E22K mutation of RLC that causes familial hypertrophic cardiomyopathy in heterozygous mouse myocardium: effect on cross-bridge kinetics

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A LARGE BODY OF EVIDENCE suggests that ventricular regulatory light chain (RLC) is one of the sarcomeric proteins associated with familial hypertrophic cardiomyopathy (FHC) (21, 36). FHC is an autosomal dominant disease that is characterized by left ventricular hypertrophy, myofibrillar disarray, and sudden cardiac death. It has been shown that, apart from mutations in ventricular RLC, FHC is caused by missense mutations of β-myosin heavy chain (22), myosin-binding protein C (51), ventricular essential light chain (LC1) (20, 21, 36), troponin T (52), troponin I (28), α-tropomyosin (48), actin (32), and titin (39) genes.

The E22K mutation in the RLC was shown to be associated with a subtype of cardiac hypertrophy defined by mid-left ventricular obstruction due to papillary muscle hypertrophy (36). This mutation was shown later to cause ventricular and septal hypertrophy (48). The three-dimensional structure of the RLC reveals that the site of mutation (Glu22) is close to the phosphorylation site of RLC (Ser15) and the Ca2+-binding site (amino acids 37–48). Earlier work, indeed, demonstrated that phosphorylation and Ca2+ binding are significantly altered by the E22K mutation (45). Studies with transgenic (Tg) mice demonstrated that this E22K mutation, when overexpressed in mouse cardiac muscle, increased Ca2+ sensitivity of myofibrillar ATPase activity and steady-state force by about ΔpCa50 ±0.1. However, the mechanisms for the E22K-mutated myocardium that might initiate changes in the Ca2+ sensitivity of force/ATPase that could potentially trigger ventricular/septal hypertrophy as seen in human patients harboring this mutation remain unclear. To shed more light on the E22K-mediated abnormalities of muscle contraction, we studied the kinetics of the cross-bridge cycle during transient contraction of cardiac muscle of Tg mice carrying the E22K mutation (Tg-m).

Muscle contraction results from interactions of myosin subfragment-1 (S1) with actin. S1 consists of the NH2-terminal globular catalytic domain and the COOH-terminal α-helical regulatory domain. The catalytic domain is responsible for binding to actin and hydrolysis of ATP. The α-helical regulatory domain is stabilized by LC1 and RLC, forming the so-called “lever arm.” The atomic structure of S1 suggests that the lever arm translates small conformational changes in the catalytic domain on ATP hydrolysis to a large linear motion of the thick filaments (37). Mechanical events after a rapid transient in ATP concentration involve rapid dissociation of cross bridges from actin followed by their slow re-binding to the thin filament. It is believed that the mechanical event is caused by a specific enzymatic event during the ATPase cycle of a cross bridge.

Spectroscopic methods offer a convenient way to measure mechanical (49) and enzymatic (14, 42) events. In particular, fluorescence polarization or anisotropy (1, 4, 6, 11, 12, 13, 15, 17, 18, 23, 27, 34, 38, 47) provides information about binding and orientation of the myosin head and the lever arm. Here, we exploit this to compare the effect of the E22K mutation in RLC on kinetics of cross bridges in Tg-m fibers with that in non-Tg fibers.
and Tg wild-type (Tg-wt) fibers. Depending on the position of the fluorescent probe on myosin or actin, its anisotropy indicates dissociation of myosin heads from thin filaments, cross-bridge rebinding to actin, or an enzymatic event on the myosin active site. The cross-bridge dissociation was determined from the increase in the rate of rotation of actin monomer to which a cross bridge was bound. Dissociation allows the actin monomer to start rotating, because its rotation is no longer restrained by myosin (8). Rebinding was determined from the rate of anisotropy change of the rhodamine-labeled recombinant essential light chain of myosin (Rh-LC1) exchanged for the native LC1 in cardiac myofibrils. Thus the lever arm of myosin contains the hyper trophy-causing mutation RLC and fluorescent LC1. The rate of ADP dissociation from the active site of myosin was determined from anisotropy of muscle preloaded with a stoichiometric amount of fluorescent ADP. ADP starts rotating rapidly after it is liberated from the immobile myosin by sudden release of excess nonfluorescent ATP. The rate of release can be monitored by measuring anisotropy of fluorescence, because free ADP rotates faster than ADP immobilized by myosin (40, 41).

