Fundamental step size in single cardiac and skeletal sarcomeres

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Yakovenko, Olga, Felix Blyakhman, and Gerald H. Pollack. Fundamental step size in single cardiac and skeletal sarcomeres. Am J Physiol Cell Physiol 283: C735–C742, 2002.—In attempting to deduce the size of the elementary molecular translation step, recent experiments using single myosin molecules translating over actin filaments have shown a consistent step size of 5.4 nm (10, 21). We have carried out parallel measurements on single myofibrils from rabbit cardiac muscle and bumblebee flight muscle. Activated specimens were released or stretched with a motor-imposed ramp, and the time course of length of individual sarcomeres was measured by projecting the image of the striations onto a linear photodiode array and tracking the spacing between A-band centroids. We confirmed the 5.4-nm step. With subnanometer precision, however, we find that this value is two times that of a more fundamental step size of 2.7 nm. Step sizes were always integer multiples of 2.7 nm, whether the length change was positive or negative. This value is equal to the linear repeat of actin monomers along the thin filament, a result that ties dynamic events to molecular structure and places narrow constraints on any proposed molecular mechanism.

single myofibril; actin; optical microscopy; quantal shortening

IN APPROACHING THE MECHANISM of motility and contraction, attention has turned toward the elementary molecular translation step. Although measurements in the past have yielded steps of broadly variable size, possibly because of challenging analytical obstacles (8, 14, 17), recent experiments with improved signals have shown steps on the order of 5–6 nm (20), and experiments on single myosin molecules translating along actin filaments have shown step size consistently of ~5.4 nm (10, 21). The value 5.4 nm is equal to the monomer repeat along a single actin strand (10, 13, 15, 20, 21).

We have carried out parallel measurements on single sarcomeres. The single sarcomere model is useful because it preserves the natural filament lattice, yet cooperativity assures that molecular translation steps are not obscured (19). Previous myofibril experiments have shown that activated sarcomere shortening occurred in steps that were integer multiples of 2.7 nm (2). Because of noise, however, detectability was limited to steps larger than 4–5 nm, so we could not establish whether the implied 2.7-nm quantum actually existed.

In the present experiments, we employed a differentially based algorithm that could suppress noise contributions sufficiently to bring the detection limit to subnanometer levels (18). With this high-resolution algorithm, we measured steps in activated sarcomeres both during shortening and during imposed stretch. Although the 5.4-nm step seen in single molecule studies is confirmed in this study, the myofibril reveals a more fundamental step size of half that value, or 2.7 nm.

METHODS

Myofibril Preparation

Isolated myofibrils were prepared from rabbit left ventricular trabecular muscles as described previously (12). Briefly, thin strips of muscle tissue were dissected for storage in rigor/glycerol solution (50/50 by volume) for a minimum of 5 days at −20°C. To obtain single myofibrils, glycerinated strips were minced, and the pieces were further skinned in a 4°C rigor solution containing 0.5% Triton X-100 for 30 min. After being washed with fresh rigor solution, the tissue pieces were homogenized in a blender (Sorvall Omni Mixer) at low speed for 5–6 s in the same buffer.

To ensure that the results were not peculiar to any particular muscle type, we also examined invertebrate specimens. Specimens of bumblebee flight muscle were prepared as described previously (2, 22). Bees were dissected at room temperature after immobilization by refrigeration. Two dorsoventral flight muscles attached to the thorax were left in situ as the rest of the body was dissected away. Muscles were chemically skinned by soaking them alternately in solution A (1% vol/wt Triton X-100 in relaxing solution containing 10 μM leupeptin and 1 mM 1,4-dithiothreitol) and solution B (50% glycerol in relaxing solution) at 4°C. Muscles were kept in solution B at −20°C for immediate use and at −80°C for longer-term storage. Myofibrils were isolated by using fine needles to disaggregate specimens on a cover glass in a drop of rigor solution.

A drop of this “cardiac” or “flight-muscle” suspension was placed in the specimen chamber (volume ~300 μl), and myofibrils were allowed to settle and stick lightly to the chamber bottom where they could later be picked up by two microneedles. Excess rigor solution and extraneous tissue were removed.
washed out of the chamber via several rinses with relaxing solution. Although we used mostly single myofibrils, cardiac doublets were sometimes selected.

