cooperative Unit Size**—We determined the cooperative unit size of actin-tropomyosin and actin-tropomyosin–troponin by two methods using tropomyosin–pyrene excimer fluorescence as a probe for the transition between Off and On thin filament states and light scatter as a probe of S-1 binding. Emission spectra of pyrene tropomyosin in the presence of actin show monomer fluorescence peaks at 390 and 400 nm and a broad excimer fluorescence peak centered on 480 nm. On addition of S-1, which switches the filament to the On state, the excimer fluorescence is increased substantially (20–60% increase; see Supplemental Material).

In steady state fluorescence titrations simultaneous light scattering and excimer fluorescence measurements show that increasing S-1 concentration results in an increase of the excimer fluorescence and light scattering and that the excimer fluorescence increase precedes the light scattering increase, reaching a maximum between 0.2 S-1 per actin (for AS-α-Tm E54K) and 0.6 (for AS-α-Tm E40K), whereas light scatter reaches a maximum at 1.0 S-1 per actin (Fig. 2, A and B). This indicates that the Off to On transition induced by S-1 is cooperative. To measure the cooperative unit size the fractional change in the excimer fluorescence ($f_{\text{on}}$) is plotted against the amount of S-1 bound to actin as determined from the light scatter reading. Fitting the data to the equation $f_{\text{on}} = 1 - (1 - f_{\text{bound}})^n$ allows the determination of the size of the cooperative unit (n) (Fig. 2C and Table 1) (17).

There were substantial differences between skeletal (a mixture of α and β) and cardiac (αα homodimer) tropomyosin with the cardiac tropomyosin having a larger cooperative unit size. The recombinant wild type AS-α-Tm had a lower cooperative unit size than native α-tropomyosin. The two DCM mutations altered the cooperative unit size in

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**FIGURE 1. Effect of E40K and E54K mutations in AS-α-tropomyosin on actin-tropomyosin-activated S-1 ATPase activity.** A, actin-tropomyosin activation of S-1 ATPase activity. The ATPase activity of 0.5 μM myosin S-1 was measured in the presence of 0–40 μM of rabbit skeletal muscle actin, with the appropriate 7:1 stoichiometric concentration of either AS-α-Tm, AS-α-Tm E40K, or AS-α-Tm E54K in ATPase buffer containing 3 mM ATP at 37 °C. ATPase activity was calculated as absolute rate in s$^{-1}$. Individual points represent the mean ± S.E. (n = 4). Lines of best fit were calculated according to the Michaelis-Menten equation. B, dependence of ATP hydrolysis rate on S-1 concentration at a fixed actin concentration, the effect of different tropomyosin species. Conditions are as follows: 4 μM actin (Tm:actin 0.4 w/w), 5 mM MgATP, 50 mM KCl, 35 mM imidazole-HCl, pH 7.4, 4 mM MgCl$_2$, 1 mM EDTA, 5 mM DTT, 1 mM CaCl$_2$. C, dependence of ATP hydrolysis rate on S-1 concentration at a fixed actin concentration; the effect of different tropomyosin species in presence of troponin (Tn) at pCa 5. Conditions are as for B with additional 7 μM cardiac troponin.
opposite directions; AS-α-Tm E40K had a cooperative unit size approximately half wild type AS-α-Tm, whereas AS-α-Tm E54K had a cooperative unit size greater than wild type. Both these differences were statistically significant (Fig. 2 and Table 1).

The cooperative unit size of actin-tropomyosin may also be determined from the kinetics of actin-S-1 dissociation by ATP. A tropomyosin-actin-S-1 complex was preformed and mixed with 20 μM ATP in the stopped-flow apparatus, which caused dissociation of S-1 from the complex. As the ATP binds and dissociates the bound S-1, the light scattering falls but the tropomyosin-actin unit remains in the On state as long as a single S-1 remains bound within the cooperative unit. Thus, the pyrene fluorescence remains constant until the last S-1 dissociates and then changes rapidly as the filament switches to the Off state. The duration of the lag depends upon the size of the cooperative unit as shown in Fig. 3A. The predicted time course of the excimer fluorescence is given by the equation relative signal $f_{on} = 1 - (1 - \exp(-k_{LS}t))^n$, where $k_{LS}$ is the experimentally observed light scattering (LS) rate constant, and $n$ is the size of the cooperative unit (17). These curves could accurately describe the observed transient, and therefore the equation may be used to determine $n$. The values for $n$ determined by this method are very similar to $n$ determined by titration (Table 1), in particular $n$ for AS-α-Tm E40K was substantially less than wild type AS-α-Tm, whereas AS-α-Tm E54K was significantly greater.