The enzymatic and mechanical events were followed in a small population of cross bridges. The characterization of motion of a small population of cross bridges in working muscle is important, because the kinetics are not averaged over a large population of myosin heads and because complications associated with molecular crowding, which may influence the structure and function of the myosin molecule studied in isolation, are avoided.

The results showed that cross-bridge dissociation from actin was slower in Tg-m than in control muscle. Rebinding of the lever arm in non-Tg muscle was different from that in Tg-m and Tg-wt muscles but similar in Tg-m and Tg-wt muscles, suggesting that the human isoform of RLC in mouse hearts alters the rate of cross-bridge rebinding. ADP dissociation from the active site of myosin in Tg-m muscle was not statistically different (at 5% level) from that in control muscle.

MATERIALS AND METHODS

Chemicals and solutions. Standard chemicals were obtained from Sigma (St. Louis, MO). 5-Dimethoxy-2-nitrobenzyl (DMNPE)-caged ATP, 5'-iodoacetamido-fluorescein (IAF), and 5'-iodoacetamidotetramethylrhodamine (IATR) were purchased from Molecular Probes (Eugene, OR). Glycerinizing solution contained 80 mM potassium acetate, 0.2 mg/ml PMSF, 2 mM β-mercaptoethanol, 4 mM MgCl₂, 5 mM ATP, 2 mM EGTA, 1 mM DTT, and 10 mM Tris·HCl (pH 7.6) in 50% glycerol. Rigor solution contained 50 mM KCl, 4 mM MgCl₂, 0.1 mM CaCl₂, 1 mM DTT, and 10 mM Tris·HCl (pH 7.6). Relaxing solution contained 50 mM KCl, 4 mM MgCl₂, 2 mM ATP, 2 mM EGTA, 1 mM DTT, and 10 mM Tris·HCl (pH 7.6). All solutions used in photolysis experiments contained 10 mM reduced glutathione.

Mice. Tg mouse models were used for the myc-wild-type and myc-E22K mutant of human cardiac RLC (46). Briefly, the cDNAs of proteins were cloned into the unique SalI site of the plasmid α-myosin heavy chain clone 26 (a gift from Jeffrey Robbins, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH). The resulting constructs contained ~5.5 kb of the mouse α-myosin heavy chain promoter, including the first two exons and part of the third exon, followed by the myc-wild-type and/or myc-E22K cDNA (534 bp) and a 630-bp 3'-untranslated region from the human growth hormone transcript. All the founders were bred to non-Tg B6SJL mice. Founders were bred several times until adequate numbers of F1 offspring were obtained for each line. Tg mice expressing human cardiac wild-type RLC showed 30% protein expression. Tg mice expressing human cardiac mutated RLC showed 60–90% protein expression.
A concentration of myosin heads in muscle of 120 μM (3) and 1–2.5% labeling translate to 400–600 cross bridges (5). Such a small number turning over once is insufficient to cause shortening of a myofibril.

**Photogeneration of ATP.** Muscle was perfused with 2 mM DMNPE-caged ATP. The UV beam was focused by the objective to a Gaussian spot with width and length equal to twice the lateral resolution of the UV beam (≈0.2 μm). The height, which equals the depth of focus of the objective, was ≈3 μm. A few seconds after the beginning of the experiment, the shutter admitting the UV light was opened for exactly 10 ms, and 0.12 mJ/s was incident on the muscle. Because of the clipping of the enlarged laser beam by the entrance aperture of the objective, loss at the mirror DM1 and loss due to absorption by glass in the objective occur. The area illuminated by UV is 0.04 μm². The energy flux at the illuminated area is 0.12 mJ·s⁻¹·μm⁻² = 0.04 μm⁻²·s⁻¹ μm⁻². ATP remains in the experimental volume, on average, for 300 μs. The energy through the illuminated area during this time is 9 × 10⁻¹⁴ J·μm⁻², which is larger than the energy flux obtainable with a frequency-doubled ruby laser (~3 × 10⁻¹⁵ J·m⁻²) (24).

