In the Drosophila flight muscle actin mutant E93K there is a charge reversal on the surface of actin close to the proposed position of tropomyosin when it is in the off state. Using a quantitative in vitro motility assay we have found that the wild type Drosophila ACT88F actin behaved like rabbit skeletal muscle actin when tropomyosin and troponin were added at pCa5 and pCa9. In contrast the effect of tropomyosin upon the E93K mutant actin filament movement was completely different from wild type and resembled the response of wild type with tropomyosin+troponin at pCa9 (i.e. the filaments were switched off). Velocity of E93K actin did not increase, and the fraction of filaments motile was reduced to less than 15% by adding up to 30 nm tropomyosin. When myosin subfragment-1 modified by N-ethylmaleimide was mixed with mutant E93K actin-tropomyosin filaments we observed that it restored motility of the filaments to the level observed with E93K actin alone. We conclude that electrostatic charge on the surface of domain 2 of actin plays a critical role in determining the state of actin-tropomyosin that is a central feature of the steric blocking mechanism of actin filament regulation.

Regulation of vertebrate striated muscle thin filament activity involves a Ca$^{2+}$-sensitive change of the state of the thin filament, which affects the interaction between actin molecules and myosin cross-bridges (1). The thin filaments can be described as a two state allosteric and cooperative binding system in which the cooperative unit consists of seven actins and one tropomyosin: troponin I and myosin heads act as negative and positive allosteric effectors respectively (2, 3). The equilibrium between the on and off states ($K_e$ in the model of Geeves and Halsall (2)) is regulated by Ca$^{2+}$ through the troponin complex and cross-binding. Troponin-tropomyosin regulation of thin filament activity is found throughout the animal kingdom, including insect striated muscles (4–6).

Tropomyosin is a dimeric α-helical coiled-coil that spans seven monomers along the actin filament (7). Tropomyosin molecules bind end-to-end, thus forming a continuous strand along the actin filament. According to current models, the tropomyosin strand may exist in two or more locations on the surface of the actin (8–10). In one position, corresponding to the biochemical on state, the negatively charged tropomyosin is located over actin subdomains 3 and 4 in a “trough” of positive charge (11, 12). The putative strong and weak myosin binding sites on actin are exposed and the myosin cross-bridges may cycle unimpeded. Recent work using actin mutants supports this model: charge change mutations within the trough of actin such as E311A/R312A in yeast (13) and K238A/E241A/E360H in Dictyostelium (14) result in weaker tropomyosin binding and destabilization of the on state.

The troponin I component of troponin, troponin I, is a positively charged protein that binds to actin and switches the tropomyosin to the off state. It is noteworthy that although troponin I controls the actin-tropomyosin state, it does not bind directly to tropomyosin, so it probably acts indirectly through actin (15). Electrostatic charges are likely to play an important part in this interaction.

The amino acids in actin involved in stabilizing the off state have not been positively identified, but there is evidence for troponin I contacts on domains 1 and 2 of actin (16). This is compatible with reports that changes in the shape of domain 2 of actin are associated with regulation (17).

A cluster of negative charge due to Asp$^{56}$, Glu$^{57}$, and Glu$^{93}$ is located at the bottom of domain 2, close to the position occupied by tropomyosin when in the off state (Fig. 1)(10). We considered the possibility that this negative charge cluster may repulse tropomyosin, thus maintaining the on state and that neutralization of the repulsion by inhibitory proteins may be an essential feature of the inhibitory mechanism. To test this hypothesis, we studied the state of actin-tropomyosin in a mutant of the Drosophila flight muscle-specific actin gene Act$^{88F}$ with a charge reversal mutation of glutamic acid 93 to lysine (E93K actin)(18, 19). The E93K mutation produces a flightless phenotype characterized by disordered sarcomeres. However in vitro motility assays have demonstrated that E93K actin filaments can move over myosin and produce force, which is not very different from the wild type (20, 21). Using in vitro motility techniques similar to those we used to characterize tropomyosin based regulation in striated and smooth muscles (22, 28, 32, 33), we can demonstrate that charge reversal of amino acid 93 on actin does indeed change the equilibrium of actin-tropomyosin toward the off state.