The kinetics of actin-S-1 dissociation can also be successfully used to measure $n$ in fully reconstituted thin filaments (Fig. 3B). We found that with all species of tropomyosin, $n$ for the regulated thin filament was about double that for actin-tropomyosin and also that $n$ was consistently greater at $pC\alpha 5$ compared with $pC\alpha 9$ ($p < 0.01$). For example, with thin filaments containing native cardiac troponin and tropomyosin $n$ was found to be $23.7 \pm 0.9$ at $pC\alpha 5$ and $16.7 \pm 0.3$ at $pC\alpha 9$. Differences between skeletal, cardiac, and AS-α-Tm were retained. The effect of DCM mutations on $n$ were also similar in the presence of troponin with $n$ for AS-α-Tm E40K less than wild type ($p =$

![Figure 2](image-url)

**FIGURE 2.** Determination of cooperative unit size by titration of tropomyosin-pyrene excimer fluorescence and light scatter. A, normalized increase of pyrene excimer fluorescence (■) and light scatter (●) because of addition of S-1 for AS-α-Tm E40K. B, normalized increase of pyrene excimer fluorescence (■) and light scatter (●) because of addition of S-1 for AS-α-Tm E54K. C, plot of fraction of filaments in the On state against fraction of S-1 bound per actin. Data are fitted to the equation $f_{on} = 1 - (1 - f_{bound})^n$. Calculated values for $n$ are summarized in Table 1. Conditions are as follows: 6 μM actin, 1 μM pyrene-labeled tropomyosin as indicated on figure titrated with 0–12 μM S-1, in 100 mM KCl, 25 mM imidazole-HCl, pH 7.4, 4 mM MgCl₂, 1 mM EDTA, 5 mM DTT.

**TABLE 1**

| Method used     | Skeletal Tm-actin | Cardiac Tm-actin | AS-α-Tm-actin | AS-α-Tm E40K-actin | AS-α-Tm E54K-actin |
|-----------------|-------------------|------------------|---------------|-------------------|-------------------|
| Equilibrium titration | 7.0 ± 0.1         | 10.7 ± 0.4       | 6.7 ± 0.2     | 3.7 ± 0.3         | 8.0 ± 0.1         |
|                 | p < 0.001         | p < 0.001        | p < 0.001     | p < 0.001         |                   |
| Kinetic measurement | 6.7 ± 0.3         | 9.7 ± 0.3        | 6.3 ± 0.3     | 4.0 ± 0.5         | 8.3 ± 0.6         |
|                 | p = 0.02          | p = 0.05         | p = 0.02      |                   |                   |
Determination of $K_T$—We determined the equilibrium constant, $K_T$, of the On-Off transition for actin-tropomyosin and actin-Tm-troponin at pCa 5 by pyrene-tropomyosin excimer fluorescence titrations (Fig. 4). Troponin-tropomyosin at pCa 9 switched off the filaments as judged by ATPase measurements and also decreased excimer fluorescence to a steady level, which we have taken as being the Off state. The addition of troponin at pCa 5 to actin tropomyosin caused an ∼2-fold increase in fluorescence, which represents the equilibrium position for actin-tropomyosin-troponin. The On state can then be achieved by the further addition of S-1 (Fig. 4). $K_T$ = (fraction in On state)/(fraction in Off state) and because the fluorescence of the On and Off states was defined it was then possible to calculate $K_T$ for actin-tropomyosin and for actin-tropomyosin-troponin at pCa 5 (Table 2). The $K_T$ for actin-α-Tm was in the region of 0.2 and was not greatly affected by mutations in tropomyosin (slight decrease). The $K_T$ for actin-α-Tm-cardiac troponin was about 0.6. The E54K mutation appeared to have no effect, but $K_T$ was reduced by 50% because of the E40K mutation.

Determination of $K_b$—McKillop and Geeves (19) have described regulation in terms of three states rather than the two states previously considered. They observed that the Off state was an equilibrium between two kinetically distinguishable states termed closed and blocked. In the blocked state myosin heads could not bind to thin filaments. The equilibrium between closed and blocked states, described by the constant $K_b$, was observed to be highly dependent upon Ca$^{2+}$; therefore, $K_b$ can be determined by measuring the rate of S-1 binding to excess thin filaments in the presence and absence of activating Ca$^{2+}$ concentrations, and values of $K_b$ in the region 0.5–0.7 have been obtained (35). It is possible that the mutations in tropomyosin alter $K_b$; we therefore measured the rates of S-1 binding to reconstituted cardiac muscle thin filaments as a function of thin filament concentration (Fig. 5). In our hands we found only about a 40% difference in $K_{obs}$ in the presence and absence of Ca$^{2+}$ for thin filaments containing skeletal muscle tropomyosin and 20% for cardiac muscle tropomyosin. Incorporation of mutant tropomyosin made very little difference to this; the ratios of $K_{obs}$ in the presence and absence of Ca$^{2+}$ were 0.89 for AS-α-Tm, 0.84 for AS-α-Tm E40K, and 0.81 for...
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AS-α-Tm E54K, corresponding to $K_b$ of 8, 5.2, and 4.2, respectively.

