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Quantitative electrophoretic analysis of myosin heavy chains in single muscle fibers

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Tikunov, Boris A., H. Lee Sweeney, and Lawrence C. Rome. Quantitative electrophoretic analysis of myosin heavy chains in single muscle fibers. *J Appl Physiol* 90: 1927–1935, 2001.—To better understand the molecular basis of the large variation in mechanical properties of different fiber types, there has been an intense effort to relate the mechanical and energetic properties measured in skinned single fibers to those of their constituent cross bridges. There is a significant technical obstacle, however, in estimating the number of cross bridges in a single fiber. In this study, we have developed a procedure for extraction and quantification of myosin heavy chains (MHCs) that permits the routine and direct measurement of the myosin content in single muscle fibers. To validate this method, we also compared MHC concentration measured in single fibers with the MHC concentration in whole fast-twitch (psoas and gracilis) and slow-twitch (soleus) muscles of rabbit. We found that the MHC concentration in intact psoas (184 μM) was larger than that in soleus (144 μM), as would be expected from their differing mitochondrial content and volume of myofibrils. We obtained excellent agreement between MHC concentration measured at the single fiber level with that measured at the whole muscle level. This not only verifies the efficacy of our procedure but also shows that the difference in concentration at the whole muscle level simply reflects the concentration differences in the constituent fiber types. This new procedure should be of considerable help in future attempts to determine kinetic differences in cross bridges from different fiber types.

single fibers; skinned fibers; cross-bridge kinetics

MUSCLES ARE ADAPTED TO PERFORM a wide variety of different motor activities and accordingly show a wide range of mechanical properties. There is considerable evidence that most of the variation in mechanical properties of muscles ultimately reflect differences in the kinetics and other properties of the myosin cross bridges (5–7, 20, 24–26). Hence, there has been an intense effort to relate the mechanical and energetic properties of skinned single fibers (fiber force, stiffness, and ATPase) to properties of the cross bridges [i.e., force per attached cross bridge, ATPase per myosin head, detachment rate constant (g), attachment rate constant (f)] within the fiber (6, 7, 20, 24–26). For instance, with the use of this approach, it was recently found that, despite a normal force per attached cross bridge, the superfast-twitch toadfish swimbladder muscle generates very low forces because of a low number of attached cross bridges [equal to $f/(f + g)$], a result of having an exceptionally fast g but slow f (22).

Cross-bridge-level analysis cannot be performed without an accurate estimate of the total number of myosin heads contained within the fibers on which the mechanics and energetics are measured. For example, the value of g is determined as the quotient of ATP turnover rate per myosin head (ATP utilization of the fiber/the number of myosin heads in the fiber) and the proportion of attached cross bridges (determined by stiffness). Likewise, the force per attached cross bridge is the quotient of fiber force and the number of attached cross bridges per half sarcomere [the number of myosin heads in the fiber × proportion of attached cross bridges/number of half sarcomeres in the fiber (determined by microscopy)].

However, there are significant technical obstacles in estimating the number of cross bridges in a single skinned muscle fiber. The number of myosin heads in a fiber is usually calculated as the product of *1*) the fiber volume and *2*) the myosin concentration in the fiber type in question. Optical measurements of skinned muscle fiber volume are prone to considerable errors. Furthermore, it is sometimes necessary to convert the skinned fiber volume to "intact" fiber volume because myosin concentration is most accurately measured biochemically in large bundles of intact cells. This conversion is complicated by the swelling that takes place in the fiber on skinning, which varies with muscle type and bathing solution (2, 3). Thus, in the best case, estimating the number of cross bridges in a skinned fiber is subject to considerable error.

Even if one knew the fiber volume accurately, the appropriate information needed to calculate the number of myosin heads usually is not available. Myosin concentration has been measured in only a very few fiber types; hence, one usually must assume a value of myosin concentration for the fiber type of interest. This

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can be problematic because early electron microscopic studies have demonstrated that myofibrillar volume can differ greatly between different types of muscle fibers (1, 8, 9, 17). Complementary biochemical measurements have demonstrated significant differences in myosin concentration between fast-twitch and slow-twitch muscles and even between white and red portions of the same muscle (10, 13). These differences can be very large. For instance, myosin heavy chain (MHC) concentration in the toadfish varied from 67 μM in the superfast-twitch swimbladder muscle to 167 μM in the fast-twitch white muscle (22).

To avoid the errors and assumptions mentioned above, a method is needed that provides an accurate and direct measure of the amount of myosin contained within single fibers used for mechanic and energetic measurements. Because no method has previously been described, we have developed such a procedure in this study. This procedure differs from routine fiber solubilization and electrophoretic techniques used to determine fiber-type because it permits *1*) the complete extraction of myosin from single fibers and *2*) the direct quantification of myosin. Both are requirements for performing the cross-bridge analysis described above. We have also performed several experiments to validate this method, including comparing MHC concentrations measured in single fibers with the MHC concentration in whole fast-twitch (psoas and gracilis) and slow-twitch (soleus) muscles of rabbit.

METHODS

Our goal was to develop a procedure by which the myosin could be completely extracted from single fibers and quantitatively measured. In the first section of METHODS, we describe this procedure. To verify this procedure, we developed a method to check the completeness of the extraction. We further verified the procedure by comparing the MHC concentration of single fibers with that of whole muscle, where it could be most accurately measured. Hence, in the later sections of METHODS, we describe the techniques used to validate our myosin quantification procedure.

