Thin Filaments Are Not of Uniform Length
in Rat Skeletal Muscle

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ABSTRACT The variation in thin filament length was investigated in slow and fast muscle from
adult and neonatal rats. Soleus (slow) muscle from adult, 3-, 7-, and 9-d-old rats, and extensor
digitorum longus (EDL; fast) muscle from adult rats were serially cross-sectioned. The number
of thin filaments per 0.06 μm² (TF#) was counted for individual myofibrils followed from the
H zone of one sarcomere, through the I-Z-I region, to the H zone of an adjacent sarcomere.
TF# was pooled by distance from the Z band or A-I junction.

In both adult muscles, thin filament length varied from 0.18 to 1.20 μm, with ~25% of the
thin filaments less than 0.7 μm in length. In 7- and 9-d soleus, thin filament length ranged from
0.18 to 1.08 μm; except for the longest (0.18 to 1.20 μm) filaments, the distribution of thin
filament lengths was similar to that in adult muscle. In 3-d soleus, thin filament length was
more uniform, with <5% of the filaments shorter than 0.7 μm. In all neonatal muscles, there
were ~15% fewer thin filaments per unit area as compared to adult muscles. We conclude: (a)
In rat skeletal muscle, thin filaments are not of uniform length, ranging in length from 0.18 to
1.20 μm. (b) There may be two stages of thin filament assembly in neonatal muscle: between
3 and 7 d when short thin filaments may be preferentially synthesized or inserted near the Z-
band, and between 9 d and adult when thin filaments of all lengths may be synthesized or
inserted into the myofibril.

A basic assumption of the sliding filament theory of muscle
contraction is that thin and thick filaments are each of uniform
length. The amount of tension generated by a muscle is thus
proportional to the degree of thin-thick filament overlap (i.e.
number of cross-bridges) (11, 13). Early studies reported that
thin and thick filaments are each of uniform lengths, as meas-
ured in electron micrographs of longitudinally sectioned muscle
(14, 17). Although the lengths of thick filaments may be easily
measured, it is not possible to discern the ends of all thin
filaments in longitudinal section. It has been suggested that
thin filament lengths can be accurately measured only in cross-
sectioned muscle in which the ends of thin filaments can be
discerned (18).

Recently, evidence has been presented that, in some muscles,
thin filaments are not of uniform lengths; for example, in
serially cross-sectioned cardiac muscle, thin filaments vary in
length by as much as 0.6 μm (19). However, in serially cross-
sectioned rabbit psoas muscle, thin filaments appear to be of
uniform length (13). This raised the possibility that variations
in thin filament length may be related to the type of muscle
(cardiac vs. skeletal) or type of fiber ("slow" vs. "fast"). This
study was undertaken to reexamine the evidence for thin
filament length directly by using serially cross-sectioned mus-
cle, with an emphasis on muscle-specific differences in thin
filament lengths. Rat soleus and extensor digitorum longus
(EDL) muscles were chosen as examples of slow and fast
muscles, respectively (1). Because muscle differentiation occurs
during the first 3 wk of postnatal life in the rat (2), the rat was
chosen as a model for investigating possible developmental
changes in thin filament lengths. These results have been
previously presented in abstract form (22).

MATERIALS AND METHODS

Male Sprague-Dawley rats were used at ages 3, 7, and 9 d and adult (10 to 15
mo). After decapitation of the rat, soleus and EDL muscles were exposed and
tied to wooden applicator sticks at rest length, removed from the rat, and fixed
at room temperature in 4% paraformaldehyde-5% glutaraldehyde in 0.2 M
Millonig's phosphate buffer, pH 7.3. After 60 min the muscles were removed
from their splints, cut into 1-mm cubes, and fixed in fresh fixative for an
additional 60 min. Specimens were rinsed four times in buffer, postfixed for 60
min in 1% OsO₄, in 0.2 M Millonig's phosphate buffer, pH 7.3, dehydrated in a
graded series of ethanol, rinsed in propylene oxide, and flat-embedded in Epon.

To ascertain whether thin filaments shrink during fixation and dehydration in
muscles cut into cubes before osmication (17), an adult soleus muscle was
maintained split into an applicator stick throughout the entire fixation and
dehydration procedures. This specimen was fixed in glutaraldehyde-paraform-
aldehyde for 3 h, postfixed in OsO4 for 1.5 h, and dehydrated in a graded series
of acetone. Acetone was used for dehydration because it does not cause thin
filament shrinkage (17). The muscle was cut into 1 x 1 x 3-mm pieces in the
final acetone rinse, and these pieces were flat-embedded. Only the outer fibers
that became black during osmication were used.

