

Effects of EDTA Treatment upon the Protein Subunit Composition and Mechanical Properties of Mammalian Single Skeletal Muscle Fibers

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ABSTRACT Considerable interest has been focussed on the role of myosin light chain LC₂ in the contraction of vertebrate striated muscle. A study was undertaken to further our investigations (Moss, R. L., G. G. Giulian, and M. L. Greaser, 1981, *J. Biol. Chem.*, 257:8588-8591) of the effects of LC₂ removal upon contraction in skinned fibers from rabbit psoas muscles. Isometric tension and maximum velocity of shortening, V_{max} , were measured in fiber segments prior to LC₂ removal. The segments were then bathed at 30°C for up to 240 min in a buffer solution containing 20 mM EDTA in order to extract up to 60% of the LC₂. Troponin C (TnC) was also partially removed by this procedure. Mechanical measurements were done following the EDTA extraction and the readditions of first TnC and then LC₂ to the segments. The protein subunit compositions of the same fiber segments were determined following each of these procedures by SDS PAGE of small pieces of the fiber.

V_{max} was found to decrease as the LC₂ content of the fiber segments was reduced by increasing the duration of extraction. EDTA treatment also resulted in substantial reductions in tension due mainly to the loss of TnC, though smaller reductions due to the extraction of LC₂ were also observed. Reversal of the order of recombination of LC₂ and TnC indicated that the reduction in V_{max} following EDTA treatment was a specific effect of LC₂ removal. These results strongly suggest that LC₂ may have roles in determining the kinetics and extent of interaction between myosin and actin.

The role(s) of the low molecular weight subunits, or light chains, of myosin in the contraction of vertebrate skeletal muscles have not been clearly resolved. The great majority of the work that has been done to investigate this problem has involved *in vitro* biochemical studies of the isolated contractile proteins, actin and myosin. Results obtained using this approach have generally been unable to indicate specific functions for the light chains. Removal of the so-called alkali light chains with NH₄Cl has been found to result in the loss of actomyosin ATPase activity (11, 15); however, these extraction conditions may denature the remainder of the myosin molecule, rendering it inactive (13). More recently, Wagner and Giniger (26) and Sivaramakrishnan and Burke (22) have shown that significant myosin ATPase activity remains even after the total removal of all light chains. Removal of up to 50% of the LC₂ light chain with dithionitrobenzene (DTNB) has been found to have little effect upon actomyosin ATPase activity

(16), though recent evidence (21) indicates that LC₂ may play a role in modulating the ATPase activity of myosin and regulated actin during Ca²⁺ activation.

Examination of the mechanical properties of single muscle cells in which the light chain (LC) composition could be manipulated and quantitated would seem to be a useful approach to the study of the physiological function(s) of these subunits. Work in this laboratory has shown that when LC₂ was partially extracted from single skinned muscle fibers (to levels that were ~70% of control) the maximum velocity of shortening (V_{max}) measured during maximal Ca²⁺ activation was reduced by about 40% (18). This phenomenon has been investigated further in the present study. LC₂ extraction conditions have been varied to yield LC₂ compositions within individual fiber segments that were 40-95% of control values measured in the same fibers. V_{max} was found to decrease as the LC₂ content of the segment was reduced. EDTA treatment of

the fiber segments also resulted in reversible reductions in tension due mainly to the loss of troponin C (TnC). V_{\max} was unaffected by decreases in tension per se, down to tension values as low as 30% of control values. Both V_{\max} and tension could be restored by readdition of LC_2 and TnC respectively. These results suggest that LC_2 may have a modulatory role in the interaction of actin and myosin in skeletal muscle.

A brief report of these results was presented at the Meetings of the Biophysical Society (19).

MATERIALS AND METHODS

Preparation: Male New Zealand rabbits (2.5–3.5 kg body wt) were sacrificed by cervical dislocation. The psoas muscles were quickly excised and placed in a skinning solution containing, in mM: KCl, 100; $MgCl_2$, 1; $ATPNa_2$, 4; EGTA, 5; imidazole, 10; pH 7.00 \pm 0.01. Small fiber bundles were dissected from the muscles, tied with surgical silk to capillary tubes, and stored in skinning solution containing 50% (vol/vol) glycerol at $-22^\circ C$ for 3–14 d before use. On the day of its use each bundle was bathed for 30 min in cold skinning solution containing 0.5% (wt/vol) Brij 58, and single fibers were then pulled free. Fiber segments were transferred to the muscle chamber containing relaxing solution (below) and mounted with small connectors (17) to wires extending between the motor arm and the force transducer. A segment of fiber between 2.0 and 5.0 mm in length remained exposed to the bathing solution between the connectors. The segments were in each case observed and photographed through a light microscope in order to obtain a measure of sarcomere length (17) both while relaxed and during Ca-activation. Initially, the end-to-end length of each segment was adjusted so that the mean sarcomere length was $\sim 2.7 \mu m$.

Solutions: The solutions that were used to relax and activate the fiber segments have been described previously (17). Relaxing solution contained, in mM: KCl, 100; EGTA, 2; $MgCl_2$, 1; $ATP Na_2$, 4; imidazole, 10; pH 7.00. The composition of the activating solution was identical to that of relaxing solution except that the concentration of EGTA was 4.0 mM and $CaCl_2$ was added to yield a concentration of 3.8 mM. Assuming an apparent stability constant of $10^{6.68}$ for the Ca^{2+} -EGTA complex, the free Ca^{2+} in the activating solution was $10^{-5.49} M$ (6). All reagents were obtained from the Sigma Chemical Co. (St. Louis, MO).

The composition of the solution used to partially extract protein subunits from the fiber segments was in most cases 20 mM EDTA, 50 mM KCl, 5 mM phosphate buffer, pH 7.0 (i.e., LC_2 extracting solution). TnC was isolated from rabbit skeletal muscle using the method of Greaser and Gergely (4) with the following modifications: (a) inclusion of 0.1 mM phenylmethylsulfonyl fluoride and 1% Triton X-100 in the washes of the muscle mince and (b) chromatography of troponin on DEAE cellulose. Total myosin light chains were removed from myosin using 4 M urea dissociation (16). The individual LC_1 and LC_2 preparations were obtained by chromatography on DEAE cellulose in 6 M urea containing 15 mM β -mercaptoethanol, at pH 6.0, using a 10 mM to 125 mM potassium phosphate gradient. The proteins were dialyzed versus 0.2 M KCl, 20 mM imidazole (pH 7.0), and were subsequently mixed with EGTA, $MgCl_2$, and ATP, and diluted to yield salt concentrations identical to those of the relaxing solution.

Physiological Apparatus: The mechanical system was similar to the one described previously (17). In the present study, the experimental troughs were made of stainless steel rather than polymer-coated aluminum. The scanning motor (Model 300 s; Cambridge Technology, Cambridge, MA) that was used could achieve a 0–100% step response time of 0.6 ms, with the segment connecting device and wire attached to the motor arm. The force transducer was a semiconductor strain-gauge device (Model AE801; Aksjeselskapet Mikro-Elektronikk, Horten, Norway) that had a resonant frequency of 1.2 kHz with connecting wire attached and a sensitivity that could be varied between 2.5 and 7.5 mV/mg. A simple wheatstone bridge in conjunction with an instrumentation amplifier (Model AD520J; Analog Devices, Norwood, MA) was used to detect and amplify force changes. Peak-to-peak noise at the amplifier output was equivalent to 0.5 mg wt. The transducer element was encased in a stainless steel cylinder to strain limit the element and to prevent water damage. A slot was cut into the side of the cylinder to allow passage of a connecting wire from the element to the fibre segment. The length and force signals were recorded on a digital oscilloscope (Model 2090-3B; Nicolet Instrument Corp., Madison, WI) and were subsequently stored on magnetic disk for later analysis.