Determination of Myosin Content in Single Fibers

Isolation of single muscle fibers. Psoas and soleus muscles were dissected from adult male New Zealand White rabbits (2.0–2.2 kg). Under a dissecting microscope, muscle bundles were pinned to a Sylgard-filled petri dish containing cold depolarizing solution (58 mM TES, 7.8 mM $MgCl_2$, 50 mM EGTA, 1.0 mM KH_2PO_4, and 6.2 mM Na_2ATP, pH 7.1). Dissected fibers were transferred to another Sylgard-filled petri dish, stretched passively, and dried for ~1 h at room temperature. Each fiber was measured and then cut in half. One-half of each fiber (3- to 6-mm long) was used for preparation of the samples for SDS-PAGE and analyzed for MHC content (see below). One group of the remaining fiber halves was used for dry weight determination, and the other group was assayed for total protein content (TPC; see below).

Preparation of samples for SDS-PAGE. One-half of each fiber was immersed into 3 μl of skinning solution (0.1 M KCl, 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, 10 mM EDTA, 10 mM $NaPP_i$, 4 mM 2-mercaptoethanol, pH 6.5, and 5% Triton X-100) in the bottom of a microtube. The tubes were then transferred onto a holder attached to a Vortex shaker and mixed for 1 h at room temperature. Seven microliters of SDS sample buffer [hereafter, referred to as single-fiber (SF) extraction buffer] in which the concentration of glycerol was doubled [62.5 mM Tris·HCl, pH 6.8, 40% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.5% (wt/vol) bromophenol blue] were added to each sample. The samples were then shaken by hand until visible separation between the two solutions disappeared and then were sonicated with a Bransonic B-12 ultrasonic cleaner (35 W at 36–46 kHz) for 1 h at 40–45°C. Samples were vortexed again for 2 h and incubated for 3 h at room temperature. The sonication and vortexing steps were then repeated. Samples were left overnight at 4°C; the next day the vortex-incubation steps were repeated two more times. These samples, containing a high concentration of myosin (hereafter referred to as concentrated samples), were then used for Coomassie-stained SDS-PAGE gels. Two microliters of each concentrated sample was diluted fourfold with SF extraction buffer to reduce the protein concentration (hereafter referred to as diluted samples) and were then used for silver-stained SDS-PAGE gels. All samples were boiled for 4–5 min before electrophoresis.

SDS-PAGE. SDS-PAGE for MHCs was performed on an SE-600 vertical slab gel unit (Hoefer Scientific Instrument) using 18 × 16-cm glass plates and 0.75-mm-thick spacers. It was performed according to the method of Talmadge and Roy (27), except that the glycerol content in the separating gel was increased from 33.7 to 36.7% and running time was extended to 42 h at 6°C (28). Concentrated and diluted samples for each individual fiber (see above) were run simultaneously on two parallel SDS-PAGE gels. Three microliters of each diluted sample (0.15–0.9 μg of total protein) were loaded per lane on the gel, which was subsequently stained with the Bio-Rad silver stain plus kit. Eight microliters of each concentrated sample (1.0–5.0 μg of total protein) were loaded per lane on a parallel gel, which was subsequently stained with Coomassie brilliant blue R-250.

Quantification of MHCs. MHCs were subsequently eluted from the bands on Coomassie-stained gels and used for direct measurement of MHC content. For that purpose, we built a miniature electroelution device with two coaxial platinum electrodes. The cylindrical cathode (5-mm inner diameter and 5-mm height) was built into a water-jacketed Plexiglas assembly, and 0.5-mm-diameter platinum wire was attached to the cover of the chamber and served as the anode. Sections containing MHC band(s) (~10 × 5 mm) were excised from the gels and destained. The gel sections were subsequently washed with 20 ml of elution buffer (25 mM Tris and 192 mM glycine, pH 8.3), formed into a ring with forceps, and placed into the chamber along the inner surface of the cathode. The chamber was filled with 60 μl of elution buffer, and MHCs were eluted for 30 min at 5–7 mA and 6°C. The completeness of protein elution was checked in two ways in control experiments. First, after elution of the protein, the gel section was silver stained and no protein staining was observed. Second, although spectrophotometric measurements of the gel sections at 280 nm revealed considerable absorption before elution, little absorption was observed following elution (i.e., the remaining absorption was <3% of the original value but could not be determined more precisely because of limitations of such optical measurements).

MHC eluate (50 μl) was subsequently diluted with 150 μl of 0.5 M Tris·HCl (pH 6.8) and assayed for protein content using the Bio-Rad protein microassay procedure; 100 μl of each sample were mixed with 25 μl of Bradford's reagent (Bio-Rad protein microassay reagent) and incubated for 10 min at room temperature. The absorption at 595 nm was then measured in a 70-μl cuvette with a path length of 1 cm.

We calibrated the protein assay using myosin purified from rabbit psoas according to the method of Perry (18). Additional purification steps included precipitation of myosin with ammonium sulfate at 50% saturation, dialysis against 0.5 M KCl and 5 mM EDTA, pH 7.0, and centrifugation (100 000 *g*) for 1 h at 4°C. The ratio of the absorbance at 280 to 260 nm was 1.69, which is a characteristic of highly purified myosin (21). Indeed, electrophoretic analysis of the protein revealed that it contained 93% myosin and 7% other proteins; hence, it could serve as an excellent standard for our protein assays. To determine the concentration of this myosin protein standard, we lyophilized a portion of this solution and weighed the remaining solid. The weight of the salts was then subtracted, giving the protein content. Our standard curve agreed within 4% of that determined using commercially available (Sigma Chemical) lyophilized myosin from rabbit back muscle.