**Amount of photoreleased ATP.** The amount of ATP produced in a single experiment was estimated by measuring the luminescence of luciferin-luciferase (LL) solution exposed to the UV beam. A 10-μl sample of rigor solution containing 2 mM caged ATP was illuminated by a 0.12-mW UV beam for 1, 30, and 60 s. Then 5 μl of 40 mg/ml LL solution was placed on an X-ray film (Kodak) and mixed with 5 μl of solution containing photogenerated ATP. The film was exposed for 10 min. The calibration curve was obtained by mixing known amounts of ATP with LL solution. The film was scanned by a Microtek scanner. Optical density was measured by Image Plus Pro (Media Cybernetics, Silver Spring, MD). During the 1-s exposure, 10⁻¹² mol of ATP were produced. Therefore, with allowance for UV absorption by the glass cover slide, ≥10⁻¹⁵ mol are produced in 0.3 μm³ during 10 ms, an amount sufficient to convert all precursor to ATP.

**Heat generation.** The most significant source of possible artifacts is heating, which is caused by absorption of UV light by caged ATP. The extinction coefficient of caged ATP at 364 nm is 4,400 M⁻¹·cm⁻¹; i.e., a 3-μm-thick section of muscle perfused with 2 mM precursor absorbs [exp(−3 × 10⁻⁷ × 4.4 × 10⁴ × 2 × 10⁻³)] = 0.00264 of the UV light. However, the heat associated with this absorption dissipates rapidly (~70 ns) from the experimental volume, because the beam diameter is small (16). The UV laser delivers 0.12 mJ/s, i.e., 0.9 × 10⁻¹¹ J in 70 ns. The experimental volume weighs ~10⁻¹² g. It takes 4.2 × 10⁻¹² J to raise the temperature of this volume by 1°C; therefore, the temperature rise during an experiment is <2°C. Three additional lines of evidence suggested that the anisotropy change did not reflect a temperature rise in the experimental volume: 1) no change in anisotropy of skeletal muscle by caged EDTA (DMNP-EDTA), 2) no change in anisotropy change of denatured skeletal muscle fibers, and 3) no change in anisotropy of skeletal muscle fibers devoid of myosin as a result of stimulation with caged ATP (not shown).

**Anisotropy of solutions.** LC1 of isolated myosin, prepared as described elsewhere (50), was exchanged with Rh-LC1, as reported by Ling et al. (30), and steady-state fluorescence anisotropy was measured in a spectrophotometer (model ISS K2). To avoid scattered light, a 500-nm cutoff filter was used in the emission channel. All measurements were done using magic-angle conditions. All experiments were performed at 0°C. For all samples used in fluorescence
measurements, absorption was <0.1. Time-resolved anisotropy was measured using Fluo Time 200 (PicoQuant, Berlin, Germany). 

**Muscle protein purification.** Porcine muscle myosin was isolated as described previously (50). Rabbit skeletal muscle actin was isolated by the method of Pardee and Spudich (35).

**Lifetime measurements.** Lifetime measurements were carried out in Fluo Time 200, which was used with a picosecond laser system. Actin was labeled with fluorescein-phalloidin at a 1:1 molar ratio.