Measurements of V_{\max} and Isometric Tension: V_{\max} was measured using the slack test method, which has been described in detail (7). Briefly, the slack test was done by rapidly decreasing the length of the fiber segment and measuring the time required to take up the imposed slack. During this time, there was zero external load and the segment shortened at V_{\max} . For each measurement, various amounts of slack were introduced in three or more successive contractions. The amount of length change was then plotted against

the duration of unloaded shortening and V_{\max} was calculated as the slope of a straight line fitted to this data using the least squares error method. If the coefficient of determination (r^2) for any fitted data was less than 0.95, that data was discarded. V_{\max} was expressed in terms of muscle lengths (ML) per second, by first normalizing the muscle length (measured in mm) to a sarcomere length of 2.4 μm and then dividing this number into the raw velocity value (calculated in mm/s). Examples of the slack test procedure are shown in Figs. 6 and 11.

Isometric tension during Ca-activation was measured from the slack test records as the difference between the steady tension values measured just prior to and immediately following the slack step. The reported tensions have been corrected for any resting tension exerted by the segments. This was done by imposing a slack step on the relaxed segments to determine the magnitude of the resting tension. This value was then subtracted from the total tension measured during activation to obtain the tension due solely to Ca-activation.

Experimental Protocol: Following dissection, each muscle fiber was divided into three segments between 3 and 5 mm in length. One of these segments was immediately dissolved in sample buffer (described below) for subsequent analysis by PAGE. Another segment was mounted into the apparatus and was used to make mechanical measurements. The third segment was stretched to the length at which it was just taut and then tied at both of its ends to the wire extending from the motor arm. Thus, although mechanical measurements were not made on the third segment, this segment was exposed to the same bathing solutions as the second segment, on which mechanical measurements were made.

The temperature of the bathing solution was lowered to $15^\circ C$ and control measurements of V_{\max} and tension (P_0) were made during maximal Ca-activation. With the fiber segments in relaxing solution, the chamber temperature was then raised to 25° , 30° , or $37^\circ C$ and the bathing solution was changed from relaxing solution to the LC_2 extracting solution, in which the segments were soaked for periods of up to 240 min. During this time, the bath solution was completely changed as often as every 5 min to minimize changes in salt concentration due to evaporation. The temperature was then lowered to $15^\circ C$ and the segment was returned to relaxing solution. The solution changes from relaxing to EDTA-containing solutions and back were usually accompanied by transient increases in tension that were as great as 0.25 P_0 . Tension in every instance was observed to return to baseline levels within 60–120 s. Following LC_2 extraction, the fiber segments were activated with Ca^{2+} to obtain measures of the isometric tension and V_{\max} . The segments were then placed in a relaxing solution containing 1 mg/ml of TnC for a period of 40 min at $15^\circ C$, after which, measurements of V_{\max} and active tension were again made. Still longer soak times in the TnC-containing solution were without additional affect on these mechanical parameters. The segment tied only to the motor arm was removed at this time and placed in the sample buffer for gel analysis.

The fiber segment mounted between the motor arm and force transducer was then transferred to a relaxing solution containing 1 mg/ml of a LC mixture containing primarily LC_2 and LC_1 . Recombination of LC_2 into the segments appeared to be complete following a 120-min incubation in this solution at $15^\circ C$. Final measurements of V_{\max} and tension were made at $15^\circ C$, before transferring the segment to sample buffer for subsequent electrophoretic analysis.

Modifications of the above protocol were required in some instances, and these are described where appropriate in the Results section.

PAGE of the Fiber Segments: The fiber segments were placed in 0.5 ml polypropylene microfuge tubes containing 10 μl of 1% (wt/vol) SDS. The samples were then ultrasonicated for 15 min in a Branson B-12 sonicator, heated at $70^\circ C$ for 2 min, and stored in the freezer ($-22^\circ C$) for 1 to 14 d before being applied to the gels. The frozen samples were thawed and 10 μl of sample buffer was added. The sample buffer consisted of 62.5 mM Tris-HCl (pH 6.8), 1.0% (wt/vol) SDS, 15.0% (vol/vol) glycerol, 5.0% (vol/vol) mercaptoethanol, and 0.01% (wt/vol) bromophenol blue. The microfuge tubes were then capped, sonicated for 5 min and finally heated at 70° for 2 min. The samples were then applied to a stacking gel with a 50- μl Hamilton syringe.

Vertical slab PAGE of the muscle samples was done using 0.1% (wt/vol) SDS gels (0.75 mm thick) cast in a Hoeffer SE-600 slab electrophoresis apparatus. Sample wells were 0.5 cm in width, and were formed with 20-well Teflon combs. The gels were cast using a modification of the system described by Laemmli (12). The acrylamide/ N,N' -methylenebisacrylamide ratio was 200:1 (wt/wt) in the separating gel and 20:1 (wt/wt) in the stacking gel. The final concentration of acrylamide in the separating gel was 15.0% (wt/vol) and 9.0% (wt/vol) in the stacking gel. The concentration of Tris (pH 9.3) in the separating gel was increased to 0.75 M (25). The electrode buffer consisted of 0.05 M Tris-0.38 M glycine, 0.1% (wt/vol) SDS, pH 8.4. The gels were run at a constant current of 20 mA/slab for ~ 5.5 h.

Because of the relative insensitivity of Coomassie Brilliant Blue for staining protein bands of microsomes such as single fibers, a modified version of the ultrasensitive silver staining technique of Oakley et al. (20) was used to stain the gels (Giulian, Moss, and Greaser, manuscript submitted for publication). Upon completion of a gel run, the gels were prefixed for 30 min in a solution containing 50% (vol/vol) ethanol (EtOH) and 10% (vol/vol) glacial acetic acid. Final fixation

was accomplished in 10% (vol/vol) glutaraldehyde solution for 30 mins, followed by overnight washing in deionized water, using a continuous water flow system. Staining was done in a freshly made ammoniacal silver solution, which was made by first adding 31.5 ml of 90 mM NaOH to 2.1 ml of cold 14.8 M NH_4OH , and then mixing in 6.0 ml of 1.14 M AgNO_3 . The solution was brought to a final volume of 150 ml with deionized water. The gel was bathed in this solution for 3.25 min with mild agitation on a rotary shaking table (modified Model G-2; New Brunswick Scientific, Edison, NJ). The gel was then washed twice for 1.0 min in deionized water and developed in a freshly prepared solution of 0.005% (wt/vol) citric acid and 0.019% (wt/vol) formaldehyde. Development of the protein bands was stopped by transferring the gels to pure water, and the gel slabs were then dried. The slabs were scanned using a laser scanning densitometer (Model SL-504-XL; Bio-Med Instruments, Chicago, IL). The areas under the protein peaks were measured from the output of the integrator section of the densitometer. The relative amount of LC_2 present in each segment was expressed as a fraction of the total alkali LCs present in the same segment (8). This ratio, $\text{LC}_2/(\text{LC}_1 + \text{LC}_3)$, was calculated as the area under the peak on the densitometric tracing corresponding to LC_2 divided by the sum of the areas corresponding to LC_1 and LC_3 . The silver stain was linear for the LC subunits in the range of fiber lengths that were used (Giulian, Moss, and Greaser, manuscript submitted for publication).