Validating the Technique: Immunoassay for Single Muscle Fibers

The completeness of myosin extraction was determined in several fibers from psoas and soleus muscle. Each fiber was divided into three fragments, each 4- to 6-mm long. The first fragment was incubated for 1 h at room temperature with 3 μl of skinning solution and pinned by one end to the bottom of a 2-cm-diameter well formed in the Sylgard-coated petri dish. The fragment was washed four times with a 50 mM Tris·HCl (pH 7.0) buffer and incubated for 1 h with 1:4 dilution of A4.1025 (IgG2a form) antibody against all types of MHCs. The supernatant was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University, School of Medicine (Baltimore, MD). After two to three washes with Tris·HCl buffer, the fiber fragment was incubated for 40 min with 1:50 diluted (final concentration of 0.02 mg/ml) fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The fiber was washed again with Tris·HCl buffer, transferred onto a microscope slide, and observed under a Zeiss Axioscope fluorescent microscope (excitation at 450–480 nm). Photographs were taken with a Kodak DC 120 digital camera and processed in Adobe Photoshop. The skinning solution was subsequently diluted with 7 μl of SF extraction buffer, boiled, and analyzed for MHC content on SDS-PAGE.

The second fragment was transferred to a microtube containing 10 μl of SF extraction buffer and underwent one sonication-vortex-incubation step (6 h) as described above. The fragment was then transferred to the Sylgard-formed well, pinned, incubated with antibodies, and photographed in the same manner as described for the first fragment above. The fragment was subsequently returned to the microtube, it underwent one more sonication-vortex-incubation step (12 h), and then it was analyzed under the microscope again. The third fragment was treated in the same way as the second fragment, except that it underwent the sonication-vortex-incubation step four times before it was analyzed for the first time (24 h). It was analyzed a second time after an additional 24-h extraction (i.e., total of 48 h). After each sonication-vortex-incubation step performed on the second and third fragments, 2 μl of the SF extraction buffer were taken and analyzed for MHC concentration as described above for the first fragment.

Validating the Technique: Comparing MHC Concentration of Single Fibers With That of Whole Muscle

Preparation of muscle extracts from whole muscle. To minimize losses during biochemical extraction of myosin, all the procedures (i.e., repeated steps of homogenization, extraction, and centrifugation) were performed in one tube. Large fresh muscle bundles (0.5–1.0 g) were crushed with scissors on ice and homogenized for 2 min with a Biospec Products "tissue tearer" homogenizer in five volumes of myosin extraction buffer (0.5 M KCl, 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, 10 mM EDTA, 10 mM $NaPP_i$, 4 mM 2-mercaptoethanol, 2 mM sodium azide, pH 6.5, plus proteolytic enzyme inhibitors: 1 mg/ml soybean trypsin inhibitor and 1 μg/ml leupeptin and pepstatin A). The homogenate was centrifuged for 30 min at 10,000 *g* and 6°C. The supernatant was retained, and the pellet was resuspended in the same amount of myosin extraction buffer and rehomogenized in the same centrifuge tube with a Teflon pestle homogenizer (3 × 20 s at 3,000 rpm). The homogenate was recentrifuged, and the whole procedure was repeated at least an additional six times. After the last centrifugation, the pellet was completely dissolved by sonication in 1 M NaOH for 1 h at 50–60°C. The combined extract (i.e., all individual extracts were pooled together) was then assayed for total protein content and used for electrophoretic analysis. To reduce KCl concentration, 1 ml of each extract was diluted five to eight times with a distilled water. Each sample was then diluted again three to five times with SF extraction buffer to obtain the final TPC of 0.1–0.25 mg/ml, run on SDS-PAGE, and stained with Coomassie R-250. The MHC bands were eluted and analyzed for MHC content as described above (see *Quantification of MHCs* above).

Skinned fiber proxies for wet weight of intact fibers. To compare MHC concentration in single fibers with those of whole muscle, a method to accurately measure fiber volume was required. As explained above, optical measurements of fiber volume are prone to considerable error. Hence, on large muscle bundles, we empirically determined two conversion factors that related muscle volume to two parameters that could be measured accurately in single fibers: dry weight and TPC.

Muscle bundles (50–100 mg) were placed on 1 × 1-cm piece of preheated, preweighed aluminum foil, weighed, and heated for 1 h at 130–160°C. Dried muscle bundles were then wrapped quickly in aluminum foil (to minimize contribution of air humidity to muscle dry weight) and weighed again. The ratio of wet weight to dry weight was determined for each muscle and thus served as a conversion coefficient. Hence, the dry weight of single fibers could be multiplied by this coefficient to give the corresponding wet weight of the fiber in question. The equivalent intact fiber volume, in turn, was determined as the quotient of the wet weight and density (1.05 gm/cm^3).

To measure dry weight of single fibers, fiber sections were picked up with forceps by one end and dried for 3–10 min at 120–130°C. Immediately thereafter, they were weighed on a Cahn microbalance (resolution = 0.1 μg). The dry weight of each fiber was calculated as the mean of 100–150 individual measurements made over 5 min by a computer interfaced with the balance. Small fibers with a dry weight of <3 μg were excluded from the experiment.