**Measurement of anisotropy of cardiac myofibrils.** Fluorescence was measured with a water immersion lens (C-Apo, ×40, 1.2 NA). Calculations showed that the high numerical aperture of the objective causes minimal distortion to the polarized intensities (12). In the definition of fluorescence intensities given below, the subscripts after intensity (I) indicate the direction of polarization of excitation and emission light relative to the myofibrillar axis. Only the myofibrils that were aligned horizontally on the stage were chosen for experiments. This introduces only a small error, because polarization varies as cos square of the angle between the myofibrillar axis and the horizontal direction. The excitation light was always perpendicular to the axis of the myofibril. It follows that photomultipliers 1 and 2 record $I_1$ and $I_2$, respectively. The microscope was operated in a "spot" mode (i.e., a single spot, located exactly in the center of the field of view, is illuminated). The beam was not scanned. To minimize photobleaching, the laser light was attenuated 100 times (power incident on the fiber was 3 μW). The experimental arrangement is described in detail elsewhere (5).

**Statistical analysis.** Data were subjected to one-way ANOVA and the Tukey-Kramer multiple comparison test using GraphPad Instat software (version 3.01). Results were considered statistically significant at $P = 0.05$.

**RESULTS**

The motions of cross bridges were synchronized by rapid (10-ms) photogeneration of ATP. During each 10-ms interval, all caged ATP is converted to ATP (10). Because the root-mean-square velocity of diffusion of ATP, determined by its diffusion coefficient $[3.7 \times 10^{-6} \text{cm}^2/\text{s}]$, is $\sim 1 \mu\text{m}/\text{ms}$, it diffuses away from the experimental volume in $\sim 300 \mu\text{s}$. Therefore, after 300 μs, there is practically no free nucleotide left in the experimental volume, and the rates represent motion caused by the turnover of a single molecule of ATP.

**Cross-bridge dissociation from thin filaments.** Thin filaments were labeled with Rh-Ph. Dissociation was triggered by rapid generation of ATP from the caged precursor. Dissociation causes rotation of actin monomer, because it is no longer immobilized by myosin (8). Muscle was initially in rigor. Figure 3A shows a typical record of perpendicular anisotropy change. On rapid (10-ms) release of ATP from the cage (Fig. 3A, arrow), anisotropy drops rapidly, indicating dissociation of cross bridges from thin filaments. The time was measured by drawing the dissociation curve on an expanded time scale (Fig. 3B) and estimating the time required for anisotropy to decay from the maximum to the minimum. Table 1 summarizes the dissociation times ($\tau$). Dissociation times were slightly higher in Tg-m fibers; the differences in $\tau$ between the three groups were statistically significant ($P = 0.0253$). Cross bridges of Tg-m (90% expression) muscle dissociate from thin filaments significantly more slowly than cross bridges of non-Tg muscle ($q = 3.813, P < 0.05$).

**Cross-bridge rebinding to thin filaments.** After dissociation, cross bridges rebind to thin filaments, as indicated by the slow exponential rise of anisotropy (Fig. 4A). The shape of the anisotropy change is similar for cardiac and skeletal muscle (5). To correct for photobleaching, the initial decline of the anisotropy curve (between points a and b) was fitted to an exponential. Subtraction of this exponential fit from the original data gives a corrected rise of anisotropy. A typical corrected curve is shown in Fig. 4B. This corrected anisotropy was fitted by a three-parameter exponential curve: 

$$y = y_0 + ae^{-kt}$$

This procedure was applied to all muscles labeled with Rh-Ph. The values of $b$ (1/$\tau_2$) are summarized in Table 2. The differences in $\tau_2$ between the three groups were statistically different ($P = 0.04$). Results show that cross bridges of Tg-m (90%) muscle rebound to thin filaments faster than cross bridges of non-Tg controls ($q = 3.767, P < 0.05$). Cross bridges of Tg-wt muscle also rebound to thin filaments faster than cross bridges of non-Tg muscle ($q = 3.623, P < 0.05$). The rate of rotation was also slightly faster in Tg-m than in Tg-wt muscle, but the difference was not statistically significant ($q = 0.1343, P > 0.05$).
Dissociation of ADP from the myosin active site. Muscle was initially in rigor. Cross bridges were labeled at the active site with Alexa-ADP. On sudden release of ATP, the cross bridges dissociated from thin filaments. Fluorescent ADP was displaced from the myosin active site by the rapidly photo-generated excess of nonfluorescent ATP. Figure 5A shows a typical record of perpendicular anisotropy change on application of ATP to Tg-wt myofibrils. The shape of the anisotropy