**Solutions**

*Cardiac specimens.* All experiments were performed at room temperature (20–22°C). Normal relaxing solution (pH 7.0) had a composition (in mM) of 10 MOPS, 64.4 K-propionate, 5.23 Mg-propionate, 9.45 Na₂SO₄, 10 ATP, 0.188 CaCl₂, 7 ATP, and 10 creatine phosphate. Rigor solution had a composition (in mM) of 750 K-propionate, 25 NaCl, 2 KCl, 2 MgCl₂, and 10 EGTA. Activating solution (pH 7.0) had a composition (in mM) of 10 MOPS, 45.1 K-propionate, 5.21 Mg-propionate, 9.27 Na₂SO₄, 10 EGTA, 9.91 CaCl₂, 7.18 ATP, and 10 creatine phosphate.

*Bumblebee specimens.* Solutions were prepared from a stock solution composed (in mM) of 750 K-propionate, 25 K₂EGTA, 15 Mg-acetate, and 25 NaN₃ at 6.8 pH. Rigor solution was obtained by mixing one part stock with four parts water. Relaxing solution (pH 6.8) was prepared by adding 5 mM Na₂ATP to rigor solution. Activation solution (pH 6.8) was prepared by adding 4.4 mM Ca-acetate to relaxing solution. Glycerol solution contained 50% glycerol and 50% relaxing solution.

**Experimental Apparatus**

Single myofibrils were isolated and mounted in a specially constructed apparatus built around a Zeiss Axiovert-35 microscope as previously described in detail (2, 22). Briefly, one end of a myofibril was attached either to the tip of a glass needle or, in some instances, to a nanofabricated tension transducer (6). The other end was mounted on the moving glass tip of the piezoelectric motor, which could impose linear length changes on the specimen. These attachment fixatures were in turn mounted on hydraulic micromanipulators (Narishige) to facilitate positioning. The myofibril’s striation pattern (Fig. 1) was projected onto a 1,024-element photodiode array, which was scanned to produce a trace of intensity vs. position along the myofibril. A bands produced positive-going signals, and I bands produced negative-going signals (Fig. 2A). Scans were made every 50 ms, a compromise between time resolution and integration time needed to reduce noise. A display of successive 50-ms scans recorded during an imposed length change similar to that used in the experiments is shown in Fig. 2B.

Sarcomere length was calculated as the span between centroids of contiguous A bands. To calculate the centroid, we developed a new algorithm based on the minimum average risk method originally developed by Kolomogorov (11) and Sokolov et al. (18).

The algorithm operates on repeated light intensity line scans of a sample, precisely quantifying movements of features of the sample between scans. The method is implemented by computing, in a limited region around the feature in question, the pointwise product of 1) the current intensity scan and 2) the first spatial derivative of the immediately previous scan. This is done by translating these scan regions relative to one another to find the optimal registration, determined as the minimum of their integrated product. The amount of shift required to achieve optimal registration is equal to the amount of feature shift.

As implemented, a two-sarcomere-wide subsection of each successive digitized scan of a myofibril is selected to bracket a given A band. The shift required to obtain the best fit, relative to the previous scan, is determined. From the relative shift between two A bands, the change of sarcomere length can be computed. By repeating this computation for each successive scan, the time course of each A band position, and thus of sarcomere length, can be obtained. Because the method is differential, high resolution is obtained.

An example of the results of A-band-centroid computation are displayed in Fig. 2B (see thin vertically oriented traces, labeled “centroids of A bands”). The horizontal span between contiguous centroids is taken as sarcomere length. Through successive sarcomere length computations, we could follow the time course of the length changes in single sarcomeres.
Protocol

All experiments were carried out at room temperature. We first checked the striation quality by stretching and releasing the specimen several times in relaxing solution. If A-band and I-band widths failed to remain invariant after several rounds of stretch and release, the specimen was discarded. Myofibrils that passed this test were stretched slightly beyond rest length, to a sarcomere length of 2.4–2.6 μm (2.9–3.2 μm for flight muscle) to reveal distinct I bands. Activation solution was then added, and a trapezoidal length change was imposed with the aid of the motor. Sometimes it was necessary to readjust needle positions to ensure that the myofibril remained parallel to the array. Other times it was necessary to readjust the lighting conditions to ensure that illumination remained uniform.

The stretch-release protocols were then carried out at moderate length (<2.6 μm for cardiac and <3.3 μm for flight muscle). The protocol consisted of imposing a trapezoidal length change of 5–7%. Speed of stretch-release was generally 3–4 nm·s⁻¹·sarcomere⁻¹. Three types of trapezoid were used: 1) a symmetrical trapezoid consisting of 13-s stretch, 2-s pause, and 13-s release; 2) an asymmetrical trapezoid in which the allowable window of sampling was largely devoted to the phase of interest (either release or stretch), i.e., 2-s stretch, 2-s pause, and 10-s release, or 10-s stretch, 2-s pause, and 2-s release; and 3) “slow stretch-release” and “fast stretch-release” protocols (average speeds were 3–4 and 10–11 nm·s⁻¹·sarcomere⁻¹, respectively) with the same types of trapezoid. As a control experiment, we applied a symmetrical “stepped ramp” trapezoid with an average step size of ~10 nm/sarcomere. For the passive cardiac stretch-release control experiments, we applied the same kinds of ramp.