To determine the wet weight-to-TPC conversion coefficient, another group of muscle bundles was weighed and homogenized in five volumes of myosin extraction buffer (see *Preparation of muscle extracts from whole muscle* above). After a 6-h extraction at 6°C, the homogenates were assayed

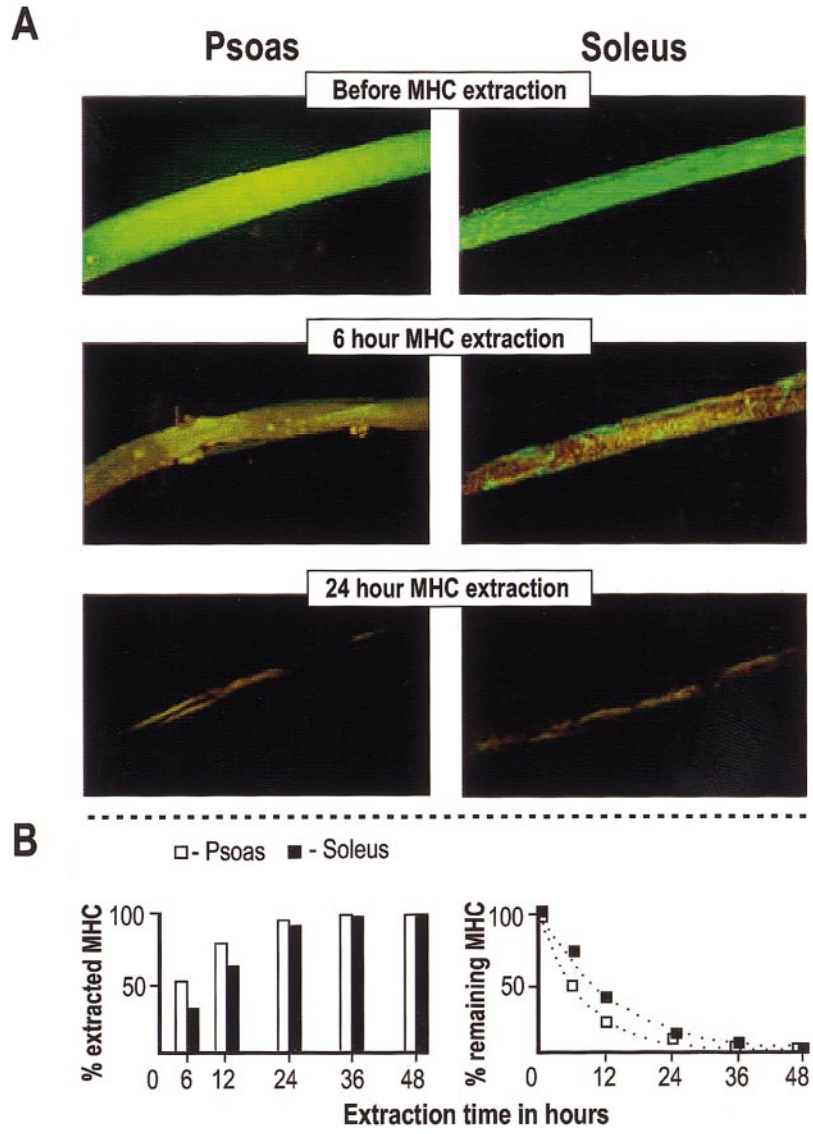

Fig. 1. *A*: immunofluorescent staining of single muscle fibers from rabbit psoas (*left*) and rabbit soleus (*right*) before and after myosin extraction. A4.1025 (IgG2a) primary antibody against all types of myosin heavy chains (MHCs) and fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG (H+L) secondary antibody were used for the immunostaining. *Top*: single fibers stained with antibody after 1-h incubation with skinning solution. *Middle* and *bottom*: fibers after 6 h of extraction (including 2 sonication-vortex-incubation steps) and after 24 h of extraction (including 4 sonication-vortex-incubation steps), respectively. Total magnification, ×83. *B*, *left*: MHC content found in the extracting solution as a function of extraction time for a single psoas fiber and a single soleus fiber. Values are normalized for the value at 48 h (i.e., complete extraction is assumed based on the finding that there is no remaining fluorescence at this time; see *A*). *Right*: myosin remaining in each fiber as a function of extraction time. Values are calculated from data set presented in *left* graph. Dotted lines represent the exponential curve fits for the psoas fiber (time constant = 7.7 h) and the soleus (time constant = 12.2 h) fiber. Together, these results suggest that there is nearly complete myosin extraction from both fast and slow fiber types using our procedure.

for TPC, by following the Bio-Rad standard protein assay procedure. The ratio between wet weight and TPC was calculated for each muscle and then used as a wet weight-to-TPC conversion coefficient. Hence, the TPC from single fibers could be multiplied by this coefficient to give the corresponding wet weight of the fiber in question.

To measure the total protein content of single fibers, the other group of fiber sections was assayed by the following procedure. Each fiber section was transferred to a microtube containing 50 μl of 1 N NaOH and dissolved by sonication for 1 h at 50–60°C. Samples were then neutralized with 50 μl of 1 N HCl, diluted with 300 μl of 0.5 M Tris·HCl (pH 6.8), and assayed for protein as described above.