<table>
<thead>
<tr>
<th>Probe</th>
<th>1/τ1, ms</th>
<th>1/τ2, Δλ (1/μs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Tg</td>
<td>Tg-wt</td>
<td>Tg-m</td>
</tr>
<tr>
<td>(n = 13)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>165±25</td>
<td>172±29</td>
<td>210±39*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, number of measurements. τ1, time of dissociations of cross-bridges from actin (see Fig. 3B); Tg, transgenic; Tg-wt, Tg wild-type; Tg-m, Tg with overexpression of E22K mutation of human regulatory light chain (60% and 90% protein expression); Rh-Ph, rhodamine B-labeled phalloidin. *P<0.05 vs. non-Tg.

Table 2. Rates of rotation of the myosin lever arm

Values are means ± SD; n, numbers of measurements. Power analysis was carried out to determine n: taking the power index of 80% and SD of measurements of b of 0.5, we need ~12 measurements to distinguish difference of b of 0.5 (33). 1/τ2, Rate of rotation of lever arm (see Fig. 4B); Rh-LC1, 5'-iodoacetamidotetramethylrhodamine-labeled essential light chain 1. *P < 0.05 vs. non-Tg.

Fig. 4. Time course of change in perpendicular anisotropy of Tg wild-type muscle labeled with Rh-LC1. Between points a and b, muscle was in rigor. Sudden pulse of UV light is applied at point b to create ATP and cause cross bridges to undergo one turnover cycle. A: orthogonal intensities and anisotropy. B: rise of anisotropy, indicating cross-bridge rebinding to thin filaments. To account for photobleaching, anisotropy between points a and b was fitted to a curve, and data were subtracted from it. Therefore, “detrended” data begin at 0. Data were fitted to an exponential rise to a maximum y = 107.8 + 13.9(1 - e^{-0.605t}).

Fig. 5. Time course of change in perpendicular anisotropy of Tg wild-type muscle labeled with Alexa-ATP. Muscle was in rigor between points a and b. Sudden pulse applied at point b generated ATP and caused contraction. A: orthogonal intensities and anisotropy. B: rise of anisotropy, indicating ADP dissociation from the active site of myosin. To account for photobleaching, anisotropy between points a and b was fitted to a curve, and data were subtracted from it. Vertical lines were fitted by inspection. Distance between them is equal to dissociation time (τ_{Alexa}).
change of cardiac muscle was similar to that of skeletal muscle (5). Correction for photobleaching was done as described in Fig. 4B: the initial decline of anisotropy (between points a and b) was fitted to an exponential, which was subtracted from original data. A typical corrected curve is shown in Fig. 5B. The dissociation time ($\tau_3$) values were estimated from this corrected anisotropy and are summarized in Table 3. Results revealed no statistically significant differences in $\tau_3$ for ADP among the three groups ($P = 0.6226$). However, $\tau_3$ was higher in Tg-m than in non-Tg and Tg-wt myofibrils, although, as stated above, the differences were not significant.

The difference between $\tau_1$ and $\tau_3$ was not statistically significant. This is consistent with the simultaneous dissociation of cross bridges from actin and release of ADP in skeletal muscle (41).