Tropomyosin Binding to Actin—To check that none of the effects observed were because of dissociation of tropomyosin from actin, we determined the binding of tropomyosin to actin in ATPase buffer as used in the $V_{max}$, $n$, and $K_T$ measurements as well as in our previously published in vitro motility experiments using these mutant tropomyosins (11). All tropomyosin species bound with an affinity in excess of $5 \times 10^6 M^{-1}$ and actin was fully saturated with tropomyosin in the micromolar range (see Supplemental Material). To expose differences in actin binding because of the DCM mutations, actin-tropomyosin binding was also measured in 200 mM KCl (Fig. 6). Wild type AS-α-Tm bound with an affinity of about $10^{-6} M^{-1}$. The affinity of AS-α-Tm E40K was the same as wild type, but the affinity of AS-α-Tm E54K was 3.5 times weaker.

We investigated whether the different effects of the same glutamic acid to lysine mutation might be because of the activity state of the actin-tropomyosin filament. Analysis of the actin-binding sequences of tropomyosin has shown that Glu-40 is in the $\beta$-band and Glu-54 is in the $\alpha$-band of the proposed actin-binding zones 1 and 2, respectively (36, 37). Because $\alpha$ zones are believed to be close to actin in switched off filaments and $\beta$ zones are close to actin in switched on filaments, the locations may account for the differing effects of the mutations on the On-Off equilibrium.

Actin tropomyosin is mostly in the Off state ($K_T = 0.1–0.2$; see Table 2); when S-1 was added to switch the filament to the On state, the pattern of binding affinity was reversed. Wild type AS-α-Tm affinity was the same as AS-α-Tm E54K, whereas AS-α-Tm E40K affinity was half this value (Fig. 6B). The results are summarized in Table 3.

Thermally Induced Dissociation of Tm-F-actin Complexes—A decrease in light scattering accompanies dissociation of tropomyosin from the surface of the actin filament. We used this to study the dissociation of tropomyosin from the actin filament at increasing temperatures.

The mutation E40K had no influence on the light scattering of the AS-α-Tm-F-actin complex or on the temperature dependence of dissociation of AS-α-Tm from F-actin. On the other hand, AS-α-Tm E54K differed from both AS-α-Tm and AS-α-Tm E40K, because dissociation of AS-α-Tm E54K from F-actin occurred with higher cooperativity than in the case of the others (Fig. 7). Dissociation of AS-α-Tm E54K occurred within a narrow temperature range (from 42 to 50 °C), whereas AS-α-Tm and AS-α-Tm E40K dissociated within a broader temperature range (from 36 to 55 °C). Hill coefficients were as follows: 17.3 for AS-α-Tm, 20.3 for AS-α-Tm E40K, and 30.2 for AS-α-Tm E54K. A very good correlation was obtained between dissociation temperature $T_{dis}$ and denaturation temperature of the actin-induced domain AT (Fig. 8B; Table 5).

CD and DSC Measurements—Measurement of the temperature dependence of CD at 222 nm showed that all three AS-α-tropomyosin species were fully folded and that their thermal stability was very similar; the CD temperature profiles and their analysis are shown in the Supplemental Material. To obtain more detail about the coiled-coil stability, we used DSC. Fig. 8 shows DSC profiles for wild type AS-α-Tm, AS-α-Tm E40K, and AS-α-Tm E54K and the results of deconvolution of DSC profiles into individual thermal transitions corresponding to separate calorimetric domains in the tropomyosin molecule. The main calorimetric parameters for these domains (the maximum temperature of the transition, $T_{max}$, and calorimetric enthalpy, $\Delta H_{cal}$, i.e. the area under the calorimetric peak) are summarized in Table 4.