RESULTS

Mechanical Characteristics of Untreated Fiber Segments

The acceptability of a particular fiber segment for use in this study was judged primarily on the basis of striation pattern uniformity at rest and during maximal activation. The mean sarcomere length in the relaxed segments was $2.72 \pm 0.08 \mu\text{m}$ ($n = 68$), as measured from light photomicrographs (Fig. 1a). Maximal activation with Ca^{2+} usually resulted in a decrease in sarcomere length in the central portions of the segments (Fig. 1b), due primarily to a slight yielding of the segment ends at the points of attachment (17). Activated segments in which sarcomere length was found to decrease by 10% or more of the control value were discarded, as were any segments in which gross striation non-uniformities were observed. Nearly 50% of all segments tried were rejected for these reasons.

The maximum Ca-activated tension developed by the fiber

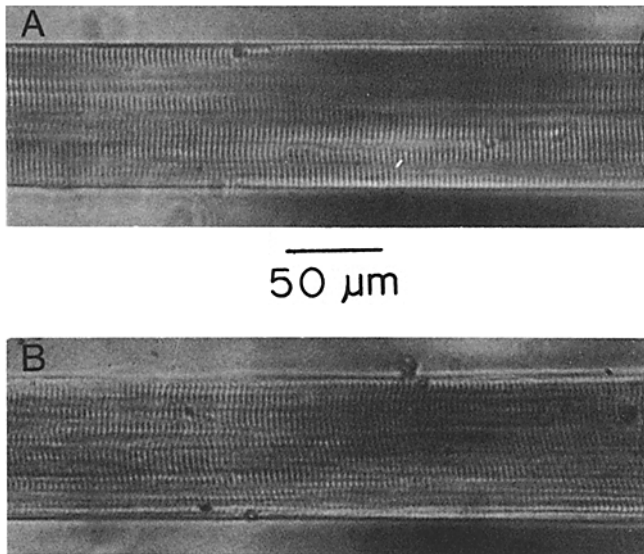


FIGURE 1 Photomicrographs of a psoas fiber segment taken while the segment was relaxed (A) and during steady activation with Ca^{2+} (B). The mean sarcomere length in A was $2.83 \mu\text{m}$, and in B, $2.81 \mu\text{m}$. Photographic procedures and calculations are described by Moss (17). Overall segment length was 4.02 mm.

segments averaged $1.34 \pm 0.21 \text{ kg/cm}$, which is in good agreement with the value of $1.61 \pm 0.77 \text{ kg/cm}$ reported by Julian et al. (8) for rabbit psoas fibers at 15°C . A mean V_{max} of 2.48 ± 0.38 muscle lengths per second (ML/s) ($n = 75$) was measured in the fiber segments prior to extraction with EDTA and encompassed a range of 1.86–3.45 ML/s. Addition of 15 mM creatine- PO_4 and creatine-phosphokinase (1 mg/ml) resulted in no significant change in either V_{max} or isometric tension in 11 fiber segments that were tested, indicating that at 15°C the ATP supplies within the segments were sufficient to sustain contraction.

Effects of EDTA Treatment on the Light Chain Composition of the Fiber Segments

Fiber segments that were freshly dissected from stored fiber bundles were found to have $\text{LC}_2/(\text{LC}_1 + \text{LC}_3)$ ratios that averaged 0.81 ± 0.08 ($n = 16$), and lay within the range 0.71–0.96 (see example, Fig. 2a). A variety of solution protocols was employed in an attempt to optimize the conditions for removal of LC_2 from the segments. By bathing segments in a solution containing 10 mM EDTA, 50 mM KCl, and 5 mM phosphate, pH 7.0 (23) at 37°C for periods of up to 120 min, it was possible to reduce the $\text{LC}_2/(\text{LC}_1 + \text{LC}_3)$ ratio to values as low as 0.07 (Fig. 2b). At this temperature, the $\text{LC}_2/(\text{LC}_1 + \text{LC}_3)$ ratio was found to decrease by >50% even with a 15-min incubation in the EDTA-containing solution.

Bathing the segments at 30°C for 120 min in the LC_2 extracting solution (see Materials and Methods) was found to reduce the $\text{LC}_2/(\text{LC}_1 + \text{LC}_3)$ ratio to values as low as 40% of control (also, see Moss et al. [18]). Increasing the duration of the treatment to 240 min had little additional effect upon this

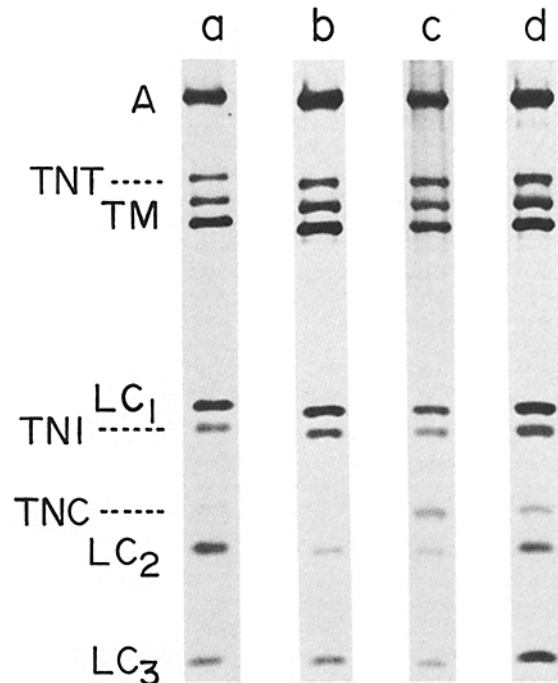


FIGURE 2 SDS polyacrylamide gels of fiber segments at different stages of the LC_2 extraction protocol. Gel a is a control fiber segment; b, following 60 min of extraction at 37°C ; c, following TnC readdition; d, following LC_2 readdition. The abbreviations used are A, actin; TM, tropomyosin; TNT, TNI, and TNC are troponin subunits T, I, and C; LC_1 , LC_2 , and LC_3 are myosin light chains 1, 2, and 3.

ratio. However, by decreasing the duration to 15 min, it was possible to reduce the amount of LC₂ extracted, in that the value of LC₂/(LC₁ + LC₃) was found to be 0.72 ± 0.09. It is likely that TnC was also extracted by this treatment, since Ca²⁺-activated tension was found to be greatly reduced, as discussed below. The amount of TnC actually lost in each case was difficult to quantitate due to its relatively low staining intensity in the silver system.

Additional experiments were done in which segments were bathed for up to 120 min at 25°C in the LC₂ extracting solution. In these cases, only slight decreases in the amounts of LC₂ and TnC present were seen. The LC₂/(LC₁ + LC₃) ratio was in all cases within 10% of control values.

Effects of EDTA Treatment on Resting Tension

The extractions performed at 37°C were usually accompanied by large increases in the tensions exerted by the fibers while in relaxing solution (Fig. 3). These tensions were in some instances as great as 25% of the maximum Ca-activated tensions (P₀) developed by the same segments prior to treatment, though the relative amounts of tension exerted in relaxing solution varied greatly in different fiber segments. The molecular basis for such increases is not presently known. Resting tensions exerted by the segments following the extractions at 30°C were increased by small amounts for soak durations of 90 and 120 min but were not significantly different from control values for shorter soak times. The amount of resting tension did not exceed 10% of P₀ in any instance, and was found in most cases to be <5% of P₀. Extractions done at 25°C resulted in no significant changes in resting tension even after incubations as long as 120 min.