In some instances, adult rat soleus and rabbit psoas muscles were glycera-
nated before fixation. Muscles were tied to applicator sticks, immersed in 50% glycerol-
50% low salt buffer (100 mM KCl, 2 mM MgCl2, 2 mM EGTA, 1 mM
dithiothreitol, 10 mM Tris-maleate, pH 6.8 [4]), and stored at -10°C until fixed.
Small fiber bundles were teased apart, splinted, and fixed as described above.
Serial 60-nm thin cross-sections were cut with a Dupont diamond knife on a
Porter-Blum MT2B ultramicrotome (DuPont Instruments, Newtown, CT), picked
up on Formvar-coated slotted grids, stained in ethanolic uranyl acetate and
Reynolds' lead citrate, and viewed in a Philips 201A electron microscope.

Muscle fibers were randomly chosen at low magnification and serially pho-
tographed at 15,000 times magnification. This magnification was always ap-
proached from the lower ranges to avoid hysteresis effects. The electron micro-
scope was calibrated regularly. Prints of final magnification 63,000 times were
used.

Three muscle fibers per rat, and 10 myofibrils per muscle (30 myofibrils per
rat), for three rats per age group, were analyzed. Each myofibril was followed
serially from the Z band of one sarcomere, through the I-Z-I region, and up
to the H zone of an adjacent sarcomere. Each myofibril profile was categorized
by its distance (i.e. number of sections) from the Z band or AI junction, expressed
as Z + 1, 2, 3, . . . or AI + 1, 2, 3, . . . sections (designated "levels") (Fig. 1). A
square with area equivalent to 0.06 μm² was drawn on each micrograph in the
center of each myofibril, and the number of thin filaments in that area was
 counted. The area equivalent to 0.06 μm² was chosen as a convenient area (1/16
μm²) that would allow only the central, and not the peripheral, region of each
myofibril to be used for filament counts. The myofibril periphery was excluded
because thin filament distribution differs between regions, thin filaments being
more closely spaced in a ring around the periphery of the myofibril. Correlations
between thin filament number counted over entire myofibrils and per 0.06 μm²
in the same myofibrils indicated that the entire myofibril was accurately repre-
 sented by the sampling area.

The number of thin filaments per 0.06 μm² (TF#) was counted for each
myofibril. TF# were pooled by level (Z + 1, 2, 3, . . . or AI + 1, 2, 3, . . .). A mean
TF# for each level was calculated for each muscle fiber (representing 10
myofibrils). The TF# means for three fibers were then used to calculate a mean
TF# for each rat, and mean TF# for three rats used to calculate a mean TF# for
each age group.

For data analysis, linear regression statistics were computed for each group
for TF# vs. distance for distances Z + 1 through Z + 9 and AI + 1 through AI
+ 8. An analysis of covariance model (ANCOVA) was then used to test for
significance of regression coefficients (slopes), and for differences in regression
coefficients and coincidence of regression lines (equality of means) between
groups. When a significant F ratio was obtained for the ANCOVA, t tests were
used to test pair-wise differences between groups (21).

RESULTS

In adult soleus, I-band TF# decreased linearly with distance from the Z-band, such that at the AI junction TF# was 25% lower (P < 0.01) than at one section from the Z-band (Z+1) (Fig. 1). A similar pattern was seen in 7- and 9-d-old soleus, with TF# also decreasing 25% from Z + 1 to the AI junction (P < 0.01). In 3-d soleus, however, TF# remained constant throughout the I-band. In adult EDL, TF# also decreased linearly from Z + 1 to AI junction (P < 0.01). The magnitude of this decrease was slightly, but not significantly, larger (30% vs. 25%) than in adult soleus.

In 7- and 9-d soleus, I-band TF# was 15% lower at each level compared to adult soleus (P < 0.01), although the slopes of the TF# distribution curves were not statistically different (Fig. 1). In 3-d soleus, TF# was 23% less at Z + 1 and 7% less at the AI junction compared to adult soleus. The regression coefficient for 3-d soleus was significantly different (P < 0.01) from all other groups. In adult soleus and EDL, TF# was virtually identical.