The DSC profile of AS-α-Tm has been decomposed into three calorimetric domains. Domain 1 at 37.7 °C has never been observed in previous DSC studies on α-tropomyosin. This

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**TABLE 2**

| Type of Tm | Cardiac | AS-α WT | AS-α E40K | AS-α E54K |
|------------|---------|---------|-----------|-----------|
| $K_T$ actin-Tm | 0.09 | 0.2 | 0.13 | 0.14 |
| $K_T$ actin-Tm-Tn | 0.07 | 0.19 | 0.11 | 0.13 |
| $K_T$ actin-Tm-Tn | 0.56 | 0.51 | 0.23 | 0.66 |

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**FIGURE 5.** Effect of tropomyosin mutations E40K and E54K on the equilibrium constant between the blocked and the closed states $K_b$. A, AS-α-Tm. B, AS-α-Tm E40K. C, AS-α-Tm E54K. $K_b$ was measured by comparing the rate of S-1 binding to pyrene-labeled actin-tropomyosin-troponin with (○) or without (□) Ca$^{2+}$ in a Hi-Tech stopped-flow at 20 °C. Buffer: MOPS 20 mM, KCl 140 mM, MgCl$_2$ 5 mM, and DTT 0.5 mM, pH 7.0, CaCl$_2$ 0.5 mM, or EGTA 1 mM.
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FIGURE 6. Tropomyosin binding to actin in the presence and absence of S-1. A, 21 µM of rabbit skeletal muscle actin was used to co-sediment 0.25–12 µM of either AS-α-Tm, AS-α-Tm E40K, or AS-α-Tm E54K. B, as for A with the addition of 3 µM NEM-treated myosin S-1. Co-sedimentation was carried out in buffer containing 200 mM KCl, Tris-HCl, pH 7.5, 3.87 mM MgCl2, and 0.5 mM DTT. Centrifugation was at 313,000 × g for 10 min at 4 °C. The concentration of bound and free tropomyosin was calculated from densitometric analysis of SDS-PAGE samples containing supernatant and pellets fractions following co-sedimentation. All calculated tropomyosin concentrations were adjusted by percentage pellet recovery, which was independently calculated using the same densitometry system. Lines are lines of best fits to the Hill equation. Results are summarized in Table 3.

FIGURE 7. Temperature dependence of dissociation of the F-actin complexes with WT, AS-α-Tm, AS-α-Tm E40K, and AS-α-Tm E54K. A decrease in the light scattering intensity reflects dissociation of the Fm-F-actin complex. The dissociation curves were obtained by subtraction of the curve for phalloidin-stabilized F-actin alone from experimental curves for the Fm-F-actin complexes (23). Conditions were the same as for DSC experiments presented in Fig. 8B. The heating rate was 1 K/min. The temperature of maximal dissociation of the Fm-F-actin complex, Tm, is the temperature at which a 50% decrease in light scattering occurs. The Tm values for AS-α-Tm, AS-α-Tm E40K, and AS-α-Tm E54K were equal to 45.3, 45.6, and 45.5 °C, respectively. Hill coefficients were 17.3 for AS-α-Tm, 20.3 for AS-α-Tm E40K, and 30.2 for AS-α-Tm E54K.

TABLE 3

| Type of Tm | Actin-tropomyosin binding constant | Actin-tropomyosin binding constant + S-1 |
|------------|-----------------------------------|---------------------------------------|
| AS-α-Tm    | 0.95                              | 0.47                                  |
| AS-α-Tm E40K | 0.95                             | 0.27                                  |
| AS-α-Tm E54K | 0.27                             | 0.50                                  |

Domain 3 has been assigned to the N-terminal part of tropomyosin molecule with Cys-190 reduced (23). It has the same position (Tm = 43.1–43.9 °C) for all tropomyosins studied, and its enthalpy is 35–40% of the total for all tropomyosins, although the ∆Hcal value of this domain is much less for AS-α-Tm E40K and AS-α-Tm E54K (∼170 kJ/mol) than for AS-α-Tm (260 kJ/mol) (Table 4).

Domain 3 has been assigned to the N-terminal part of tropomyosin (23). As expected, both DCM mutations, E40K and E54K, strongly affect the calorimetric parameters of this domain. Both mutations decrease the Tm value of domain 3 by more than 2 °C. Furthermore, the mutation E40K strongly decreases the enthalpy of this domain in comparison with AS-α-Tm (135 versus 280 kJ/mol), and this is the main reason why the total calorimetric enthalpy of AS-α-Tm E40K (405 kJ/mol) is much less than that of AS-α-Tm (665 kJ/mol) (Table 4).

The most pronounced changes in domain 3 are observed with AS-α-Tm E54K. In this case, domain 3 is very small (its enthalpy is only 15% of the total), and a new domain at 55 °C appears on the thermogram (domain 4). This domain, which is present only in AS-α-Tm E54K, possesses more than 25% of the total enthalpy (Table 4). Taken together, domains 3 and 4 in AS-α-Tm E54K comprise 41% of the total enthalpy, equivalent to domain 3 in AS-α-Tm (42%) (Table 4). We can propose from these data that the E54K mutation has two opposite effects on the N-terminal region of the AS-α-Tm molecule; it destabilizes some part of this region but stabilizes (i.e. significantly increases the thermal stability) some other part in this region of tropomyosin.