Active Tension Development following EDTA Extraction

The steady isometric tension developed by the EDTA-treated fiber segments was measured at 15°C in a solution

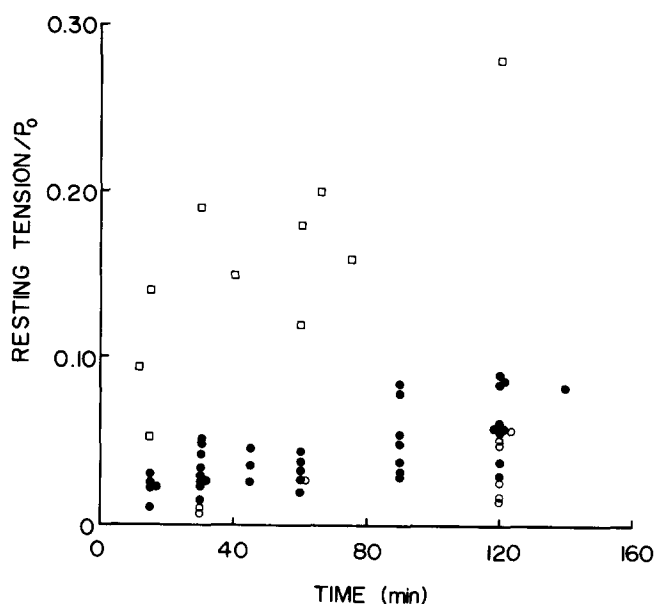


FIGURE 3 Plot of resting tension developed by the segments as a function of the duration of EDTA extraction at 37°C (□), 30°C (●), and 25°C (○). In each case, resting tension was expressed as a fraction of the maximum isometric tension (P₀) developed by the same fiber segment before EDTA treatment.

containing maximally activating levels of Ca⁺⁺, i.e., pCa 5.49 (8). In the segments that had been bathed in EDTA at 37°C, large decreases in Ca-activated tension were observed. The magnitude of this decrease was not greatly influenced by the duration of the EDTA soak, though slightly lower active tensions were seen at longer times. After 60 min of EDTA treatment at this temperature, tension was found to range between 8 and 26% of control values. The observed decreases in tension were reversed only slightly by soaking the segments at 15°C in a relaxing solution containing 1 mg/ml of TnC (Fig. 4a), even though TnC re-uptake by the segments was apparent (Fig. 2c). Subsequent soaks in relaxing solution containing 0.5 mg/ml of LC₂ (Fig. 4b) had no further effect on the tension developed by the segments. Re-uptake of LC₂ is demonstrated in the gel of Fig. 2d. In no instance was the amount of tension recovery following the addition of these subunits >20% of the control value. The total tension developed by these segments (i.e., the sum of the resting and Ca-activated tensions), never exceeded 40% of P₀.

The losses in active tension that followed extractions with EDTA at 30°C (Fig. 5) were consistently smaller than those seen when the extractions were done at 37°C, though in every instance tension was found to be less than the value obtained prior to the EDTA treatment. Generally, longer duration soaks

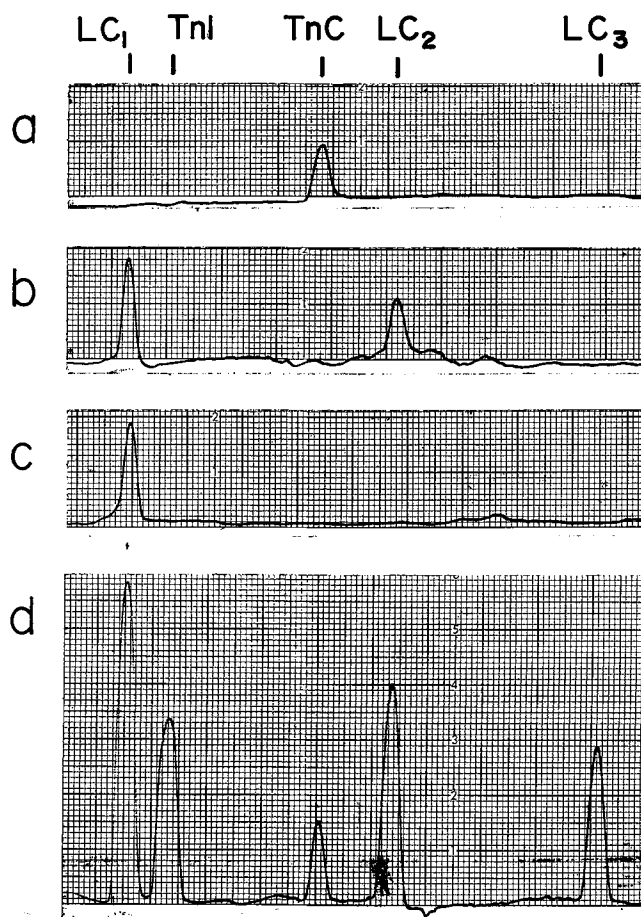


FIGURE 4 Densitometric scans of the SDS polyacrylamide gels of the addback solutions containing TnC (a), LC₂ (b), and LC₁ (c). Approximately 40 ng of protein was applied to the gel in a, and 20 ng each in b and c. Note the contamination of the LC₂ preparation (b) with approximately equivalent amounts of LC₁. A scan of an SDS gel of an untreated fiber segment is included (d) for reference.

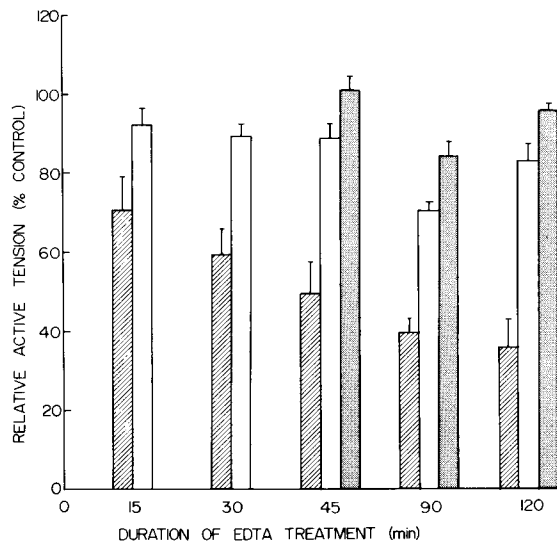


FIGURE 5 Relative active tension versus the duration of EDTA treatment at 30°C. At each time point, the tension was measured just following the soak (diagonal cross-marking), and following the TnC (clear bars) and LC₂ (stippled bars) recombinations. Tensions were in each case expressed as a percent of the active tension measured in the same fiber before treatment, and are reported here as the mean ± 1 SD ($n = 4-6$).

in EDTA resulted in greater losses in developed tension: following 15 min of extraction, tension averaged $70 \pm 9\%$ of control while an extraction period of 120 min yielded tensions that were $34 \pm 14\%$ of control. Recombination of TnC into these segments resulted in substantial reversals of active tension (Fig. 5) to values that averaged 85% of control. The absolute value, as percent control, to which tension finally recovered following the readdition of TnC was not noticeably influenced by the duration of the EDTA soak. Further, much smaller increases in Ca-activated tension were observed (Fig. 5) when these same segments were then bathed in relaxing solution containing myosin LC₂ (Fig. 4b).

At 25°C, EDTA-extraction for 120 min resulted in a relatively small decline in active tension, to $76 \pm 15\%$ of the control value. Addition of TnC to these fiber segments resulted in the recovery of tension to $93 \pm 7\%$ of control. No additional effects on tension were observed when these segments were bathed in the LC₂-containing relaxing solution.

