Biochimica et Biophysica Acta, 449 (1976) 310–326

BBA 47186

MYOSIN AND ACTOMYOSIN FROM HUMAN SKELETAL MUSCLE

GARY BAILIN

Department of Physiology and Biophysics and Department of Medicine, Mount Sinai School of Medicine, the City University of New York, Fifth Avenue and 100th Street, New York, N.Y. 10029 (U.S.A.)

(Received May 10th, 1976)

SUMMARY

Human skeletal natural actomyosin contained actin, tropomyosin, troponin and myosin components as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Purified human myosin contained at least three light chains having molecular weights (±2000) of 25 000, 18 000 and 15 000. Inhibitory and calcium binding components of troponin were identified in an actin-tropomyosin-troponin complex extracted from acetone-dried muscle powder at 37 °C. Activation of the Mg-ATPase activity of Ca^{2+}-sensitive human natural or reconstituted actomyosin was half maximal at approximately 3.4 μM Ca^{2+} concentration (CaEGTA binding constant = $4.4 \cdot 10^5$ at pH 6.8). Subfragment 1, isolated from the human heavy meromyosin by digestion with papain, appeared as a single peak after DEAE-cellulose chromatography. In the pH 6–9 range, the Ca^{2+}-ATPase activity of the subfragment 1 was 1.8- and 4-fold higher that the original heavy meromyosin and myosin, respectively. The ATPase activities of human myosin and its fragments were 6–10 fold lower than those of corresponding proteins from rabbit fast skeletal muscle. Human myosin lost approximately 60 % of the Ca^{2+}-ATPase activity at pH 9 without a concomitant change in the number of distribution of its light chains. These findings indicate that human skeletal muscle myosin resembles other slow and fast mammalian muscles. Regulation of human skeletal actomyosin by Ca^{2+} is similar to that of rabbit fast or slow muscle.

INTRODUCTION

Muscle contraction is now believed to be effected by the interaction of myosin, actin, tropomyosin and three components of the troponin complex [1–4]. The interaction between actin and myosin is generally recognized to be directly responsible for the contractile process. The regulatory proteins, tropomyosin and troponin, participate in the regulation of contraction and calcium plays an important role in these interactions [1–5]. Most of these results were obtained using rabbit skeletal muscle.

Abbreviations: AM, actomyosin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

The level of our knowledge of the contractile proteins of human skeletal muscle, however, has not paralleled the extensive research on rabbit muscle. Studies on human skeletal muscle have been reported with less frequency for reasons that are directly related to difficulties in obtaining fresh tissue in sufficient quantities and to problems associated with the purification and identification of the separate protein components. The major proteins, myosin, actin, tropomyosin and troponin, have reportedly been identified in human skeletal muscle (see e.g., refs. 6–12). The substructure of the troponin complex, however, has not been elucidated and the light-chain pattern of human myosin is not fully understood, though it has been suggested that the light-chain composition of myosin from human skeletal muscle (vastus lateralis) may resemble that of myosins from slow and fast twitch muscles [13].

This paper will show that human skeletal actomyosin, myosin and troponin are composed of protein components similar to those found in rabbit skeletal muscle. Evidence will also be presented indicating that the ATPase activity and alkali lability of human skeletal myosin and its proteolytic fragment, subfragment 1, more closely resemble the properties of a slow muscle.