Using the Bio-Rad protein reagent, we found that a given concentration of MHC gave 1.24-fold higher absorbance than the same concentration of BSA. This could be a result of higher content of basic amino acids (particularly arginine) in myosin compared with BSA. For TPC determination, we used BSA as a calibration standard because these values could be compared more easily to previous measures of muscle protein content. Importantly, the TPC values for the muscle extracts obtained with NI-protein assay (Geno Technology), in which the protein-to-protein variation in staining is reduced, gave TPC values consistent with those we obtained with the Bio-Rad method.

RESULTS

Myosin Extraction From Single Fibers

To validate our procedure for measuring myosin content, we first had to demonstrate that all the myosin could be extracted. Immunofluorescent staining of the fiber against MHCs was performed to evaluate the completeness of myosin extraction from single muscle fibers. In intact (i.e., not skinned) fibers, only a small portion from each end of the fiber was stained with antibody. Hence, to permit the antibodies to enter the cell, we skinned the fiber for 1 h with 5% Triton X-100, which enabled extensive staining along the fiber's length (Fig. 1*A, top*). Our method of myosin extraction was highly effective, as evidenced by the dramatic reduction in staining intensity (*middle* and *bottom*) of fast-twitch (*left*) and slow-twitch (*right*) fibers. After

48 h of extraction, there was no visible staining of fibers (not shown).

Further evidence for the completeness of extraction came from the increase in the MHC content found in the extracting solutions with increasing time (Fig. 1*B*, *left*). These values are consistent with the fiber myosin content exhibiting an exponential decay with a time constant of ~8.59 ± 0.47 h ($n = 6$) for the psoas fibers and 11.03 ± 0.50 h ($n = 6$) for the soleus fibers (Fig. 1*B*, *right*). At the 24-h mark, ~94 and 90% of the myosin had been extracted from the psoas and soleus fibers, respectively. Very little myosin was extracted in the subsequent 12 h and even less between the 36- and 48-h marks. Together with the immunofluorescent-staining measurements, these results suggest that there was essentially complete myosin extraction from both fast and slow fiber types using our procedure.

Repeatability of Myosin Quantification

For this procedure to be useful, the myosin extraction and myosin quantification steps must be repeatable. To assess the overall repeatability of our procedure, we performed another set of experiments in which we cut fibers in half, ran each half (1- to 7-mm long) independently through the extraction and quantification steps, and compared the measured myosin content. As can been seen in Fig. 2*A*, values of MHC content from one-half plotted against its mate fall very close to the line of unity. For fibers containing more than 0.7 μg of MHC (length ≥4.5 mm), the average deviation of individual observations compared with the mean value for each pair was only ± 2.6%. The repeatability appeared to decline as MHC content (and fiber length) was reduced (Fig. 2*B*): for fibers containing 0.5 μg (~3-mm long), the average deviation was ±4.3% and for fibers containing 0.28–0.38 μg (2- to 2.5-mm long) the average deviation was ±9.7%. For a fiber containing 0.2 μg (fiber length ≤ 1.5 mm), the deviation can be substantial.

One source of this variation is in the assay itself. Repeated protein assays with the same sample showed the following: with high MHC contents (~1 μg MHC), the assay was reproducible to ±1%; with MHC contents of 0.3–0.5 μg, the assay reproducibility was ±2%; and, with MHC contents of 0.1–0.2 μg, the assay reproducibility was ±5%. Although the fiber variation is similar to the assay variation for long fibers (i.e., with a high MHC content), the fiber variation increases much more dramatically at short fiber lengths than does the assay variation. We do not believe that the large discrepancy between the fiber variation and assay variation at short lengths reflects additional variation in MHC extraction and quantification but rather that this increased variation simply reflects inaccuracies in cutting short fibers into two pieces of precisely the same length. The repeatability measurement we employed depends on the fibers being cut exactly in half, so that each fiber half starts off containing precisely the same amount of MHC. Although one can accurately cut a 10-mm-long fiber into two pieces having lengths that do not vary by more than a few percent, as the fiber lengths become shorter, there is an increasing and unavoidable variation in length (and hence MHC content) of the two fiber "halves." Because this variation is present in the samples before the extraction even begins, it artifactually inflates the measured "fiber variation" at short lengths. Because cutting fibers in half is not part of our procedure for quantifying the fiber MHC content, we expect that the