**Fluorescent lifetimes.** To determine whether Tg-m myosin is intrinsically different from Tg-wt myosin, we compared fluorescent lifetimes of fluorescent-phalloidin attached to G-actin to which myosin was bound. Lifetime is a sensitive measure of conformation, because it is sensitive to environmental changes (29). If the mutation altered binding characteristics of myosin, the fluorescent lifetime of fluorescein on actin would be affected. Figure 6 shows typical time-resolved fluorescence decay of phalloidin on actin (phalloidin-to-actin ratio = 1). For the best fit, two lifetimes, $1.420 \pm 0.017$ and $3.592 \pm 0.015$ ns, were used. On binding of equimolar Tg-wt myosin, the fast lifetime increased by 0.7% to $1.431 \pm 0.037$ ns and the slow lifetime did not change. Similarly, binding of equimolar Tg-m myosin did not significantly change either lifetime. The complete analysis is shown in Table 4. Changes in short and long lifetimes were smaller than the standard deviation of the measurement. In an effort to measure change in the binding constant, similar measurements were carried out at 0.5–2.5 molar ratios of actin to myosin. In each case, no change in lifetime was observed. Thus the mutation does not change fluorescent lifetime. The affinity was also assessed by measuring changes of anisotropy of actin on myosin binding. Binding of myosin induces conformational change in myosin (25) and actin (8). Figure 7 shows a typical decay of anisotropy of phalloidin on actin. The decay was dominated by a single decay process, characterized by $\sim 1$-ns characteristic time.

### Table 3. Times of ADP dissociation from myosin active site

<table>
<thead>
<tr>
<th>Probe</th>
<th>$\tau_3$, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Tg (n = 14)</td>
<td>102.5 ± 38.8</td>
</tr>
<tr>
<td>Tg-wt (n = 14)</td>
<td>117.0 ± 43.4</td>
</tr>
<tr>
<td>Tg-m (90%) (n = 16)</td>
<td>131.3 ± 56.5</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, numbers of measurements. Taking the SD as 50, we need $\sim$12 measurements to detect 0.5% difference with the power of 80% (33). $\tau_3$, time of ADP dissociation.

### Table 4. Effect of binding of Tg-wt and Tg-m myosins on fluorescent lifetimes of phalloidin-actin

<table>
<thead>
<tr>
<th>Probe</th>
<th>Life time, ns</th>
<th>$f_{\text{amplitude}}$, %</th>
<th>$f_{\text{anisotropy}}$, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin alone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>1.420 ± 0.017</td>
<td>34.73</td>
<td>57.35</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>3.592 ± 0.015</td>
<td>65.27</td>
<td>42.65</td>
</tr>
<tr>
<td>Actin + Tg-wt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>1.431 ± 0.018</td>
<td>34.36</td>
<td>56.80</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>3.593 ± 0.017</td>
<td>65.64</td>
<td>43.20</td>
</tr>
<tr>
<td>Actin alone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>1.400 ± 0.017</td>
<td>36.37</td>
<td>59.34</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>3.580 ± 0.017</td>
<td>63.63</td>
<td>40.66</td>
</tr>
<tr>
<td>Actin + Tg-m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>1.408 ± 0.018</td>
<td>35.74</td>
<td>58.60</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>3.382 ± 0.018</td>
<td>64.26</td>
<td>41.40</td>
</tr>
</tbody>
</table>

Values represent changes after binding of equimolar Tg-wt myosin and Tg-m myosin (means ± SD). f, percentages of the total Signal intensity and amplitude contributed by processes decaying with lifetimes $\tau_1$ and $\tau_2$.

Fig. 6. Fluorescent lifetime of fluorescein-phalloidin labeling of 0.2 μM actin; 10,826 data points were analyzed. Top: decay of fluorescence after excitation with 470 nm light pulse. Bottom: autocorrelation of differences between data and fitting (black line).