**EXPERIMENTAL PROCEDURES**

*Actin Preparation—Drosophila WT* indirect flight muscle-specific actin was made from a rosy$^{506}$ homozygous strain (rosy$^{506}$ is an eye color

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* This work was supported by the Biotechnology and Biological Sciences Research Council (to A. R. and J. C. S.) and by the Wellcome Trust and Clinical Research Committee (Brompton Hospital) (to S. B. M. and W. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: WT, wild type ACT88 actin; E93K
Fig. 1. Surface model of the actin monomer showing the location of the carboxyl oxygens of glutamic acid 93 relative to the positions of tropomyosin in the off and on (Ca$^{2+}$ + cross-bridges) states. Actin structure was drawn from the atomic coordinates published by Lorentz et al. (43) using Molview molecular graphics software. The tropomyosin positions are based on the models of Holmes (11) and Lehman et al. (10).

mutation) and E93K mutant actin from a rosy506 Act88Fsneakersp. [Act88F$^{roxy506}$] strain, which has a single copy of the E93K mutant Act88F gene inserted into an Act88F null genome by P-element-mediated transformation (18). Flies were grown and then stored at -20 °C prior to processing.

A myofibrillar preparation was made from 50 g (50,000) of flies according to the method of Saide et al. (23). After a high salt extraction (20 mM KPO$_4$, 500 mM KCl, pH 7.0) on ice for 10 min, the myofibril pellet was dehydrated by resuspending first in 6 volumes of ice-cold 50% acetone, then in 6 volumes of ice-cold 100% acetone, and finally allowed to air dry overnight at room temperature.

Actin extraction from the acetone powder was performed essentially after the methods of Bullard (24) and Pardee and Spudich (25) in ACEX (2 mM Tris-Cl, 0.2 mM CaCl$_2$, 0.2 mM ATP, 1 mM dithiothreitol, pH 8.0) at 4 °C for 60 min. After spinning the extract at 43,000 rpm for 30 min, the actin stock was clarified by first spinning at 90,000 rpm for 20 min at 4 °C, a tenth of the extract volume of 10 mM KCl was added and polymerization allowed to proceed for 2 h at 4 °C. Solid KCl was added to a final concentration of 800 mM. After incubation for 10 min at 37 °C to dissociate the contaminating Drosophila thin filament proteins, the preparation was spun at 90,000 rpm for 20 min at 4 °C in a Beckman TLA100.3 rotor. The F-actin pellet was resuspended in 3 ml of ACEX, homogenized, and then dialyzed for 24 h in 3 liter changes of ACEX. The optical density at A$_{280}$ to A$_{310}$, with an extinction coefficient of 0.62 cm$^2$/mg, was used to estimate the molarity of the G-actin stock.

Anion exchange chromatography employing a 1-ml Mono Q column (Amersham Pharmacia Inc.) was used to isolate the indirect flight muscle-specific actin isoform from the mixture of the six Drosophila isoforms constituting the G-actin stock. Before application to the column, the actin stock was clarified by first spinning at 90,000 rpm for 20 min in a Beckman TLA100.3 rotor and then filtering through 0.2-mm filters (Millipore). A 28-min segmented gradient from 0 to 500 mM NaCl in 20 mM MOPS, pH 6.5, was then used to resolve the actin mixture into four different isoform peaks (Fig. 2).

The peak B fractions from chromatographic runs of WT (or E93K) actin were pooled and polymerized as before for 2 h at room temperature and then spun at 100,000 rpm for 10 min at 4 °C in a Beckman TLA100.3 rotor. Six separate paired preparations of WT and E93K actin were prepared; yield was 30–100 μg per preparation. E93K does not polymerize as readily as WT actin, and in one preparation it was necessary to add heavy meromyosin at 1:500 mol/mol in order to promote polymerization. The F-actin pellets were resuspended in ACEX to a final concentration of about 5 μM actin.

In Vitro Motility Assay—The in vitro motility assay was performed as described by Fraker and Marston (22) using 100 μg/ml skeletal muscle HMM on cover glasses coated with silicone by soaking in 0.2% dichloromethylsilane in chloroform. F-actin was labeled with rhodamine-phalloidin (ϕ) as described by Kron et al. (26). F-actin-ϕ-tropomyosin and actin-ϕ-tropomyosin-troponin complexes were formed at 10 °C in the presence of troponin C, troponin I, and troponin T. The complexes were then diluted to 0.1% shear. The actin in the assay was assayed for motility at 20 °C. A constant force was applied to the motility assays, and all measurements were made by video taping the movement of actin filaments. The results were quantitated as a function of time by computer software.
infusion into the motility cell. We have demonstrated that tropomyosin does not dissociate during the experiment under these conditions and that additional tropomyosin in buffers C and D is not necessary (22).