V_{max} following EDTA Extraction

Treatment of the fiber segments with EDTA resulted in a decrease in the measured value of V_{max} (see example, Fig. 6). EDTA extraction at 37°C resulted in V_{max} values that in most segments ranged between 11 and 34% of control values. In several additional segments, the shortening velocity was too low to be measured accurately. Recombinations of TnC and especially LC₂ into these segments were not complete and were usually without effect with regard to V_{max} , though in three instances recoveries of V_{max} to within 30–41% of control were observed. The 30°C extraction in EDTA (Fig. 7) led to decreases in V_{max} that were dependent on the duration of the soak (Fig. 8). Small though significant decreases in V_{max} , to values that were $75 \pm 6\%$ ($n = 4$) of control, were observed following 15 min of extraction at this temperature; while at 120 min, V_{max} had decreased to $48 \pm 4\%$ ($n = 6$) of control values. Addition of TnC to these segments usually, but not always,

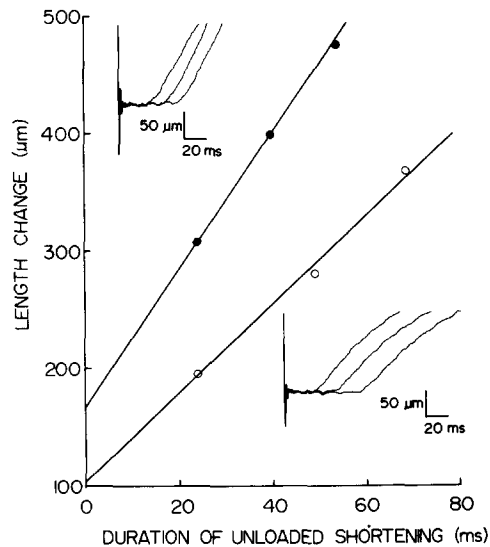


FIGURE 6 Slack test determinations of V_{max} prior to EDTA treatment (●) and following 60 min of extraction at 30°C (○). The actual force records, with expanded scales, obtained under each condition are shown adjacent to each set of experimental points. V_{max} prior to treatment was 2.43 ML/s and following treatment was 1.66 ML/s. $ML = 2.50$ mm; sarcomere length was $2.57 \mu\text{m}$. Fiber #11161.

resulted in small increases in V_{max} relative to the same segments just following the EDTA extractions. Finally, recombination of LC₂ into the segments (Fig. 7) resulted in increases in V_{max} to values near control. The level to which V_{max} recovered following LC₂ addition appeared to be independent of the duration of the EDTA soak.

The Relationship between V_{max} and Light Chain Content

V_{max} is plotted, as percent control, in Fig. 9 vs. the LC₂/(LC₁ + LC₃) ratio obtained from gel analysis of the same fiber segments. This plot includes data only from segments in which the control value of LC₂/(LC₁ + LC₃) was greater than 0.70. This criterion was used in order to avoid possible errors in quantitation of the LC ratio due to ineffective staining of the gels with silver. A clear correlation between V_{max} and the LC₂/(LC₁ + LC₃) ratio is apparent for both the EDTA treated segments and following readdition of LC₂, with V_{max} decreasing as the light chain ratio was reduced. However, the conclusion that there is a functional relationship between V_{max} and the LC content of a fiber segment does not necessarily follow from this data, since active tension is also greatly reduced by the EDTA treatments (Fig. 5). Thus, the decline in V_{max} following EDTA extraction may, at least in part, be attributable to the concomitant fall-off in tension. For example, the presence of a substantial internal load within skinned fibers, which has been suggested previously (24), might result in progressively decreasing V_{max} values as tension declined. Possible interactions between tension changes and EDTA treatment to alter V_{max} have been investigated for the fiber segments that were extracted for 30–120 min at 30°C. Under these conditions, EDTA treatment resulted in decreases in tension to $46 \pm 10\%$ control values and in V_{max} to a value that was $55 \pm 9\%$ of control (Fig. 10). Addition of TnC to the treated segments resulted in an increase in tension from $46 \pm 10\%$ to $80 \pm 12\%$ of control. The mean value of V_{max} , on the other hand, remained virtually unchanged, though in individual instances, small increases in

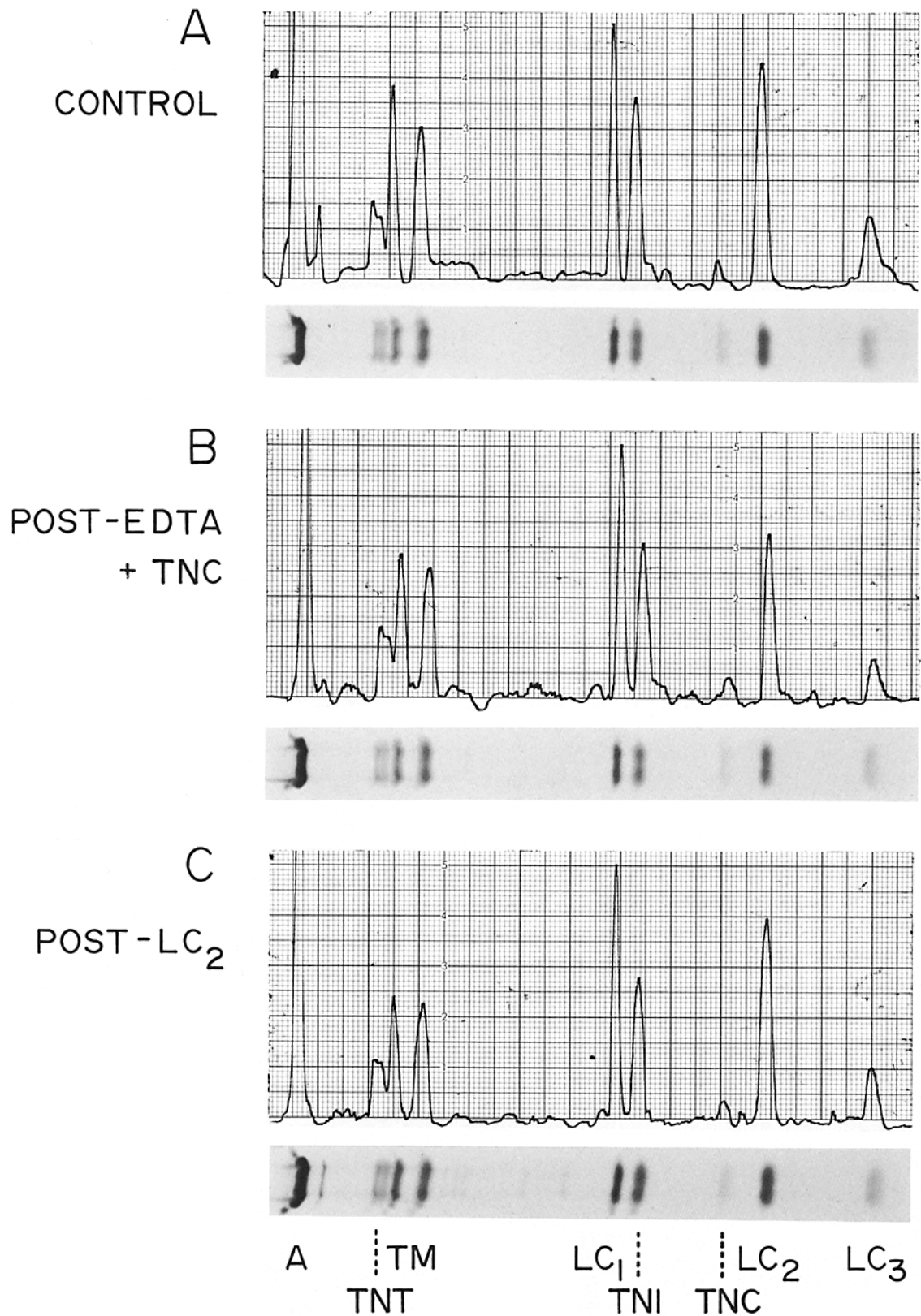


FIGURE 7 SDS polyacrylamide gels and corresponding densitometric scans of segments of the same fiber at different stages of the LC₂ extraction protocol at 30°C. (a) An untreated control segment, $LC_2/(LC_1 + LC_3) = 0.83$. (b) A segment following LC₂ extraction and TnC readdition, $LC_2/(LC_1 + LC_3) = 0.61$. (c) A segment following LC₂ readdition, $LC_2/(LC_1 + LC_3) = 0.89$. The abbreviations used are listed in the legend of Fig. 2.