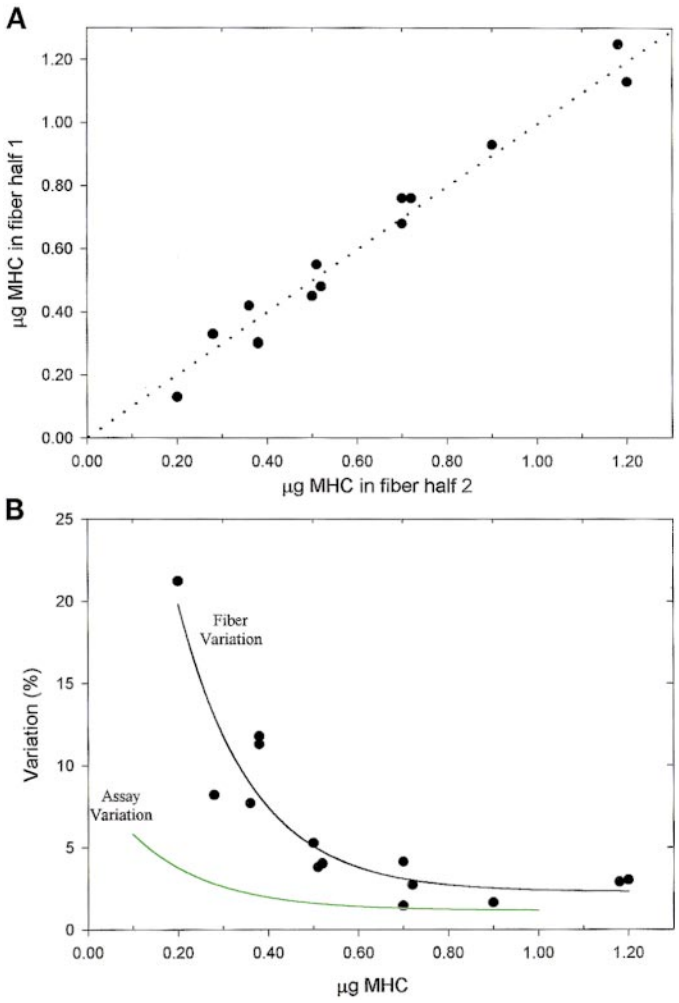

Fig. 2. *A*: myosin content of one-half of the fiber from rabbit psoas plotted against the myosin content of its mate. Fibers were stretched and cut in half, and the myosin in the two halves was independently extracted and quantified by the normal procedure outlined in the text. Line of unity (dotted) is shown for reference. All points fell close to the line of unity. Average deviation of individual observations for a given sample was determined and plotted in *B*. Average deviation was very small for fibers with high myosin contents, but the variation increased with smaller fibers (fiber variation line). The variation as a function of myosin content was well fit by an exponential plus linear function ($2.29\% + 59.76\% \times e^{-6.13 \times \text{MHC content}}$; *top line*). Part of this variation was due to variation in the assay itself (assay variation), which was also well fit by an exponential plus linear function ($1.16\% + 8.31\% \times e^{-5.77 \times \text{MHC content}}$; *bottom line*; individual data points have been omitted for clarity). Most of the remainder of the variation is likely due to imprecision in cutting short fibers exactly in half. Because fibers are not cut during the normal quantification procedure, errors involved in normal quantification are likely similar to those of the assay variation.

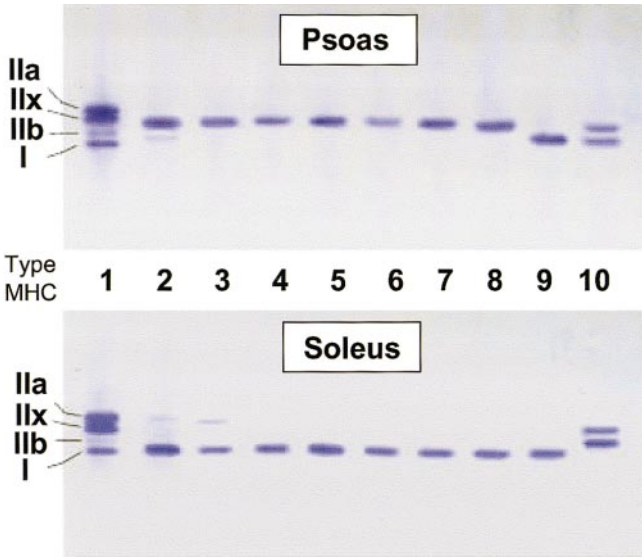

Fig. 3. High-molecular-weight portions of Coomassie-stained SDS gels for the MHCs from rat diaphragm (*lane 1* in each gel), whole rabbit psoas and soleus muscles (*lane 2* in *top* and *bottom* gels, respectively), and single muscle fibers from rabbit psoas and soleus (*lanes 3–9* in *top* and *bottom* gels, respectively). *Lane 10* in each gel represents MHC standard from rabbit muscle (Sigma Chemical).

variation of our MHC quantification to be lower than the fiber variation values shown in Fig. 2*B* (*top line*) and close to the much lower assay variation values (Fig. 2*B*, *bottom green line*). Support for this conclusion comes from measurements of dry weight of fiber "halves." In short fibers, there is substantial variation of approximately the same magnitude as the difference between the fiber variation and assay variation values (Fig. 2*B*). Hence, we conclude that, for fibers with a myosin content of at least 0.28 μg (≥2 mm long), our procedure for myosin extraction and quantification is quite reproducible.

Comparing Myosin Concentration in Whole Muscle and Skinned Single Fibers

To further assess the accuracy of our procedure, we compared the concentration of myosin determined from whole muscle with that determined from single fibers. Because myosin concentration varies with fiber type, we chose muscles that were nearly pure in fiber type. Whole psoas muscle extracts revealed two MHC bands on the SDS gels (Fig. 3, *lane 2*). On the basis of its electrophoretic mobility, the major band corresponded to the fast MHC IIx in rat diaphragm and the minor band corresponded to the fast MHC IIb. On the basis of scans with a laser densitometer (LKB), the relative percentages of these MHC isoforms in four muscles were 92 ± 3 and 8 ± 3%, respectively. The total MHC concentration was 184 μM, which was 27 ± 1% of total protein. Whole soleus muscle extracts also revealed two MHC bands on the SDS gels (Fig. 3, *lane 2*). On the basis of their electrophoretic mobility measured in four muscles, the major band (97 ± 1%) corresponded to slow MHC I and the minor band (3 ± 1%) corresponded to MHC IIa. These results are in good agreement with previous histochemical studies showing that young adult (4–8 mo) rabbit soleus contains up to 98% slow-twitch type I fibers (12, 14). The total concentration of MHCs in whole soleus muscle (144 μM) was 22% lower than that in the whole psoas muscle (Table 1). In soleus, myosin comprised 24 ± 1% of total protein.