In all muscles studied, A-band TF# decreased monotonically from the AI junction to the H zone (Fig. 1). However, in 3-d soleus the slope of the regression line was significantly different (P < 0.01) from that in all other groups; for 7- and 9-d soleus, the slopes were also significantly different (P < 0.01) from those in all other groups. In adult soleus and EDL, the longest thin filaments were ~0.1 μm longer than in neonatal soleus, as evidenced by the two additional A-band sections (AI + 7 and AI + 8).

Thick filaments were also counted by the same method used for counting thin filaments. Thick filament number per 0.06 μm² remained constant throughout all regions of the A-band, exhibiting no age or fiber type differences (data not shown).

Because TF# was measured per unit area and not for the entire myofibril, it was possible that thin filament counts were influenced by changes in myofibril cross-sectional area with location in the sarcomere. For example, a smaller myofibril cross-sectional area close to the AI junction would allow only the central, and not the peripheral, region of each myofibril to be used. To investigate this possibility, serial myofibril cross-sectional areas were measured in each muscle by planimetry using a HIPAD digitizing board (Houston Instruments, Austin, TX) interfaced to a Radio Shack TRS-80 microcomputer. I-band myofibril cross-sectional area was greatest closest to the Z-band, decreasing slightly with distance from the Z-band (data not shown). Thus the differences observed in TF# with increasing distance from the Z-band were actually somewhat underestimated.

These data showing changes in thin filament number by location in the sarcomere in rat soleus and EDL are not consistent with an earlier report of constant thin filament number throughout the entire sarcomere in glycinated rabbit psoas muscle (13). To examine whether these differences were due to tissue processing (fresh fixation vs. glycination before fixation) or to species or muscle differences (rat soleus and EDL vs. rabbit psoas), TF# was measured in adult rat soleus and rabbit psoas that had been glycinated before fixation. In glycinated adult soleus, TF# decreased from Z + 1 to the AI junction at the same rate as in fresh fixed adult soleus (Fig. 2). In glycinated rabbit psoas, TF# did not vary with location in the sarcomere, a finding consistent with Huxley's data (13) on glycinated rabbit psoas. Thus, there appear to be species
or muscle-related differences in thin filament number distribution by location in the sarcomere.

The splinted soleus experiment was performed to determine whether selective shrinkage of thin filaments was responsible for the observed decrease in TF# with distance from the Z-band (17). I-band TF# distributions were similar between soleus muscles that had been splinted throughout fixation and dehydration and soleus that had been cut into cubes before osmication and dehydration (Fig. 3). However, thin filaments appeared to be ~0.12 μm longer in splinted soleus, as evidenced by the extra sections in the I- and A-bands (Z + 10 and AI + 9). Thus in muscles not splinted throughout fixation and dehydration, thin filament shrinkage was ~10%, consistent with an earlier report (17). Nevertheless, this experiment indicates that thin filament shrinkage was not responsible for the observed decreases in TF# with distance from the Z-band or AI junction.

In summary, in adult soleus and EDL, and 7- and 9-d soleus, I-band TF# decreased linearly from one section from the Z-band (Z + 1) to the AI junction. In all muscles studied, A-band TF# decreased monotonically from the AI junction to the H zone. A wide range of thin filament lengths, 0.18 to 1.20 μm, was thus observed in these muscles. These results provide evidence that thin filaments are not of uniform length in rat skeletal muscle.

In an earlier study reporting uniform lengths of thin filaments in various frog muscles (17), thin filament lengths were estimated from the length of the I band plus A-band overlap region as seen in longitudinal section, in which the ends of shorter or longer thin filaments may be obscured and thus not included. It has been suggested that thin filament lengths can be measured accurately only in cross-sectioned muscle in which the filament ends can be discerned (18).

Thin filaments appear to be selectively susceptible to shrinkage if the muscle is not splinted during osmication and ethanol dehydration (17). It is possible that thin filament shrinkage is responsible for the variation in thin filament length observed in the present study. However, shrinkage appears to be limited to no more than 10% of thin filament length (17; our results). To account for the consistent age-related differences in thin filament number in the present study, thin filaments from adult and 3-, 7-, and 9-d soleus would all have to have been affected differently, but each age group in a consistent manner, by the tissue processing procedures. Moreover, in glycerinated rabbit psoas fixed by the same methods used on rat soleus and EDL, thin filaments were of uniform length. If glycerination before fixation provided protection against thin filament shrinkage, then, in glycerinated rat soleus, thin filaments should also have been of uniform lengths. However, thin filament length distributions were similar between glycerinated and fresh-fixed rat soleus. Furthermore, in adult soleus splinted throughout fixation and dehydration to prevent thin filament shrinkage (17), thin filament number distribution was similar to that observed in adult soleus fixed by the standard procedures. It thus seems unlikely that thin filament shrinkage is responsible for the variation in thin filament lengths observed in rat muscle.