A flow cell was prepared from a freshly siliconized coverslip and a microscope slide as described by Kron et al. (26). Assay components and buffers were infused into the flow cell in 30–60-s intervals. Two 50-μl aliquots of HMM at 100 μg/ml were infused in buffer A (50 mM KCl, 25 mM imidazole-HCl, 4 mM MgCl₂, 1 mM EDTA, 5 mM dithiothreitol, pH 7.4) to provide a coating of immobilized HMM on the coverslip. This was followed by 2 × 50 μl of buffer B (buffer A + 0.5 mg/ml bovine serum albumin) then 2 × 50 μl of 10 mM actin-φ2 = associated tropomyosin–troponin in buffer A. 50 μl of buffer C (buffer B + 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 3 mg/ml glucose, 0.5% methylcellulose, pH 7.4) to provide a coating of immobilized HMM on the coverslip. This was followed by 2 × 50 μl of buffer D (buffer C + 1 mM ATP) were then infused. Ca²⁺ concentration was varied by incorporating Ca²⁺-EGTA buffers in the final assay buffers C and D.

The movement of actin-φ–tropomyosin filaments over the immobilized skeletal muscle HMM was observed under a Zeiss epifluorescence microscope (63×/1.4 objective) with a DAGE-SIT-68 camera and recorded on video tape. Videos were digitized, and the movement was analyzed to determine fraction of filaments moving and velocity of motile filaments using either the automatic tracking program described by Marston et al. (27) or the manual tracking procedure in the cases where filaments were not easily detected (22, 28).

RESULTS AND DISCUSSION

Comparison of Rabbit Skeletal and Wild Type Drosophila Flight Muscle Actin Filament Motility—Actin filament movement over immobilized heavy meromyosin was studied in an in vitro motility assay. With this assay it is possible to measure a number of parameters in place of the single parameter of ATPase. These are fraction of actin filaments moving, velocity of the filaments that are moving, and the number of filaments attached to myosin per unit area. It has been shown that Ca²⁺ can regulate all three of these parameters independently through tropolin-tropomyosin (22, 28, 29).

In previous work it has been shown that addition of arthropod (Limulus) tropomyosin to actin increases filament velocity (4), but this is not observed with vertebrate striated muscle tropomyosin. We therefore decided to test the Drosophila actin with vertebrate smooth muscle tropomyosin (from sheep aorta) as well as with vertebrate striated muscle tropomyosin (from rabbit). Smooth muscle tropomyosin tends to activate actomyosin ATPase, while skeletal muscle tropomyosin is usually inhibitory (30, 31). In the in vitro motility assay smooth muscle tropomyosin gives an increase in filament velocity similar to Limulus tropomyosin (4, 32, 33). This has been suggested as indicating that actin-tropomyosin is predominantly in the on state, since smooth muscle tropomyosin has a higher Kᵣ and size of cooperative unit compared with skeletal muscle tropomyosin (28, 30, 31).

The regulation of Drosophila flight muscle actin filament movement by tropolin and tropomyosin has not been studied before, and it is possible that Drosophila actin could have regulatory characteristics quite different from vertebrate actin. Table I compares Drosophila actin and the well studied rabbit striated muscle actin filaments (22, 28, 33). The two types of actin filaments moved at the same velocities with a high proportion of the filaments motile. Smooth muscle tropomyosin increased the velocity of both types of actin filaments by around 25%, while skeletal muscle tropomyosin had no effect on velocity, although cosedimentation experiments showed it did bind under the conditions of the motility assay (22). The fraction of filaments motile was not affected by either tropomyosin.

In previous experiments we have demonstrated in our system that addition of up to 16 nm troponin at Ca9 results in a marked decrease in the fraction of filaments moving accompanied by a modest decrease in the velocity of the filaments that remain motile (22, 25). Addition of skeletal muscle troponin to wild type 88F actin-skeletal muscle tropomyosin filaments produced the same decrease in the fraction of filaments motile (half-maximal effect at about 4 nm troponin) and the same small decrease in velocity of motile filaments (16% at 16 nm troponin) as was observed previously with skeletal muscle actin filaments (compare Fig. 3 with Fig. 3 in Ref. 22). This behavior was observed irrespective of the type of tropomyosin used and is characteristic of actin-tropomyosin filaments in the off state (28). Different technical approaches to measuring troponin control of filament motility have produced differing patterns of results in some experimental circumstances (these are discussed in Ref. 28); however, all the published analyses agree that reduction in the proportion of filaments that are motile is a characteristic feature of actin-tropomyosin filaments switched off by troponin or other inhibitory proteins (22, 32, 46, 47). At pCa5 troponin did not alter the fraction of filaments motile or the velocity.