A major problem we faced in determining the concentration of myosin from single fibers was obtaining an accurate estimate of their volume. To avoid inaccuracies, we empirically determined two conversion coefficients, linking dry weight and TPC of the psoas and soleus muscles with their wet weight (Table 1). We then used dry weight and TPC as proxies for intact fiber volume. Because the whole muscle measurements came from bundles nearly pure in fiber type, in each case we compared MHC concentration in whole muscle with that of single fibers with the predominant fiber type.

When we used TPC as a proxy, MHC concentrations in single fibers were very close to the corresponding values obtained for whole muscle extracts (Table 1). When dry weight was used as a proxy, myosin concentration values for single fibers from both muscles were ~8% lower than when they were calculated on the basis of TPC, but this was not statistically different.

DISCUSSION

Quantitative Measurement of Myosin Content in Single Skinned Fibers

Data from this study show that the procedure we developed represents an effective means for measuring total myosin content in single muscle fibers. First, using immunochemistry, we observed essentially complete extraction of myosin from single fibers after 48 h of extraction, and extraction was nearly complete after 24 h. Additional evidence in support of complete extraction is that myosin content in the extraction buffer rises rapidly and reaches a near-maximal value in the

Table 1. *Wet weight-to-dry weight and wet weight-to-total protein content conversion coefficients*

Conversion Coefficients				
Wet weight/ dry weight	Wet weight/ TPC	Sample	MHC Concentration, μM	*n*
		Psoas		
		Whole muscle	184 ± 13	4
4.24 ± 0.22		Single fibers	169 ± 6	32
	5.22 ± 0.41	Single fibers	180 ± 6	28
		Soleus		
		Whole muscle	144 ± 13	4
4.10 ± 0.31		Single fibers	130 ± 6	26
	6.22 ± 0.38	Single fibers	140 ± 8	22

Values are means ± SE. Wet weight-to-dry weight and wet weight-to-total protein content (TPC) conversion coefficients were determined in bundles from rabbit psoas (n = 7) and soleus (n = 6) muscles. Molarity of myosin heavy chains (MHCs) was calculated using a molecular mass of 223,000 Da for MHC and 1.05 g/cm^3 as the density for muscle.

first 24 h (Fig. 1*B*, *left*). These data are consistent with an exponential decay of fiber myosin content with average time constants of 8.59 and 11.03 h for the psoas and soleus fibers, respectively (Fig. 1*B*, *right*). Taking our measurement at 48 h (equivalent to 4–6 time constants) ensures that essentially all the myosin is extracted.

Further evidence of the efficacy of the extraction and quantification of the myosin is the reproducibility of the technique. We found that, when fibers were of sufficient length to be cut in half accurately, the average difference from the mean in the myosin extracted and quantified was only ± 2.6%. It seems unlikely that such a small error could be achieved unless there was a definitive end point to extraction (i.e., complete extraction).

Final evidence that myosin from single fibers can be completely extracted and quantified comes from comparisons of myosin concentrations in single fibers with those in whole muscles. The "all in one tube" method we used for biochemical extraction of myosin from intact muscle reduces the possibilities of loss of protein and thus is probably the most accurate method for measuring myosin concentration. Although the myosin concentration from the two muscle types were different from one another, the myosin concentration in single fibers agreed very well with that measured in the respective whole muscle. The fact that there was excellent agreement based on both the use of dry weight and TPC as proxies for fiber volume provides yet further evidence that there is complete extraction and quantification of myosin by our procedure. For instance, if there was a similar but incomplete extraction of protein and myosin from single fibers, then the concentration based on TPC would be the same as in the whole muscle, even though the procedure would not provide an accurate measure of myosin content. However, we obtained a similar result based on dry weight, which is not susceptible to problems of extraction. This provides strong evidence of complete extraction and quantification of myosin.

Although the values were not statistically different between the two methods, the consistently lower (~8%) values obtained when volume was based on dry weight probably represent a small overestimation of fiber dry weight. We sometimes observed during the first 20–30 s after removal of the fibers from the heater that their dry weight increased by 5–10% (depending on room air humidity). From this viewpoint, using TPC as a proxy for intact fiber volume seems to be more reliable than using dry weight.

Different Myosin Concentrations in Psoas and Soleus Muscles

The results from our experiments not only show that this procedure permits accurate determination of myosin content in single fibers but also provides some insight into why myosin concentration varies from one muscle to another. The differences in myosin concentration in the two muscles could be the result of differences in myosin concentration of the predominant fiber type, or, in theory, the content of the major types might be the same but there could be large differences in myosin concentrations of a minor fiber type. All psoas and soleus fibers included in Table 1 are from the predominant fiber type in both cases (type IIx and type I, respectively). The close agreement between values from the whole muscle and values from the constituent single fibers and the large percent made up by the major fiber type (92 and 97%, respectively) suggest that the myosin concentration of the whole muscle is a simple manifestation of the myosin concentration of the predominant fiber type. This conclusion is further supported by our measurements of MHC concentration in the whole gracilis muscle. The gracilis, which is 99% type IIB (12), has almost the same MHC concentration (188 ± 15 μM; $n = 5$) as the psoas. This suggests that the myosin concentration of the type IIB fibers, which make up 8% of the psoas, is nearly the same as in the type IIx fibers, which make up 92% of the psoas. Thus potential differences in the MHC concentrations of the minor fiber-type population are not responsible for differences in the MHC concentration between the psoas and soleus.