Fig. 7. Anisotropy of 0.2 μM fluorescein-phalloidin-0.2 μM actin complex. Note decay of vertical fluorescence after excitation with 470-nm light pulse, decay of horizontal fluorescence, and decay of anisotropy. $\chi^2 = 1.1445$; 8,117 data points were analyzed.
DYNAMICS OF CROSS BRIDGES CARRYING E22K MUTATION OF RLC

Table 5. Effect of binding of Tg-wt and Tg-m myosins on anisotropy of phalloidin-actin

<table>
<thead>
<tr>
<th></th>
<th>$a_{0.05}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>1.08±0.28</td>
</tr>
<tr>
<td>Actin + Tg-wt</td>
<td>0.95±0.22</td>
</tr>
<tr>
<td>Actin + Tg-m</td>
<td>0.81±0.25</td>
</tr>
</tbody>
</table>

Values are means ± SD of changes after binding of equimolar Tg-wt myosin and equimolar Tg-m.

Table 5. Effect of binding of Tg-wt and Tg-m myosins on anisotropy of phalloidin-actin

The fact that ATPase of Tg-m is the same as that of Tg-wt myosin, we conclude that mutation does not alter the major properties of myosin.

Control. It is possible that the observed changes in anisotropy of the lever arm do not reflect rotation but, instead, reflect changes in anisotropy of the probe due to 1) binding of cross bridges to thin filaments or 2) ATP-mediated release of LC1 from the lever arm. To check for possibility 1, we compared excitation anisotropies of Rh-LC1 solutions incorporated into myosin with anisotropy of actin-Rh-LC1-myosin complex (Fig. 8). The limiting anisotropy of immobilized Rh-LC1-myosin at 540 nm was 0.364. The anisotropy of Rh-LC1 bound to myosin in the absence of actin at 540 nm was 0.289. Because myosin at low ionic strength is filamentous and does not execute any nanosecond-time-scale motions, this decrease indicates collective motion of the probe and of myosin-bound light chain. Addition of increasing concentrations of actin had no effect on the anisotropy. This suggests that the anisotropy change is probably not due to actin dissociation. In principle, it is possible that the anisotropy remains unchanged because of compensating changes in rotational correlation time of rhodamine and fluorescent lifetime of rhodamine (Perrin’s equation). However, this is not the case here: fluorescent lifetimes in the absence and presence of actin were the same (Table 4). This proves that anisotropy of rhodamine is unaffected by formation of the actomyosin complex and that anisotropy change in fibers reflects rotation of the lever arm.

To check for possibility 2, we compared excitation anisotropies of Rh-LC1-S1 in the presence and absence of ATP. Addition of 2 mM ATP to 0.5 μM S1 exchanged with Rh-LC1 had little effect on excitation anisotropy at 500–530 nm and no effect on anisotropy at 540 nm (Fig. 8B).

The limiting anisotropy of immobilized Rh-LC1-myosin (Fig. 8A) and Rh-LC1-S1 (Fig. 8B) at 540 nm was 0.364 and 0.371, respectively. This translates to an ~13° angle between absorption and emission dipoles of rhodamine, which is consistent with earlier work (9).

DISCUSSION

Characterization of cross-bridge motion in working muscle is essential, because behavior of proteins in vivo may be quite different from that in vitro. In solution, proteins are loosely packed; in vivo, they are crowded. Molecular crowding influences protein solubility and conformation in solution (31). The effect of crowding is particularly severe in muscle, where actin and myosin are present at hundreds of micromolar concentration (3). Actin and myosin are meant to operate in such crowded environments, as reflected by the fact that their $K_m$ is in the micromolar range, but crowding may impose constraints affecting structure and function of enzymes, so that their properties in dilute solutions are different from those in muscle (2). To examine whether the point mutation in RLC leads to changes in cross-bridge kinetics in muscle, we followed mechanical and enzymatic events in Tg-m muscle. Mechanical events were measured by anisotropy of F-actin labeled with Rh-Ph and myosin labeled at the lever arm with Rh-LC1. Enzymatic events were followed by measurement of dissociation of ADP from the active site. The measurements were done on a small population of cross bridges in contracting Tg-m and Tg-wt cardiac myofibrils.