Actin-induced Changes in the Thermal Unfolding of Tm and Its DCM Mutants—Fig. 8B shows DSC profiles for AS-α-Tm, AS-α-Tm E40K, and AS-α-Tm E54K, complexed with phalloidin-stabilized F-actin. It is seen that in the presence of actin a new, very sharp peak appears on the thermogram. The maximum temperature of this peak (Tm) correlates well with dissociation temperature, Tm, determined from light scattering experiments (Fig. 7). Fig. 8B also shows the results of deconvolution of DSC profiles. The main calorimetric parameters for calorimetric domains (Tm and ΔHcal) are summarized in Table 5.

Let us consider the actin-induced changes in the domain structure of tropomyosin and its DCM mutants. Domain 1 remains unchanged in the presence of F-actin for all tropomyo-
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sins studied. This confirms the assumption that this domain represents some damaged part of the tropomyosin molecule (or some admixture), which cannot interact with actin.

The new sharp peak, indicating cooperative unfolding (the actin-induced peak labeled AT in Fig. 8), originates from domain 2 whose enthalpy strongly decreases in the presence of F-actin. The calorimetric parameters of domain AT are very similar for all tropomyosin species. It is important to note that both DCM tropomyosin mutants, AS-α-Tm E40K and AS-α-Tm E54K, differ from AS-α-Tm in respect of conversion of domain 2 into domain AT (compare Tables 4 and 5). This conversion “domain 2 to domain AT” significantly increases, by more than 1.5 times, the \( \Delta H_{\text{cal}} \) value for AS-α-Tm E40K and AS-α-Tm E54K but not for AS-α-Tm. This is in favor of our assumption that, in the case of DCM Tm mutants, some part of the tropomyosin molecule unfolds noncooperatively with no contribution to the thermal transition, and that the “unseen” enthalpy of such noncooperative unfolding becomes visible only in the presence of F-actin, after dissociation of tropomyosin from the surface of the actin filament. A very similar effect was recently reported in DSC studies with non-muscle tropomyosins (21).

Interaction with actin has no significant influence on the position of domain 3 (and also domain 4 in AS-α-Tm E54K) because these domains unfold above the temperature for dissociation from actin. The enthalpy of these domains somewhat increases in the presence of F-actin, the effect being the most pronounced for AS-α-Tm E40K (compare Tables 4 and 5). However, this effect may be caused by overlapping the peak of domain 3 with the peak AT.

**DISCUSSION**

In this study we have investigated the effect of different isoforms and mutations in tropomyosin on the cooperative allosteric regulatory mechanism. For these studies we have

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**TABLE 4**

Calorimetric parameters obtained from the DSC data for individual thermal transitions (calorimetric domains) of WT AS-α-Tm, AS-α-Tm E40K, and AS-α-Tm E54K

| Tm        | Domain 1 (?) | Domain 2 (C-terminal region with Cys-190 reduced) | Domain 3 (N-terminal region) | Domain 4 (?) | \( \text{Total } \Delta H_{\text{cal}} \) |
|-----------|--------------|---------------------------------------------------|-------------------------------|--------------|----------------------------------|
|           | \( T_m^a \)  | \( \Delta H \) (°C kJ/mol % total)                | \( T_m^a \)  | \( \Delta H \) (°C kJ/mol % total) | \( T_m^a \)  | \( \Delta H \) (°C kJ/mol % total) | \( T_m^a \)  | \( \Delta H \) (°C kJ/mol % total) | \( \Delta H_{\text{cal}} \) (kJ/mol) |
| WT        | 37.7         | 125 (39%)                                         | 43.9                         | 260 (39%)    | 50.8                             | 280 (42%)     | 55.1                         | 129 (26%)    | 500 |
| E40K      | 37.8         | 99 (24.5%)                                        | 43.2                         | 172 (42.5%)  | 48.5                             | 135 (33%)     | 405 |
| E54K      | 37.8         | 126 (25%)                                         | 43.1                         | 170 (34%)    | 48.7                             | 75 (15%)      | 405 |

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*a* The error of the given values of transition temperature \( T_m \) did not exceed ±0.2 °C.

*b* The relative error of the given values of calorimetric enthalpy, \( \Delta H_{\text{cal}} \), did not exceed ±10%.