In total, 253 sarcomeres from 53 activated myofibrils and 33 sarcomeres from 9 relaxed myofibrils were analyzed in cardiac muscle, and 332 sarcomeres from 46 activated myofibrils were analyzed in bumblebee flight muscle.

Sarcomere Length Measurement

Analytical details follow largely along lines already presented (2, 22). To define a step, it was necessary to define the pair of pauses surrounding it. The pause was taken provisionally as a region of the trace whose estimated best fit by eye was nominally parallel to the horizontal axis. The region had to contain a minimum of five consecutive sample points to qualify; most contained more. After beginning and end points of the pause were assigned, a best fit line was computed. This fit provided a guide for slight adjustment of the break points to yield pauses with slope closest to zero. Step size was then computed as the vertical span between midpoints of two successive best fit line segments. The procedure was repeated for other pauses and steps.

Although considerable attention was paid to resolution of sarcomere length change, the absolute values of sarcomere lengths were measured with accuracy no better than 1–2%.

Controls

To check for potential artifact, many controls have previously been carried out (2, 22). The effects of discreteness of the photodiode array were checked in two ways: 1) by using two magnifications and obtaining similar results, and 2) by replacing the photodiode array with a nondiscrete sensor, consisting of a scanning mirror and photomultiplier, and seeing comparable steps. Specimen-translation artifacts were checked by testing whether smoothly translating A bands could produce steps. Analytical procedures were checked by testing whether each of two independent algorithms yielded similar step distributions. All tests proved negative for artifact.

In addition, controls were run to check for subnanometer detectability in the presence of noise (2). Although baseline noise superimposed on a linear ramp could produce occasional steplike features, we found that any such steps had no consistent size or pattern. Furthermore, by imposing a motor waveform that forced sarcomeres to step at a size slightly different from that of naturally occurring steps, we were able to distinguish step-size distributions with peaks separated by ~0.4 nm. Thus resolution was adequate to detect steps on a subnanometer scale.

Additional controls have been run in the present experiments. In the first control, the experimental conditions remained identical, but the specimen was placed in relaxing solution instead of activating solution to determine whether steps of similar size might show up. In a second control to check for resolution, we applied a stepped ramp to the myofibril and investigated the resulting sarcomere length changes. Results of these controls are presented below.

RESULTS

Bumblebee Myofibril Experiments

Figure 3 shows the time course of sarcomere length change in single sarcomeres of single bumblebee myofibrils. The activated specimen was allowed to shorten or was stretched by a motor-imposed ramp. Sarcomeres generally followed the ramp on a coarse scale. On a finer scale, the pattern was staircaselike, with short pauses (arrows) interspersed between steps. The steps were typically 2–3 nm in size.

The results of analysis of these contractile steps are shown in Fig. 4. Step size was computed in the following way. Beginning and end points of each pause were identified, and an algorithm computed the best-fit line between these points (see METHODS). The algorithm then computed the vertical spacing between successive pauses, which gave the size of the step. Sizes obtained from many steps were plotted as a continuous histogram. For low-velocity ramps (nominally 1 nm·s⁻¹, sarcomere⁻¹), the histogram shows a single peak, the maximum of which falls at 2.68 nm. For higher-velocity ramps (nominally 8 nm·s⁻¹·sarcomere⁻¹), the main peak fell at 2.67 nm, with a secondary peak at twice that value and a hint of a peak at approximately three times the value. Figure 4, inset, shows lower-resolution data obtained earlier (2), where multiple peaks likewise fell at approximately integer multiples of 2.7 nm.

Cardiac Myofibril Experiments

The results of parallel experiments carried out on cardiac myofibrils are shown in Figs. 5 and 6. Figure 5 shows representative sarcomere length-change traces. Ramp speed was nominally ~3–4 nm·s⁻¹·sarcomere⁻¹, midway between the two values used for bumblebee samples. The traces are noisier than those of the bumblebee because the striation patterns were less regular (Fig. 1). On the other hand, many of the pauses were of longer duration and were, therefore, relatively more prominent.

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Analytical results obtained from shortening steps are shown in Fig. 6. They show a primary peak at 2.71 nm, indistinguishable from the bumblebee results. Several additional peaks were seen at approximate integer multiples of the primary value. (Little significance should be attributed to the relative amplitudes of the peaks, because these may change with loading conditions and with noise. Nor should any significance be assigned to the width of each peak, which also depends on noise.) Hence, the cardiac histogram illustrates in a single figure that the size of the shortening step is an integer multiple of 2.7 nm.