The results showed that the E22K mutation had a small effect on the time of cross-bridge dissociation. The difference
was not statistically significant, with 60% of myosin molecules carrying the mutation (P > 0.05, q = 2.86), and marginally significant, with 90% myosin (P < 0.05, q = 3.813). It is unlikely that this disparity reflects an intrinsic difference in binding of mutated myosin to actin: isolated Tg-m and Tg-wt myosin induced the same change in fluorescent lifetime and anisotropy of actin. This, together with the fact that mutated and wild-type myosins have the same ATPase activity (45), suggests that the difference in τ₁ is not due to intrinsic differences in cross bridges. We speculate that it is due to myofibril disarray in the transgenic hearts (46).

We observed no effect on the rate of cross-bridge rebinding to thin filaments. Control experiments excluded the possibility that anisotropy changes had nothing to do with rotation of the lever arm and, instead, reflected changes in fluorescent dipole moment of rhodamine caused somehow by binding of cross bridges to thin filaments. Similarly, the effect had nothing to do with differential release of LC1 by ATP from myosin. Although anisotropy of free Rh-LC1 (0.256 at 540 nm; Fig. 8A) was increased to 0.289 for Rh-LC1-myosin in the absence of actin (Fig. 8A), suggesting that rotation of LC1 on myosin is restricted by the interactions with the heavy chain, it is unlikely that this interaction is loosened by ATP. Anisotropy of Rh-LC1 on S1 was unaffected by 2 mM ATP (Fig. 8B). The same was true of Rh-LC1 on myosin. This lack of effect is consistent with the fact that loosening of LC1 from myosin requires high ATP concentration or TFP and high temperature (43).

Finally, we saw no effect of mutation on the time of ADP dissociation from the myosin active site. The absolute values of cross-bridge dissociation and rebinding, as well as ADP release, reported here are lower than in solution (53), because cross bridges executed only a single turnover. In a single-turnover experiment, the cross bridge detaches and reattaches only once; i.e., after reattachment, the probability of detachment becomes 0. A cross bridge that finished the turnover cycle acts as a load for cross bridges that did not complete the cycle. This load slows the contraction and the rate of anisotropy change.

As shown previously, the E22K mutation of RLC resulted in a 20-fold decrease in the ability of isolated RLC to bind Ca²⁺ (45). This mutation also altered the Ca²⁺ sensitivity of ATPase and force development in Tg mouse cardiac muscle preparations (46). In addition, the E22K mutation did not affect the rate of force development and only slightly decreased the rate of force relaxation measured with caged Ca²⁺ chelators (46). The results of the present study showing that the mutation had no major effect on mechanical performance of the cross bridges are consistent with this earlier work.

Kinetics of lever arm rebinding in Tg hearts are significantly different from those in non-Tg hearts. There can be two reasons for this observation. 1) Tension is an average of contributions from large populations of cross bridges. However, each cross bridge has different kinetics, depending on its position relative to the actin-binding site (19). Therefore, kinetics of a single cross bridge may be different from kinetics of the whole assembly. If a single cross bridge were monitored, the anisotropy of the lever arm would be expected to change in four discrete steps, corresponding to cross-bridge release from actin, free rotation, weak binding, and transition to the strongly bound state. However, if the number of cross bridges is large, these steps become obscured, and their kinetics become altered. In our experiment, the number of cross bridges was estimated to be 400. Thus the tension response, being an average of trillions of cross bridges in a muscle bundle, can be quite different from the response of a few hundred myosin heads. 2) The non-Tg mouse expresses endogenous mouse RLC, whereas the Tg (Tg-wt or Tg-m) mouse expresses human RLC. There is a 95% homology between human and murine vascular RLC (vRLC). Of the eight amino acids that are different between the human and mouse vRLC, six are located in the NH₂-terminal region of the RLC (at positions 4, 5, 10, 11, 13, and 14). The NH₂-terminal domain wraps around the COOH-terminus of myosin heavy chain. It is possible that any structural alterations in this NH₂-terminal region of myosin would produce difference in rates of rotation of the lever arm.

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