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**FIGURE 8.** DSC analysis of tropomyosin and actin-tropomyosin. A, temperature dependence of the excess heat capacity \( (C_p) \) of WT, AS-α-Tm, AS-α-Tm E40K, and AS-α-Tm E54K are shown in the reduced state. **Black solid lines** represent the experimental curves after subtraction of instrumental and chemical base lines. **The protein concentration was 30 μM. Other conditions are as follows:** 30 mM HEPES, pH 7.3, 100 mM NaCl, and 1 mM MgCl₂. The heating rate was 1 K/min. **Dotted lines** represent the individual thermal transitions (calorimetric domains 1–4) obtained from fitting the data to the non-two-state model. **Solid lines** represent the individual thermal transitions (calorimetric domains 1–4) obtained from fitting the data to the non-two-state model. **Domains 1–4** as well as transition AT are described in the text. **The heating rate was 1 K/min.** Concentration of all tropomyosins was 10 μM. Other conditions are as follows: 46 μM F-actin, 70 μM phalloidin, 30 mM HEPES, pH 7.3, 100 mM NaCl, 1 mM MgCl₂, and 1 mM β-mercaptoethanol. **A temperature region above 65 °C corresponding to irreversible thermal denaturation of phalloidin-stabilized F-actin is not shown.**
used recombinant human α-tropomyosin with an N-terminal Ala-Ser extension, commonly added to bacterially expressed α-tropomyosin to mimic acetylation. We have found that this recombinant tropomyosin gives a significantly lower cooperative unit size compared with native N-acetylated α- tropomyosin. The DCM-causing mutation E40K further reduces the size of the cooperative unit, whereas the other DCM-causing mutation, E54K, has the opposite effect. This diversity of molecular effects is mirrored by measurements of the structural effects of the mutations using CD and DSC. Correlation of functional and structural data allows us to explain the mechanism of the E40K mutation in terms of destabilization of the On state of the thin filaments; however, the E54K mutation has a more complex effect on tropomyosin structure and function.

Cooperative Parameters of Native and AS-α-Tropomyosin Isoforms—The cooperative unit size, n, represents the average number of actin monomers that can be switched On or Off by the binding or dissociation of a single strong-binding myosin head. Measurement of n by two different methods showed that for cardiac muscle actin-tropomyosin (100% αα-tropomyosin) it is about 10, which is substantially greater than for skeletal muscle tropomyosin (about 7). This result is in accord with previous measurements that demonstrated that n for skeletal muscle αβ-tropomyosin was less than all other tropomyosin species tested (17, 38) (Table 1). In the experiments described in this study and in the majority of published studies on tropomyosin mutants, bacterially expressed tropomyosin has been used with an additional Ala-Ser at the N terminus in place of the acetyl group present in native tropomyosin. Most functions of AS-α-Tm have been found to be indistinguishable from native α-tropomyosin extracted from muscle (6, 11, 23, 39), although Bing et al. (38) suggested that AS-α-Tm had a reduced cooperativity. Our direct measurements show AS-α-Tm has a cooperative unit size of about 6.5, similar to skeletal muscle tropomyosin and substantially smaller than native α-tropomyosin (Table 1). It is well established that end-to-end interactions between tropomyosin dimers are essential for the cooperative control of the thin filament, and the structure of the N terminus is critical for this interaction (39, 40). Our results indicate that, although the N-terminal Ala-Ser extension can substitute effectively for the N-acetyl group of native tropomyosin for most of its functions, end-to-end interactions are sufficiently different to affect cooperativity.

We have made comprehensive measurements of the effect of sheep cardiac muscle troponin on the cooperative unit size. Troponin increases the cooperative unit size of actin-tropomyosin with all of the tropomyosins tested (Fig. 3). n was also Ca2+-sensitive, being on average 2.2 times the actin-tropomyosin value in the presence of Ca2+ and 1.6 times in its absence. The increased n has been reported before and was shown to be because of tropomyosin-troponin T (T1 domain) interactions (41), but there have been conflicting reports about the effect of Ca2+. Geeves and Lehrer (17) using the same techniques as us did not observe any difference because of Ca2+, whereas Maytum et al. (35) used curve fitting to S-1 binding titrations and found that the cooperative unit size of actin-tropomyosin-troponin in the absence of Ca2+ was equal to actin-tropomyosin in the absence of troponin. The differences between our data and other published data may be related to the measurement techniques, the troponin species used, or the phosphorylation state of the troponin. The sheep cardiac troponin used in this study was largely dephosphorylated (troponin I and troponin T) (42), although it has been reported that troponin phosphorylation does not influence n (35).

Effect of DCM Mutations in Tropomyosin on Thin Filament Function—The mutations E40K and E54K in α-tropomyosin are reported to cause DCM, and in vitro both mutations lead to decreased Ca2+ sensitivity that may be the origin of the hypotrophic DCM phenotype (8, 11). Although indirect experiments suggest that thin filament cooperativity is altered by the DCM mutations (Fig. 1, B and C), most of the parameters we have measured are altered in different ways by the E40K and E54K mutations. The E40K mutation reduces cross-bridge turnover rate in in vitro motility and ATPase assays, but E54K mutation increases it slightly (Fig. 1A) (11), and the effect of the two mutations on cooperativity relative to wild type AS-α-Tm likewise is opposite (Fig. 1, B and C). This indicates the likelihood of multiple mechanisms by which mutations can cause the disease phenotype; we should therefore consider the effects of E40K and E54K mutations on the mechanism of cooperative thin filament regulation separately.