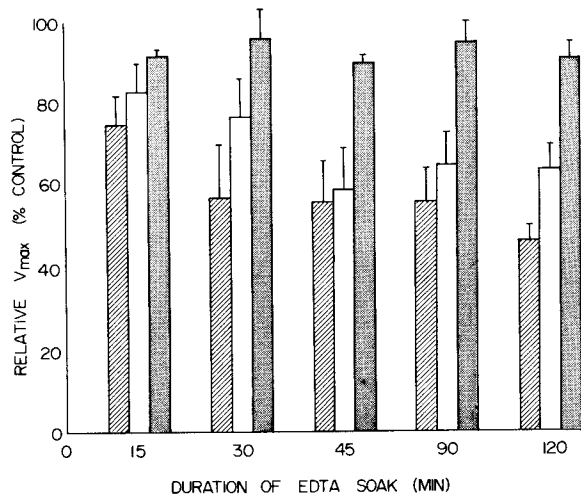


FIGURE 8 Relative V_{max} vs. the duration of EDTA soak at 30°C. At each time point, V_{max} was measured just following the EDTA soak (diagonal cross-marking), and following the TnC (clear bars) and LC₂ (stippled bars) recombinations. V_{max} was in each case expressed as a percent of the V_{max} measured in the same fiber prior to treatment (i.e., control) and these values are reported here as the mean ± 1 SD ($n = 4-6$ in each instance).

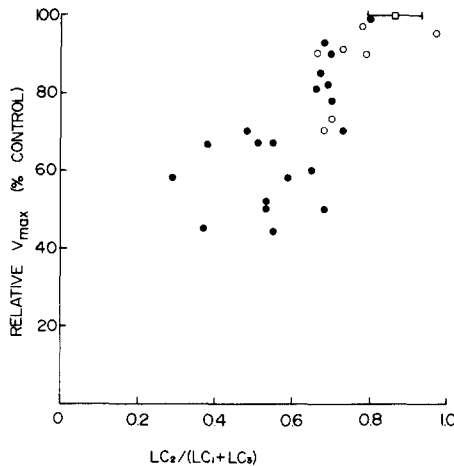


FIGURE 9 Plot of relative V_{max} (percent control) as a function of the LC₂ content measured in the fiber segment. The following symbols were used: (\square) measurements obtained in control segments (i.e., prior to EDTA treatment); (\bullet) measurements obtained following EDTA treatment for various times at either 25°C or 30°C; (\circ) measurements obtained following LC₂ readdition to EDTA extracted segments.

V_{max} of $<10\%$ were frequently observed. Subsequent addition of LC₂ to these segments resulted in a small, though significant ($P < 0.01$; paired Student's t test), additional increase in the mean developed tension, to $91 \pm 13\%$ of the control values; however, V_{max} was found to increase substantially, to $92 \pm 8\%$ of control. For these extraction conditions, then, the observed decreases in Ca-activated tension appear to be due mainly to the loss of TnC, with smaller additional decreases due to the partial extraction of LC₂, while alterations in V_{max} resulted from reductions in the LC₂ content of the segments.

For further investigation of the relationships between the mechanical properties of the fiber segments and protein subunit composition, the order of TnC and LC₂ recombinations was reversed in several cases. Results from one fiber segment are shown in Fig. 11. In this particular case, the decline in V_{max}

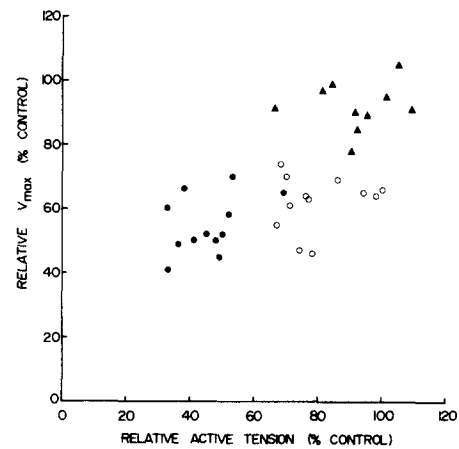


FIGURE 10 Relative V_{max} plotted vs. relative tension measured in the same segments following EDTA extraction at 30°C (\bullet), TnC recombination (\circ), and LC₂ recombination (\blacktriangle). V_{max} and tension are each expressed as a percent of the respective control values measured prior to EDTA treatment.

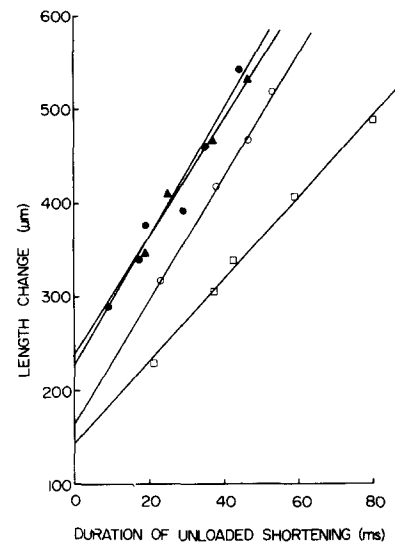


FIGURE 11 Experiment to determine the effect of a reversal in the order of readdition of LC₂ and TnC to an EDTA-extracted fiber segment. V_{max} was measured prior to EDTA treatment (\bullet) and, in order, following EDTA treatment (\square), LC₂ readdition (\circ) and TnC readdition (\blacktriangle). V_{max} prior to treatment was 1.99 ML/s; following EDTA, 1.30 ML/s; following LC₂, 1.96 ML/s; following TnC, 1.90 ML/s. Tension prior to treatment was 79.5 mg wt.; following EDTA, 35.2 mg wt.; following LC₂, 39.8 mg wt.; and following TnC, 78.8 mg wt. ML = 3.84 mm; sarcomere length = 2.73 μ m. Fiber #11112.

following EDTA extractions was nearly completely reversed by readdition of LC₂, while at the same time active tension increased from 44% of control immediately following LC₂ extraction to 53% of control following LC₂ readdition. Subsequent addition of TnC to the fiber segment had little further effect on V_{max} but resulted in virtually complete recovery of tension. Qualitatively similar results were obtained in three additional fiber segments.