We conclude that the motility parameters of actin from rabbit and Drosophila are indistinguishable, and they are regulated by tropomyosin and troponin in the same way. This justifies the use of mammalian regulatory proteins in our investigation of mutant Drosophila actin.

Comparison of Wild Type and E93K Mutant Actin—Although the E93K mutation has a flightless phenotype E93K actin, filaments have been reported to be capable of movement in the in vitro motility assay and of exerting force (20, 21). We found that wild type and E93K actin moved at almost the same velocity at 30 °C. At 28 °C wild type moved 26% faster than E93K actin, and it has been reported that at 23 °C wild type moved 50% faster than E93K actin (20). Thus our results are in broad agreement with previous experiments using E93K actin and confirm that the mutation does not seriously compromise the actin–myosin interaction. Some differences are to be expected, since we have used a different surface (siliconized glass), and we performed experiments at higher ionic strength than previously and used 0.5% methyl cellulose (20, 21).

We have found that silica surfaces seem to provide less resistance to movement than nitrocellulose and also that the addition of methylcellulose to the medium enables weakly bound filaments to remain attached to the myosin surface (28). Thus the conditions we have adopted would minimize differences in motility...
performance between wild type and mutant actin.

When smooth or skeletal muscle tropomyosin were added to wild type actin filaments, the fraction of filaments that were motile did not change. In contrast, when either smooth or striated muscle tropomyosin was added to the mutant E93K actin filaments, the fraction of filaments motile was greatly reduced (Fig. 4). Experiments were done with six paired preparations of wild type and E93K actin. The fraction of wild type actin filaments motile was 79 ± 3.6% (S.E., n = 7) and of E93K actin 71 ± 2% (S.E., n = 7). Tropomyosin progressively reduced the fraction of E93K actin filaments motile reaching 14 ± 2% (n = 5) with 16 nM skeletal muscle tropomyosin and 17 ± 2% (n = 3) with 16 nM smooth muscle tropomyosin; in contrast, tropomyosin did not affect WT actin filament motility (Fig. 4).

Skeletal and smooth muscle tropomyosin had different effects upon filament velocity. The skeletal muscle tropomyosin had no effect on velocity of the wild type actin as observed previously with rabbit actin (Table I) and slightly decreased velocity of E93K actin filaments (Fig. 4). The decrease was greater at 25 °C (up to 37%, Fig. 4) than at 27 °C (up to 19%). Smooth muscle tropomyosin enhanced the velocity of wild type actin filaments by 25% at 30 °C (Fig. 4, Table I), but this was not observed with E93K actin filaments, instead velocity declined in the same way as it did with skeletal muscle tropomyosin (Fig. 4).

It will be noted that the the large decrease in the fraction of E93K actin filaments motile and the small decrease in velocity when tropomyosin was added (Fig. 4) resembles the effect of adding troponin at pCa9 to wild type actin-tropomyosin filaments (Fig. 3). As has been discussed, this effect is characteristic of actin-tropomyosin filaments in the off state; thus it appears that the E93K mutation in actin causes tropomyosin to bind to actin only in the off state (32).

To confirm that the “switch off” of E93K actin filament movement was due to the tropomyosin-mediated mechanism, we added NEM-S-1 to E93K actin-tropomyosin. NEM-S-1 forms strong bonds to actin, even in the presence of ATP, thus it switches actin-tropomyosin to the on state independently of regulatory proteins (29, 34). We have shown previously that adding NEM-S-1 to actin-tropomyosin resulted in an increase in filament motility (28). When NEM-S-1 was mixed with E93K actin-smooth muscle tropomyosin filaments, we observed that it restored both the fraction of filaments motile and their velocity to the level observed with E93K actin when tropomyosin was absent (Fig. 5). This reversal of inhibition required less than 0.4 NEM-S-1 per actin, thus confirming that it was acting cooperatively upon tropomyosin in the same way as it does with wild type striated muscle actin-tropomyosin (28, 34). E93K actin-tropomyosin filaments were also reactivated by adding troponin in the presence of Ca2+ (Fig. 5). This indicates that the effect of the E93K mutation is to shift the on/off equilibrium of the thin filament toward the off state to a certain degree, but the Ca2+-activated troponin complex is capable of shifting the equilibrium back to the on state. In contrast a deletion mutation in tropomyosin has been shown to produce thin filaments that cannot be activated by Ca2+ and troponin (36), although they can be switched on by rigor S-1.