It is likely that differences in myosin concentration observed in this study are due to differences in the volume occupied by myofibrils, as suggested by earlier quantitative electron microscopic stereographic studies (1, 8, 9, 17). It has been noted by Rome and Lindstedt (23) that there is a competition for volume among the myofibrils, sarcoplasmic reticulum (SR), and mitochondria (the volume of fat and glycogen, usually a small percent, is ignored for this discussion). Different functions require increased volumes of different intracellular components at the expense of the others. In this particular case, differences in MHC concentrations in single fibers from psoas and soleus muscles are likely to be due to differences in their mitochondrial volume. According to Jackman and Willis (12), whole muscle mitochondrial content in rabbit soleus is 14.5 mg/g of wet weight, whereas, in the fast-twitch gracilis muscle (99% fiber type IIB), it was only 5.3 mg/g of muscle wet weight.

This difference between myosin concentration associated with aerobic and anaerobic fibers is probably quite general. For instance, the red portion of latissimus dorsi in birds contained 25% less myosin than its white portion (10). However, the difference in myosin concentration can also reflect differences in the volume of the SR. For instance, the toadfish swimbladder muscle used to produce high-frequency sound has a very high SR content but low myosin concentration, which is less than half that of the toadfish fast-twitch white muscle (22). Finally, absolute values of myosin content can also vary between the species. For instance, Everett et al. (10) have reported that the myosin concentrations in red and white muscles of chickens are 180 and 240 μM, respectively, which is higher than the corresponding values in rabbit red (soleus) and white (psoas) muscles presented in this study.

Comparison to Other Values of MHC Concentration in Psoas

Our value for MHC concentration in rabbit psoas is in good agreement with that of Yates and Greaser (29) (188 ± 9 μM), who used amino acid analysis of MHC bands on SDS gels from fresh bundles of intact fibers. There is considerable variation in values obtained by others who measured MHC concentration in glycerinated single fibers. The variation in values may be attributable to differences in techniques for MHC quantification and determination of fiber volume. Bagshaw and Reed (4), using electron paramagnetic resonance spectroscopy, obtained a value of 190 μM. Ferenczi et al. (11) reported a lower value for MHC concentration, only 154 ± 16 μM. In that study, the authors calculated fiber volume based on caged ATP binding analysis, which is more accurate than most other determinations but may have also slightly overestimated the fiber volume. There are likely systematic differences that would lower MHC concentration in glycerinated fibers compared with that of intact muscle. First, the glycerination procedure may extract some of the myosin. Second, fibers swell when they are skinned, thereby increasing the volume used in the calculation of concentration. Hence, it is unclear why Marston and Treagear (15, 16) observed higher MHC concentrations in glycerinated fibers (220–230 μM) than we obtained in intact fibers.

For comparison with these glycerinated fiber values, we estimated MHC concentration in glycerinated psoas fibers by measuring myosin content and using our wet weight-to-dry weight conversion coefficient (determined for intact fibers). We obtained a value of 158 ± 7 μM (n = 20), which is similar to other values stated above. However, because we have not determined (and used) the appropriate conversion coefficient for glycerinated fibers, the significance of the comparison of this value to the others should be viewed with caution.

In conclusion, we have developed a procedure for complete myosin extraction from single muscle fibers. In combination with SDS-PAGE, it represents a very reproducible method for MHC quantification in single muscle fibers of the size typically used for skinned fiber mechanics and energetics experiments. Although there are simpler techniques that are routinely used for fiber typing and for qualitative determination of myosin content, we believe that the outlined procedure is valuable because it provides the requisite level of accuracy for meaningfully expressing the mechanics and energetics of muscle fibers in terms of cross bridges and cross-bridge kinetics (25). Determinations of such biophysical parameters as the force per attached cross bridge, ATP turnover per myosin head, and attachment and detachment rate constants are becoming increasingly important to understanding the molecular basis for muscle diversity, for understanding the functional significance of mutations of the muscular systems, and for relating cross-bridge behavior within the myofibril lattice to behavior of single cross bridges in in vitro motility and laser trap studies.

The high accuracy of this myosin quantification procedure depends on complete extraction of myosin from the fiber. This must be determined in different types of muscle. For instance, in this study, myosin extraction was significantly slower (t-test, P = 0.005) for the slow-twitch soleus fibers (time constant = 11.03 h) than it was for the fast-twitch psoas fibers (time constant = 8.59 h). Furthermore, fish muscle myosin does not extract completely under the experimental conditions suitable for extraction of myosin from mammalian muscles (19). Finally, our results indicate that slow-twitch fibers from rabbit soleus contain a significantly lower amount of myosin than fast-twitch fibers from psoas muscle. These data should be taken into consideration in calculations of force and ATP utilization per cross bridge in comparative and biophysical studies.

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