### TABLE 5
Calorimetric parameters obtained from the DSC data for individual thermal transitions (calorimetric domains) of WT AS-α-Tm, AS-α-Tm E40K, and AS-α-Tm E54K complexed with phalloidin-stabilized F-actin

| Tm       | Domain 1 (C-terminal region with Cys-190 reduced) | Domain 2 (N-terminal region of Tm*) | Domain 3 (or domains 3 and 4 in E54K-Tm) |
|----------|---------------------------------------------------|-----------------------------------|------------------------------------------|
|          | Tm° cal (°C) | ΔH kJ/mol (% total) | Tm° cal (°C) | ΔH kJ/mol (% total) | Tm° cal (°C) | ΔH kJ/mol (% total) |
| WT       | 37.4       | 95 (13%) | 43.9   | 75 (10%) | 46.0       | 240 (33%) | 50.3   | 325 (44%) | 735 |
| E40K     | 37.7       | 87 (12%) | 43.3   | 76 (10.5%) | 45.5       | 268 (37.5%) | 48.3   | 284 (40%) | 715 |
| E54K     | 37.8       | 103 (15%) | 42.4   | 65 (9%) | 45.5       | 271 (38%) | 48.1   | 271 (38%) | 710 |

* The error of the given values of transition temperature (Tm) did not exceed ±0.2 °C.
* The relative error of the given values of calorimetric enthalpy, ΔH, did not exceed ±10%.
* Domains 3 and 4 of AS-α-Tm E54K were not separated in this case as their unfolding was independent of the presence or absence of F-actin.
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Structural and Functional Consequences of the E40K Mutation.—The presence of the AS-α-Tm E40K mutation in Ca\(^{2+}\)-activated cardiac muscle thin filaments leads to a reduction of 17% in filament sliding speed and 26% in thin filament-activated S-1 ATPase activity (11), and in this study we have shown that this is because of an 18% reduction in \(V_{\text{max}}\), which is observed both in the presence and absence of troponin (Fig. 1A). In addition the E40K mutation affects thin filament cooperativity. The apparent cooperative unit size, \(n\), of actin-tropomyosin is reduced by 39% from 6.5 to 4 (Fig. 2 and Table 2) with corresponding decreases of 20% for fully reconstituted thin filaments in the presence of Ca\(^{2+}\) and 36% in the absence of Ca\(^{2+}\) (Fig. 3B). The On-Off equilibrium constant, \(K_{\text{p}}\), is also lower for Ca\(^{2+}\)-activated thin filaments containing the E40K mutation (Fig. 4 and Table 2), whereas the closed to blocked equilibrium constant, \(K_{\text{c}}\), is not affected by the mutation.

These observed differences may be sufficient to account for the effect of the E40K mutation on thin filament Ca\(^{2+}\) sensitivity (EC\(_{50}\) for Ca\(^{2+}\) is increased on average by 166% (8, 11)). A lower \(n\) and \(K_{\text{p}}\) would produce a lower fraction of thin filaments in the On state, and consequently more Ca\(^{2+}\) would be needed to switch thin filaments on, leading to reduced Ca\(^{2+}\) sensitivity. The coupling of the cooperative switching equilibrium to Ca\(^{2+}\) binding and thin filament activation was first demonstrated by Bremel and Weber (12) and has been confirmed for DCM and HCM mutations by Robinson et al. (43) who showed that the Ca\(^{2+}\)-binding affinity of troponin C in reconstituted thin filaments was reduced by 0.58 pCa units because of the E40K mutation.

CD and DSC measurements have been used to determine the effect of mutations on the stability of the tropomyosin coiled coil. There is a small, nonsignificant, increase in the melting temperature in the CD profile (see supplemental Fig. D) in contrast to the substantial destabilization of the N terminus reported for HCM mutations in tropomyosin (–3.7 °C for A63V, –10.4 °C for K70T (10), and –1.1 °C for E180G (9)). DSC measures the unfolding with a greater complexity than CD and also detects transitions that do not give a spectral change. The DSC measurements of AS-α-Tm E40K show a substantial loss of stability in “domain 3” assigned to the N-terminal half of tropomyosin, which is apparent both in the presence and absence of actin (Fig. 8 and Tables 4 and 5). This is likely to be because of the breaking of the inter-strand salt bridge, formed between Glu-40 and Arg-35, by the charge-reversal mutation (4).