Molecular Interpretation of the Effect of the E93K Mutation—Recent structural work has indicated that tropomyosin is loosely associated with actin and that its location may be dependent upon the charge distribution on the surface of actin. The neg-

![Image](https://example.com/image1.png)

**FIG. 3. Regulation of wild type Drosophila flight muscle actin filament movement by skeletal muscle troponin-tropomyosin.** The effect of 0–16 nM skeletal muscle troponin on skeletal muscle actin-S-tropomyosin filament velocity (upper panel) and fraction of filaments motile (lower panel) was measured at pCa9 (closed circles) and pCa5 (open circles) at 25 °C. The movement of all filaments over a 0.65-s time interval was determined in 10 fields of view, and the data were plotted as a frequency histogram of velocities. There were two populations of filaments, moving and not moving. Any movement of less than 0.4 NEM-S-1 per actin, thus confirming that it was acting cooperatively upon tropomyosin in the same way as it does with wild type striated muscle actin-tropomyosin (28, 34). E93K actin-tropomyosin filaments were also reactivated by adding troponin in the presence of Ca2+ (Fig. 5). This indicates that the effect of the E93K mutation is to shift the on/off equilibrium of the thin filament toward the off state to a certain degree, but the Ca2+-activated troponin complex is capable of shifting the equilibrium back to the on state. In contrast a deletion mutation in tropomyosin has been shown to produce thin filaments that cannot be activated by Ca2+ and troponin (36), although they can be switched on by rigor S-1.

![Image](https://example.com/image2.png)

**FIG. 4. In vitro motility analysis of Drosophila wild type actin and E93K actin filament movement over skeletal muscle HMM. Left panels, the effect of sheep aorta tropomyosin on actin-7 filament movement, measured at 30 °C. Right panels, effect of skeletal muscle tropomyosin on actin-7 filament movement measured at 25 °C. Upper panels, actin filament velocity, μm/s. Lower panels, fraction of actin filaments motile. Closed symbols, wild type 8SF actin; open symbols, E93K actin. Six paired preparations of actin were used, and the results were pooled. Each point represents the mean and S.E. of three to six measurements using up to four separate preparations.
The critical nature of this region to muscle function is highlighted by the fact that the E93K mutant produces a flightless phenotype, and the E93A/R95A mutation in yeast is lethal (37). Mutations in this region affect sarcomere assembly (18), and this may be a consequence of the poor polymerizability of E93K actin or due to an altered interaction with tropomyosin. The motility parameters of E93K actin are not greatly different from WT, but the interaction of E93K with tropomyosin is altered as shown by these experiments (Fig. 4). The observation that a charge reversal mutation of glutamic acid 93 to lysine results in tropomyosin binding to actin in the off state, as defined by the motility parameters (low fraction of filaments motile with little change in velocity) suggests one aspect of actin structure that may be important for regulation. Alterations of other amino acids in this charged cluster (D56A/E57A) seem to have similar effects upon actin-tropomyosin state (48), but charge changes at the nearby surface helix (E99A/E100A) or in the middle of domain 2 (D24A/D25A) have no effect upon regulation (49), indicating a specific role for this site.

We propose that electrostatic charge on the surface of domain 2 of actin plays a critical role in determining the equilibrium between states of actin-tropomyosin that is a central feature of the steric blocking mechanism of actin filament regulation (8, 42). Concentrated negative charge in this region, as found in wild type actin, promotes the on state, and the absence or reversal of such charge, as found with the E93K actin mutant and actin-troponin I complex, permits tropomyosin to move toward domain 2 and thus promotes the off state. It is possible that the ability of structurally unrelated proteins such as troponin and caldesmon to inhibit actin-tropomyosin interaction with myosin may simply be due to neutralizing negative charge on the surface of actin, thus permitting tropomyosin to move to the off position on actin. It would also be compatible with the general observation that inhibitory proteins bind to actin rather than to tropomyosin.

Acknowledgment—We thank Anne Lawn for excellent technical assistance in purifying the actins.

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Glu\textsuperscript{93} \rightarrow \text{Lys} \text{ Mutation in Actin Changes Tropomyosin to Off State}