While we cannot rule out the possibility of depolymerization of thin filaments during tissue processing, it seems unlikely that this was responsible for the observed results. Rat and rabbit muscle thin filaments would have to exhibit different susceptibilities to depolymerization during identical treatments, since thin filament number distributions were different in these muscles. Similarly, thin filaments in soleus muscle from rats of different ages would also have to exhibit different susceptibilities to thin filament depolymerization during identical treatments. Moreover, two very different procedures—glycerination before fixation and fresh fixation—resulted in similar thin filament number distributions in the same muscle.

In the present study, there were two age-related differences in 1-band thin filament number distribution in soleus muscle. The first occurred between ages 3 and 7 d, when thin filament number increased in sections closest to the Z-band (Z + 1 through Z + 4). This may reflect an increase in the synthesis or insertion into the sarcomere of very short thin filaments, 0.18 to 0.36 μm in length. The second age-related difference appeared between ages 9 d and adult when 1-band thin filament number increased 14% at all levels. This suggests a uniform increase in synthesis or insertion of thin filaments of all lengths. There also appeared to be an increase in the length of the longest thin filaments with age.

**DISCUSSION**

There have been other reports of nonuniform lengths of thin filaments in striated muscles. Robinson and Winegrad (19) found that, in serially sectioned frog and rat atrial muscle, thin filaments varied in length by as much as 0.6 μm. Page (18) reported exceptionally long (>1.1 μm) thin filaments in kitten papillary and frog atrial muscles. The existence of exceptionally long thin filaments in frog semitendinosus muscle has also been suggested, based on physiological data (8).

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The function of the short filaments is unclear at present. The short, thin filaments may represent filaments that function to maintain the Z lattice structure. These short filaments may also be involved in maintaining the thin filament lattice, perhaps at the level of the N lines (6). It is also possible that these short filaments are not thin (i.e. actin containing) filaments but some other types of filament, for example, intermediate filaments. However, in skeletal muscle, intermediate filaments appear to be oriented transversely around the periphery of the myofibril and in the intermyofibrillar spaces (9, 10, 16). Intermediate filaments have not been shown to run longitudinally inside the myofibril. Although in the present study we cannot clearly designate all filaments as thin filaments, these filaments were of uniform diameter (7–8 nm, as measured at \( \times 285,000 \)) in all regions of the sarcomere. It is thus unlikely that our thin filament counts include intermediate filaments.

Very short thin filaments could contribute to tension generation only at short sarcomere lengths, while exceptionally long thin filaments would function in tension generation at both long and short sarcomere lengths. The classical theory of cross-bridge behavior in muscle contraction and its relationship to the length-tension curve assumes uniform lengths of thin filaments (11, 12). This assumption may be correct for the muscles used in these studies—frog sartorius and semitendinosus and rabbit psoas. Indeed, thin filament counts in rabbit psoas support this assumption (13; our results). However, there may be other muscles in which the assumption of uniform thin filament lengths may not be valid, for example, frog and rat atrial and rat papillary muscles (19), and rat soleus and EDL. Robinson and Windgrad (19) suggest that, in cardiac muscle, the length-tension relationship must take into account not only sarcomere length (overlap of thin and thick filaments) but the distribution of thin filament lengths as well. This statement also seems relevant for rat skeletal muscle.

In mouse biceps brachii muscle, the shape of the length-tension curve changes during early postnatal development, exhibiting a wider plateau with increasing age (7). With maturation, tension development becomes less dependent on sarcomere length, especially at sarcomere lengths below rest length. This finding is compatible with our data showing an increase in variation of thin filament length with age. Adult muscle, having more of the short thin filaments, should be better able to generate tension at short sarcomere lengths than neonatal muscle which has fewer short thin filaments.

The short thin filaments may represent directional growth of thin filaments from the Z-band toward the center of the sarcomere. Thin filaments do exhibit polarity with respect to the Z-band (14, 20), although the actual direction of growth is unclear. Electron microscopy autoradiography indicates that, in growing muscle, Z-band protein incorporation is much higher than in other components of the sarcomere (3). The Z-band may serve as a nucleation site for thin filament insertion and elongation during growth.