Effect of LC₁ Addition on the Mechanical Properties of EDTA-treated Segments

Recent work (27) has shown that, at 37°C, solutions containing 10 mM EDTA will partially extract the alkali light chains

from skeletal muscle myosin. Thus, it seemed possible that EDTA extractions done at 30°C may remove small amounts of LC₁ and/or LC₃. Since LC₁ is present in the LC₂ addback solution (Fig. 4*b*), it is conceivable that the reversible decrease in V_{max} seen following EDTA treatment may be related, at least in part, to the partial loss of alkali LCs. To test this possibility, several fiber segments, following the EDTA treatment at 30°C and the subsequent TnC recombination, were bathed in a relaxing solution containing purified LC₁ (Fig. 4*c*) prior to the LC₂ recombination. Following this treatment, no uptake of LC₁ into the fiber segments was apparent from the gels of the segments, and there was no effect on either V_{max} or isometric tension. Upon bathing the segment in relaxing solution containing LC₂, V_{max} was restored to approximately the control value. This result indicated that if some portion of the alkali light chains was lost from the segments during the EDTA treatment, the amount lost was not sufficient to affect the mechanical properties of the segments.

(Note: The ordinal intercept of the straight line fitted to the slack test data is a measure of the extension of series elastic elements during tension development [7]. Thus, in Fig. 11, the data obtained prior to EDTA treatment and that obtained following TnC readdition yielded greater intercepts than the data obtained following EDTA treatment and LC₂ readdition. These differences are due to the greater tensions developed by the segment in the former two cases. This same argument applies to the observed difference in ordinal intercepts in Fig. 6.)

Addition of LC₂ and TnC to Untreated Control Fibers

In several instances, untreated fibers were bathed in relaxing solution containing either TnC or LC₂. This was done in order to determine whether the mechanical properties of this particular skinned fiber preparation were somehow limited by an incomplete complement of either of these protein subunits. Diminished amounts of these subunits could occur due to losses during storage in the glycerol-relax solution. Also, the relative amounts of the subunits present might vary between animals. In five fiber segments from two different rabbits, V_{max} following the TnC soak was 101 ± 6% of the pre-soak value, while active tension was 100 ± 5% of control. In an additional five fiber segments, the LC₂ soak resulted in tension and V_{max} values that were 102 ± 5% and 102 ± 3%, respectively, of the control values. Thus, in no case was a significant difference found upon addition of LC₂ and TnC to segments that had not been previously extracted with EDTA.

Effect of Trifluoperazine upon Mechanical Properties of EDTA-treated Fiber Segments

In light of the recent report by Guerriero et al. (5) that myosin LC kinase appears to be bound within the I-bands of myofibrils isolated from skeletal muscle, it was conceivable that some part of the observed mechanical changes following the LC₂ extraction and readdition procedures were due to changes in myosin LC kinase activity, and hence in the degree of LC₂ phosphorylation, in these fiber segments. As a test of this idea, a specific kinase inhibitor, trifluoperazine (TFP; Smith-Kline and French, Philadelphia, PA), was applied to the fiber segments in concentrations of up to 50 μM (10). TFP had no effect upon either tension or V_{max} in the EDTA-treated segments during maximal Ca²⁺-activation or in the same segments following readdition of LC₂. This result indicates that

the mechanical effects of LC₂ extraction and subsequent readdition of LC₂ are unlikely to be due to differences in the levels of LC₂ phosphorylation.

DISCUSSION

The main results of this study indicate clearly that the V_{max} of maximally Ca-activated skinned skeletal muscle fibers decreases as the LC₂ content of the fibers is reduced by extraction with EDTA. This effect of LC₂ extraction can be reversed by readdition of LC₂ to the fiber segments (18). The procedure used to remove LC₂ also resulted in the dissociation of a significant amount of TnC from the segments, as reported previously (18), which mechanically was reflected in the much lower Ca-activated tension developed by the EDTA-treated fiber segments. Readdition of TnC to the segments resulted in the recovery of most but usually not all of the control tension. Subsequent recombination of LC₂ into these fibers led to an additional smaller recovery of tension. A conclusion to be drawn from these results is that myosin LC₂ in skeletal muscle is involved in the interaction between actin and myosin, and appears specifically to have roles in determining the kinetics and, to a lesser degree, the extent of this interaction.

Biochemical measurements have shown that the ATPase activity of myosin and regulated actin in the presence 10⁻⁵M Ca²⁺ was reduced by about one-half following the extraction of ~1 mol LC₂/mol myosin (21). These results are consistent with the effects on V_{max} following of LC₂ removal from skinned skeletal muscle fibers reported here. The present study extends the earlier biochemical findings by indicating that the modulation of the actomyosin ATPase by LC₂ occurs in a preparation in which the thick and thin filament lattices were maintained intact and the cross-bridges were under load during mechanical measurements, conditions that are difficult to simulate in solutions of actin and myosin.

The effect of LC₂ removal upon V_{max} appears to be unrelated to removal of alkali light chains by the EDTA treatment. Wikman-Coffelt et al. (27) have found that, at 37°C, 5–10% of the alkali light chains are removed following a 10-min incubation in a solution containing 10 mM EDTA. Wagner and Giniger (26) reported significant reductions in the actin-activated ATPase activities of myosins from which most of the alkali LCs had been removed, though it is unclear whether such reductions were the result of LC removal per se or instability of the alkali LC-deficient myosin. In the present study, addition of LC₁ to TnC-treated, EDTA-extracted segments resulted in no significant changes in V_{max} or tension. Recovery of V_{max} in these segments occurred only after readdition of LC₂. Thus, if alkali light chains were extracted in the EDTA solutions, the amounts removed were apparently insufficient to affect mechanical properties.

Extraction conditions similar to those of the present study have been used previously to remove so-called regulatory LCs from scallop myosin (9, 23). At 0°C, incubations in solutions containing 10 mM EDTA resulted in the loss of ~50% of the normal 2 mol regulatory LC/mol myosin (9). At the same time, the Ca regulatory mechanism for the interaction of scallop myosin with actin was lost. At higher temperatures, 25°C or 35°C depending on the species of scallop, treatment with 10 mM EDTA led to the complete dissociation of the regulatory LCs (3). The effect of the removal of these LCs on the actomyosin ATPase in the presence of Ca²⁺ were qualitatively similar to the effects on V_{max} reported in the present study. Removal of the regulatory LC resulted in a decrease in the ATPase to ~20% of the value obtained for intact myosin.

Dependence of V_{max} on LC_2 Content

The correlation between relative V_{max} and LC_2 content of the fiber segments is striking (Fig. 9). Tension also decreased with EDTA treatment (Fig. 5), due mostly to the loss of TnC and in part to the removal of LC_2 . In some cases, tension in the EDTA-treated segments recovered to near the control value when TnC was added, with little or no change in V_{max} . Thus, there is no appreciable dependence of V_{max} on isometric tension, at least following extractions at 30°C, and decreases in V_{max} following EDTA treatment therefore appear to be causally related to the reduction in the LC_2 content of the segments.

EDTA extractions at 37°C led to losses in both tension and V_{max} that were largely irreversible upon the readdition of TnC and/or LC_2 . This high temperature was required in order to lower the $LC_2/(LC_1 + LC_3)$ ratio below about 0.3. The irreversibility of the losses in tension and V_{max} may indicate that components other than TnC and LC_2 were removed during the EDTA treatment at 37°C, or that alterations in the structure of proteins such as myosin or those in the Z-disks may have occurred, either because of the extraction conditions or the activation of proteases. None of these possibilities is immediately apparent from the SDS gels of these fiber segments. Conclusions regarding the possible dependence of V_{max} on tension, as opposed to reductions in LC_2 content, are not possible following high temperature extractions due to an inability to regain physiological values for tension and V_{max} .