Lengthening steps were analyzed in a similar way. Pauses and steps were marginally less distinct than those observed during shortening (Figs. 3 and 5) but were clear enough to allow analysis. A series of histogram peaks was observed, each lying at an integer multiple of ~2.7 nm (Fig. 7). For comparison, bumblebee muscle data are shown. A narrow peak was observed at 2.68 nm. Thus lengthening steps have the same character as shortening steps.

Experiments were also carried out to determine the effect of ramp speed. This was done both for stretch and for release. Results are shown in Fig. 8, A and B. For stretch (Fig. 8A), slower speed slightly increased the fraction of smaller steps relative to the larger ones. For release, the result was the same. It appears that for low speed, smaller steps are relatively more abundant, although over the speed range studied, the effect is modest.

Finally, we addressed the question of whether the larger, higher order steps are real or whether they

Fig. 3. Time course of sarcomere length change during motor-imposed trapezoid in single sarcomere of single bumblebee myofibril. A: slow lengthening ramp. B: slow shortening ramp. Arrows denote representative pauses.

Fig. 4. Histogram of shortening step size in bumblebee flight muscle obtained by imposing shortening ramps. Low-speed ramps are shown by solid line (907 steps from 142 sarcomeres in 28 myofibrils); high-speed ramps are shown by shaded line (1,011 steps from 190 sarcomeres in 40 myofibrils). Inset: similar data obtained at lower resolution, from Blyakhman et al. (2). In all histograms, peaks are situated at approximate integer multiples of 2.7 nm.

Fig. 5. Representative traces of sarcomere length change during motor-imposed trapezoid in rabbit cardiac muscle. First, second, and fourth traces show segments of record; third trace shows entire record.
might arise because intervening pauses went unrecognized. If occasional pauses are “missed” because they are buried in the noise, step size will be misinterpreted to be a multiple of the real size. To check this possibility, we plotted the duration of measured pauses. Figure 9 shows the result. The distribution is Gaussian, centered around 498 ms with an SD of $\pm 120$ ms. The instrumentation can detect pauses down to 250 ms, or two SDs below the mean. In other words, if larger steps arose out of missed pauses, the distribution of pause duration would have to have been distinctly bimodal.

Control Experiments

A series of control experiments to check for artifact has already been reported (2, 3, 22). Two additional controls in cardiac specimens were carried out here. In the first, we applied a stepped ramp to the myofibril to check resolution (Fig. 10). Imposed step size was 10–15 nm/sarcomere. These steps appear faithfully in the sarcomere length trace. In addition, smaller steps gen-

Fig. 6. Histogram of shortening steps obtained from activated rabbit cardiac muscle. Histogram includes 493 steps from 75 sarcomeres in 25 myofibrils.

Fig. 7. Histograms of lengthening steps in bumblebee flight muscle (shaded line) and rabbit cardiac muscle (solid line). Number of steps: bumblebee, 908; cardiac, 378.

Fig. 8. A: histogram of step size in cardiac muscle obtained by imposing stretch ramps. Low-speed ramps are shown by shaded line (378 steps from 96 sarcomeres in 26 myofibrils); high-speed ramps are shown by solid line (370 steps from 88 sarcomeres in 26 myofibrils). B: similar data for release: High-speed (solid line, 350 points from 70 sarcomeres in 13 myofibrils) and low-speed (shaded line, 360 steps from 95 sarcomeres in 26 myofibrils) shortening ramps imposed on cardiac myofibrils.

Fig. 9. Distribution of pause durations measured during shortening in activated cardiac specimens.
erated spontaneously are interspersed between the larger steps (see arrows). Results of measurement of these steps are shown in Fig. 11. For lengthening and shortening alike, the size pattern of the spontaneously occurring steps is the same as for the steps seen during imposition of the ramp.