The observation that thin filaments are of various lengths in rat skeletal muscle but of uniform length in rabbit skeletal muscle suggests the possibility that in these muscles different factors control thin filament length. The developmental changes in thin filament length also suggest alterations in factors regulating thin filament length during postnatal development. Factors controlling thin filament length, at least in vitro, include Ca\(^{2+}\) concentration, critical concentration of actin, bound nucleotides (5), and \( \beta \)-actinin (15).

In conclusion, the results presented in this study provide strong evidence that in rat slow and fast skeletal muscles thin filaments are of various lengths (0.18 to 1.20 \( \mu \text{m} \)). The distribution of these lengths changes during early postnatal development. These findings should be considered in interpreting length-tension relationship data from rat skeletal muscle, especially in growing rat muscle.

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REFERENCES

1. Ariano, M. A., R. B. Armstrong, and V. R. Edgerton. 1973. Hindlimb muscle fiber populations of five mammals. J. Histoch. Cytochem. 21:51–55.
2. Close, R. I. 1972. Dynamic properties of mammalian skeletal muscles. Physiol. Rev. 52:129–197.
3. Dodoune, J. P. 1980. Protein turnover in muscle cells as visualized by autoradiography. Int. Rev. Cytol. 67:215–257.
4. Engler, J. D., R. Zak, and D. A. Fischman. 1976. Compositional studies of myofibrils from rabbit striated muscle. J. Cell Biol. 68:123–141.
5. Engel, J. H. Pasold, F. W. Huxley, and A. Wegener. 1977. The polymerization reaction of muscle actin. Mol. Cell. Biochem. 18:3–13.
6. Franzini-Armstrong, C. 1970. Details of the I band structure as revealed by the localization of ferritin. Tissue Cell. 2:327–339.
7. Goldspink, G. 1968. Sarcomere length during postnatal growth of mammalian muscle fibers. J. Cell Sci. 3:539–548.
8. Gordon, A. M., A. P. Hussey, and F. J. Julian. 1967. Tension development in highly stretched vertebrate muscle filaments. J. Physiol. 180:143–169.
9. Granger, B. L., and E. Lazardies. 1978. The existence of an insoluble Z-disc scaffold in chicken skeletal muscle. Cell 15:1253-1262.
10. Granger, B. L., and E. Lazardies. 1979. Desmin and vimentin coexist at the periphery of the myofibril Z-disc. Cell 19:1053–1063.
11. Huxley, A. F., and R. Niedergerke. 1954. Structural changes in muscle during contraction. Interference microscopy of living muscle fibers. Nature (Lond.) 173:971–973.
12. Huxley, H. E., and J. Hanson. 1954. Changes in the cross- striations of muscle during contraction and stretch and their structural interpretation. Nature (Lond.). 173:975–976.
13. Huxley, H. E. 1960. Muscle Cells. In The Cell IV. J. Bradet, and A. E. Mirsky, editors. Academic Press, Inc., New York, 356–481.
14. Huxley, H. E. 1963. Electron microscopic studies on the structure of natural and synthetic protein filaments from striated muscle. J. Mol. Biol. 7:281–308.
15. Maruyama, K. 1976. Actinins, regulatory proteins of muscle. Adv. Biophys. 9:157–185.
16. Nandi, M. G., and C. Franzini-Armstrong. 1980. Trabeicular network in adult skeletal muscle. J. Ultrastr. Res. 73:21–26.
17. Page, S. G., and H. E. Huxley. 1963. Filament lengths in striated muscle. J. Cell. BioL 19:369–390.
18. Page, S. G. 1974. Measurements of structural parameters in cardiac muscle. Ciba Found. Symp. 24:13–25.
19. Robinson, T. F., and S. Winegrad. 1977. Variation in thin filament length in heart muscle. Nature (Lond.) 267:74–75.
20. Shimada, Y., and T. Obinata. 1977. Polarity of actin filaments at the initial stage of myofibril assembly in myogenic cells in vitro. J. Cell Biol. 72:773–785.
21. Snedecor, G. W., and W. G. Cochran. 1971. Statistical Methods. Iowa State University Press, Ames, Iowa.
22. Traeger, L., and M. A. Goldstein. 1982. Thin filaments are of uniform length in rabbit skeletal muscle. Biophys. J. 37:127 a.