It is possible that destabilization of the coiled coil may be directly responsible for the reduced \(n\) and \(K_{\text{p}}\), although, besides tropomyosin-tropomyosin interactions, the E40K mutation also affects the tropomyosin-actinin interaction. Interestingly, the E40K mutation has no effect on tropomyosin affinity for actin alone, where it would bind predominantly in the Off state (\(K_{\text{p}}\) = 0.1–0.2; Fig. 4 and Table 2), but it does weaken binding of tropomyosin to actin in the presence of sub-stoichiometric NEM-S-1, which switches actinin-tropomyosin to the On state (Fig. 6 and Table 3). This result may be explained in terms of the structure of tropomyosin. It has been proposed on the basis of analysis of the amino acid sequence that there are 14 19–20-residue actin-binding motifs in tropomyosin that are divided into two types of sequences (α and β) that alternate (34). Fig. 9 shows the first four actin-binding motifs of α-tropomyosin. McLachlan and Stewart (36) suggested that the α- and β-bands may represent actin-binding sites for two different thin filament states that could correspond to On and Off. Holthauzen et al. (37) have recently demonstrated that this is indeed the case and that the α-bands form an interface with actin in the absence of Ca\(^{2+}\), whereas in the presence of Ca\(^{2+}\), the tropomyosin rolls 90° so that the β-bands present an interface with actin. Glu-40 is in the first β-band, and therefore the E40K mutation would be expected to destabilize the On state with consequent effects upon actin affinity in the On state and hence the On-Off equilibrium. This may also account for the changes in cross-bridge cycling rate, Ca\(^{2+}\)-binding affinity, Ca\(^{2+}\) sensitivity, and DSC profiles.

The available data on the molecular mechanism generating the DCM phenotype permit a self-consistent hypothesis based on the destabilization of the On state of the cardiac muscle thin filament by the E40K mutation in α-tropomyosin, although alternative mechanisms are not excluded. In contrast the effects of the E54K mutation are much more complex.

Structural and Functional Consequences of the E54K Mutation.—The effect of the E54K mutation in α-tropomyosin on the cooperativity of cardiac muscle thin filaments is different from the E40K mutation even though both mutations involve the same Glu to Lys amino acid substitution at the “e” position of the heptad repeat (Fig. 9), and both mutations cause the same DCM phenotype (5, 11). The different pattern of results may be related to the fact that Glu-54 is located in the second α-band rather than the first β-band. Unlike E40K, the E54K mutation weakens tropomyosin binding to actin alone (Off state) but not in the presence of NEM-S-1 (On state) suggesting destabilization of the Off state. The light scatter data (Fig. 7) show that the thermal dissociation of E54K tropomyosin from actin in the Off state is also more cooperative than wild type or E40K tropomyosin. Consistent with this is the absence of any reduction in \(V_{\text{max}}\) cooperative unit size, \(K_{\text{p}}\), or \(K_{\text{c}}\). These aspects of the E54K mutation, in fact, resemble the results reported for HCM mutations, which also decrease actin-tropomyosin binding affinity (6, 9, 10) and increase Ca\(^{2+}\) affinity for troponin C; however, other properties of the E54K mutation are similar to other DCM mutations, notably the reduced Ca\(^{2+}\) sensitivity (EC\(_{50}\) increased by an average 167% (8, 11)).

The complexity of the effects of the E54K mutation are particularly apparent in the DSC measurements that show a dual effect. The domain 3 peak that derives from the N-terminal half
of tropomyosin is much reduced, representing only 15% of the total enthalpy compared with 42% for wild type tropomyosin, which would indicate destabilization of the N-terminal region of the tropomyosin as with the E40K mutation (Fig. 8 and Tables 4 and 5). In addition there is a new domain, unfolding at 55 °C on the thermogram, possessing 25% of the total enthalpy that has not previously been observed in any tropomyosin species (23). We can propose from these data that the E54K mutation has two opposite effects on the stability of the N terminus of tropomyosin; it destabilizes some part of the region, like E40K, but it stabilizes some other part of the tropomyosin structure.

The two opposite structural effects detected by DCS may correspond to the two opposite types of functional changes induced by the E54K mutation. With the data available it is not possible to explain how the E54K mutation causes the DCM phenotype; clearly it is different from the E40K mutation, and this emphasizes again that the end result of the mutations, namely the decreased Ca2+ sensitivity, is the critical factor in causing the DCM phenotype rather than the mechanism by which it is produced. The unique stabilized domain induced by the E54K mutation may be the key to understanding how the mutation affects regulation and is worthy of further investigation.

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