Because the steps appear so consistently at integer multiples of 2.7 nm, suspicion arises that the origin of the steps could lie in some unsuspected feature of the apparatus, notwithstanding extensive controls. We therefore carried out experiments identical to those described above, except that the specimens were unac-

**DISCUSSION**

The results show a high level of consistency among specimens for both shortening and lengthening: step values of 2.7 nm and integer multiples thereof. The observations confirm and extend earlier observations of step size values at integer multiples of 2.7 nm and establish that the previously implied quantum does indeed exist. Because the same quantal value is found in invertebrate and vertebrate specimens alike, the value may be taken as general. Thus activated sarco-

A third finding of interest is that contractile steps can be both small and large. The small step, or quantum, is 2.7 nm, but steps as large as five times that value were not infrequent. Such large steps could have arisen from inadvertently missed pauses only if the pause-duration distribution (Fig. 9) was bimodal, with peak separation more than two times the SD, a feature that seems unlikely. Hence, the contractile machinery apparently executes steps not only of 2.7 nm but also of
5.4 nm and of larger size as well. The capacity to generate such larger steps is also seen in various single molecule experiments (10, 13, 15, 21). It implies a paradoxical combination of deterministic and stochastic components: a component that produces steps that are exact integer multiples of 2.7 nm, n × 2.7 nm, and a component that determines the value of n for each step, seemingly unpredictably, although no systematic attempt has been made to determine whether the size sequence might follow some subtle pattern.

A fourth finding of significance is that length changes occur with a high degree of cooperativity. The myofibril contains many hundreds of filaments in parallel. If length changes occurred randomly in each filament unit, the sarcomere shortening trace would be smooth, not stepwise. The consistently sharp transitions between pauses and steps implies a high degree of cooperativity among parallel filaments.

The result of highest significance, perhaps, is the correspondence between dynamics and structure. Active shortening occurs in steps that are integer multiples of 2.7 nm. The 2.7-nm value is equal to the linear repeat of actin monomers along the thin filament. Correspondence between dynamics and structure is seen similarly in the microtubule-kinesin system (18), wherein the observed 8-nm translation step is equal to the axial repeat of tubulin along the protofilament. "Backwards" steps are also integer multiples of 8 nm. The striking parallelism between the two systems implies a high level of mechanistic similarity: whatever mechanism governs kinesin-microtubule translation probably governs active translation in the myosin-actin system. In both cases, active translation corresponds to molecular structure.

The results also show correspondence with at least some experiments in isolated actomyosin systems. The results reported by Kitamura et al. (10) and Yanagida et al. (21) by using single myosin molecules translating along actin filaments showed consistent step sizes of ~5.4 nm. Also, recent optical trap experiments from the Molloy laboratory (13) show steps on the order of 5–6 nm. In the experiments of Guildford et al. (8) employing an optical trap, on the other hand, the step size was ~11 nm, almost exactly twice the Kitamura/Yanagida/Molloy value. Both these values were seen in our results (Figs. 4, 6, and 7); they show up as two and four times the more primary value of 2.7 nm. Whether such correspondence is more than coincidental awaits improvement in precision in the single molecule experiments. If the correspondence is not coincidental, the smaller quantal value seen here might arise because the parallel filament array allows more opportunities for interaction than a single filament; hence, every potential actin-myosin interaction can be realized, and the steps will be smaller.

On the other hand, the kind of multipeaked distributions seen here are obtainable only with resolution on the order of 1 nm or better. Without such resolution, the separation between peaks will vanish, and the distribution will look smooth and broad. It is therefore necessary to exercise caution when comparing literature data of lower resolution. In such situations, the peak of the distribution will reflect the relative heights of the multiple peaks, which could well change with loading conditions. Nevertheless, there appears to be reasonable agreement with published data.

The results also show correspondence with measurements from whole muscle fibers. When tetanically contracting fibers are abruptly released to shorten against a constant load, the shortening trace shows a series of oscillations that gradually damp out (4). That these oscillations may correspond to steps at the sarcomere level was first suggested by Huxley and colleagues (1, 9), and the correspondence has since been confirmed (7). The sharp transient that occurs at the onset of the load clamp apparently synchronizes the steps, which show up as inflections in the fiber length trace. The amplitudes of these inflections were recently measured by Edman and Curtin (5) and were found to be 2.7 nm/half-sarcomere, the same as the step size measured here. Thus the same fundamental step size is arrived at by two independent approaches.

What kind of mechanism fits these signaturelike results? Steps that are integer multiples of the actin monomer repeat are inevitable if each projecting myosin head is fixed in space and sticks transiently to one or another actin monomer as thin filaments translate past thick filaments. Step size will then be n × 2.7 nm. This feature would be the same for lengthening and for shortening. The larger steps (n > 1) would correspond to collectively “missed” opportunities for attachment.

Details of how such translational motion might be produced are open to speculation, but a simple mechanism based on appreciable evidence has been suggested (16). In this mechanism, the actin filament undergoes inchwormlike motion, crawling step by step over the thick filament. This produces a string of steps, the size of which is always an integer multiple of 2.7 nm. Other mechanisms may be possible, but such mechanisms will need to produce the multipeaked distribution reported here, which imposes a rather severe constraint.

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