It should be noted that because the ratio $LC_2/(LC_1 + LC_3)$ references the LC_2 content of the fiber segments to the alkali LCs present, the values of this ratio in EDTA-treated segments probably overestimate the amounts of LC_2 present. The extraction procedure likely removes small amounts of the alkali LCs (27), which will tend to increase the calculated ratio. On the other hand, expressions of the absolute amounts of LC_2 present, as a function of the total protein, are not presently feasible due to the small sample size, i.e., $<0.1 \mu\text{g}$ total protein. A good indication that substantial amounts of alkali light chains were not removed by this procedure is that the ratio of the areas under the LC_1 and actin peaks changed little in most segments relative to control following the EDTA soak.

The role of LC_2 in Skeletal Muscle Contraction

The effect of LC_2 removal to alter V_{max} indicates that this LC may modulate the interaction kinetics of actin and myosin. Biochemical evidence consistent with such a suggestion has only recently begun to emerge. Lehman (14) has reported that carefully prepared myofibrils, from which troponin and tropomyosin had been removed with low salt solutions, have an ATPase activity that is Ca^{2+} sensitive. More recently, Pemrick (21) has reported that, in solutions of myosin and regulated actin, removal of ~50% of LC_2 with DTNB (i.e., 5,5'-dithiobis(2-nitrobenzoic acid)) resulted in an ~50% decrease in the actin-activated MgATPase measured in the presence of $10^{-5} M Ca^{2+}$ and physiological Mg-ATP concentrations. These results are in good agreement with the findings of the present study. One possible hypothesis that would explain these results is that Ca^{2+} binding to LC_2 (1), which occurs in the physiological range of Ca^{2+} concentrations (2), alters the ATPase activity.

LC_2 appears as well to play a role in determining the extent of interaction between myosin and actin, a conclusion which is based upon the small though significant increase in tension development that is observed following readdition of LC_2 to the EDTA-extracted, TnC-treated fiber segments. Pemrick (21) found that, in solutions of myosin and regulated actin present in ratios of 1.0:2.5 or less, the Ca^{2+} sensitivity of the MgATPase

was decreased when 50% of LC_2 was removed with EDTA, i.e., the Ca^{2+} for half-maximal activation of the ATPase was shifted from ~0.09 μM for native myosin to 0.2–0.4 μM for LC_2 -deficient myosin. Pemrick's interpretation of these results was that in the presence of LC_2 the interaction of myosin with the thin filament is enhanced and the Ca^{2+} concentration necessary to activate the thin filament is decreased. Further work using the skinned fiber preparation, in which V_{max} and tension could be measured as functions of $[Ca^{2+}]$ preceding and following LC_2 extraction, would help to determine whether such a mechanism is operable under physiological conditions.

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REFERENCES

1. Bagshaw, C. R. 1977. On the location of the divalent metal binding sites and the light chain subunits of vertebrate myosin. *Biochemistry*, 16:59–67.
2. Bagshaw, C. R., and G. H. Reed. 1977. The significance of the slow dissociation of divalent metal ions from myosin "regulatory" light chains. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 81:386–90.
3. Chantler, P. D., and A. G. Szent-Györgyi. 1980. Regulatory light-chains and scallop myosin: full dissociation, reversibility and cooperative effects. *J. Mol. Biol.* 138:473–492.
4. Greaser, M. L., and J. Gergely. 1971. Reconstitution of troponin activity from three troponin components. *J. Biol. Chem.* 246:4226–4233.
5. Guerrero, V., Jr., D. R. Rowley, and A. R. Means. 1981. Production and characterization of an antibody to myosin light chain kinase and intracellular localization of the enzyme. *Cell*. 27:449–458.
6. Julian, F. J. 1971. The effect of calcium on the force-velocity relation of briefly glycerinated frog muscle fibers. *J. Physiol. (Lond.)*, 218:117–145.
7. Julian, F. J., and R. L. Moss. 1981. Effects of calcium and ionic strength on shortening velocity and tension development in frog skinned muscle fibres. *J. Physiol. (Lond.)*, 311:179–199.
8. Julian, F. J., R. L. Moss, and G. S. Waller. 1981. Mechanical properties and myosin light chain composition of skinned muscle fibers from adult and new-born rabbits. *J. Physiol. (Lond.)*, 311:201–218.
9. Kendrick-Jones, J., A. G. Szent-Györgyi, and W. Lehman. 1970. Regulation in molluscan muscles. *J. Mol. Biol.* 54:313–326.
10. Kerrick, W. G. L., P. E. Hoar, P. S. Cassidy, L. Bolles, and D. A. Malencik. 1981. Calcium regulatory mechanisms. Functional classification using skinned fibers. *J. Gen. Physiol.* 77:177–190.
11. Kominz, D. R., W. R. Carroll, E. N. Smith, and E. Mitchell. 1959. A subunit of myosin. *Arch. Biochem. Biophys.* 79:191–199.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage 14. *Nature (Lond.)*, 227:680–685.
13. Leger, J. J., and F. Marotte. 1975. The effects of concentrated salt solutions on the structure and the enzymatic activity of myosin molecules from skeletal and cardiac muscles. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 52:17–21.
14. Lehman, W. 1978. Thick-filament-linked calcium regulation in vertebrate striated muscle. *Nature (Lond.)*, 274:80–81.
15. Lowey, S., and D. Risby. 1971. Light chains from fast and slow muscle myosins. *Nature (Lond.)* 234:81–85.
16. Lowey, S., and A. G. Weeds. 1971. Substructure of the myosin molecule. II. The light chains of myosin. *J. Mol. Biol.* 61:701–725.
17. Moss, R. L. 1979. Sarcomere length-tension relations of frog skinned muscle fibres during calcium activation at short lengths. *J. Physiol. (Lond.)*, 292:177–192.
18. Moss, R. L., G. G. Giulian, and M. L. Greaser. 1982. Physiological effects accompanying the removal of myosin LC_2 from skinned skeletal muscle fibers. *J. Biol. Chem.* 257:8588–8591.
19. Moss, R. L., G. G. Giulian, and M. L. Greaser. 1982. The mechanical effects of myosin LC_2 removal from rabbit skinned skeletal muscle fibers. *Biophys. J.* 37(2, Pt. 2):365A. (Abstr.)
20. Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105:361–363.
21. Pemrick, S. M. 1977. Comparison of the calcium sensitivity of actomyosin from native and LC_2 -deficient myosin. *Biochemistry*, 16:4047–4053.
22. Sivaramakrishnan, M., and M. Burke. 1982. The free heavy chain of vertebrate skeletal myosin subfragment 1 shows full enzymatic activity. *J. Biol. Chem.* 257:1102–1105.
23. Szent-Györgyi, A. G., E. M. Szentkiralyi, and J. Kendrick-Jones. 1973. The light chains of scallop myosin as regulatory subunits. *J. Mol. Biol.* 74:179–203.
24. Thames, M. D., L. E. Teichholz, and R. J. Podolsky. 1974. Ionic strength and the contraction kinetics of skinned muscle fibers. *J. Gen. Physiol.* 63:509–530.
25. Thomas, J. O. 1978. Determination of the subunit structures of proteins. *Techniques in Protein and Enzyme Biochem.* B106:1–22.
26. Wagner, P. D., and E. Giniger. 1981. Hydrolysis of ATP and reversible binding to F-actin by myosin heavy chains free of all light chains. *Nature (Lond.)*, 292:560–562.
27. Wikman-Coffelt, J., S. Srivastava, and D. T. Mason. 1979. Dissociation and reassociation of rabbit skeletal muscle myosin. *Biochimie*, 61:1309–1314.