METHODS AND MATERIALS

Human skeletal myosin was prepared as follows: fresh skeletal muscle (a mixture of vastus medialis and vastus lateralis) was obtained from tissue removed from patients 16–33 years of age undergoing leg amputation because of bone cancer. There was neither clinical nor laboratory evidence of disease in the muscle tissue used for this study [12]. In a typical preparation, the muscle tissue was minced twice in a precooled grinder. The minced muscle was extracted with 2.25 l of a solution containing 0.3 M KCl, 0.075 M KH_2PO_4, 0.075 M K_2HPO_4, 0.01 M ATP and 0.001 M dithiothreitol, pH 6.6, at 4 °C for 30 min with mechanical stirring. The mixture was centifuged at 13 200 $\times g$ for 10 min and the supernatant was filtered through a paper pad which had been washed with 0.6 M KCl. The filtrate was diluted with 10 vols. of cold distilled water, and the precipitate which formed was allowed to settle overnight and then collected by centrifugation at 13 200 $\times g$ for 10 min. The pellets were suspended in a solution containing 0.04 M KCl, 0.001 M dithiothreitol and centrifuged. In this way, most of the hemeproteins could be removed. The precipitate was dissolved in 0.6 M KCl, 0.001 M dithiothreitol and an equal volume of cold distilled water was added. The pH was adjusted to 6.5 and the suspension was centrifuged at 13 200 $\times g$ for at least 30 min. The supernatant (1.1 l) was diluted with 20 vols. of cold distilled water containing 0.001 M dithiothreitol. The precipitated myosin was collected and dissolved in 0.6 M KCl at 25 mg/ml. About 5 g of myosin were isolated by this procedure from 750 g of muscle mince. The myosin was stored at 13 mg/ml in 50 % glycerin in a deep freeze. In general, further purification of this myosin was accomplished by precipitating the myosin (70 mg) in 10 vols. of cold distilled water after removing all of the glycerin. To this suspension, dithiothreitol was added to 0.001 M final concentration and ribonuclease at a ratio of 500 : 1 (myosin : ribonuclease, w/w) at pH 6.8. The mixture was stirred overnight in the cold room. The myosin was collected and dissolved in 0.4 M KCl, 0.04 M Tris · HCl (pH 7.8) and 0.001 M dithiothreitol. The protein solution at 10 mg/ml was applied to a 40-ml bed of Whatman DE-52 (DEAE) cellulose equilibrated with 0.4 M KCl, 0.04 M Tris · HCl (pH

7.8) and 0.001 M dithiothreitol. The myosin was eluted from the cellulose bed with the same solution at 4 °C [14]. The eluant containing the myosin was precipitated in 10 vols. of 0.01 M KCl, 0.001 M dithiothreitol, collected and dissolved in 0.6 M KCl, 0.001 M dithiothreitol, and it was clarified in a Spinco preparative ultracentrifuge at 100 000 $\times g$ for 1 h. This myosin (35 mg) contained less than 1 % RNA contamination (w/w) as determined by the orcinol method of Schneider [15] and the $A_{280\,nm}/A_{260\,nm}$ ratio was 1.4.

Heavy meromyosin from human skeletal muscle was prepared by digesting the myosin in 0.6 M KCl, 0.001 M dithiothreitol with trypsin at a ratio of 100 : 1 (myosin : trypsin, w/w) at room temperature, pH 6.2 for 15–30 min [16].

Sugfragment 1 from human skeletal muscle was prepared by digestion of human skeletal heavy meromyosin at 10 mg/ml (26 ml) in a 0.04 M KCl, 0.001 M dithiothreitol with papain at a ratio of 25 : 1 (heavy meromyosin : papain, w/w) for 30 min, pH 7.85, at room temperature [14, 17]. To stop the reaction, a 50-fold excess of iodoacetate over papain was added followed by a twofold excess of dithiothreitol [17, 18]. The mixture was centrifuged at 17 300 $\times g$ for 20 min to remove papain which had become insoluble under these conditions.

Column chromatography of human muscle subfragment 1. The crude subfragment 1 (110 mg) was applied to a Sephadex G-200 column equilibrated with 0.05 M KCl, 0.1 M Tris · HCl (pH 7.6) and 0.001 M dithiothreitol. The column (2.5 $\times$ 40 cm) was eluted with the same solvent [18]. The major fractions containing high ATPase activity were concentrated by 65 % $(NH_4)_2SO_4$ precipitation at 4 °C. Following dialysis against 0.05 M Tris · HCl (pH 7.9), 0.001 M dithiothreitol the protein was purified further by chromatography on a Whatman DE-52 column according to the method of Lowey et al. [17]. Elution was accomplished with a linear gradient made with 200 ml of 0.05 M Tris · HCl (pH 7.9), 0.001 M dithiothreitol originally in the mixer and 200 ml of 0.5 M KCl, 0.05 M Tris · HCl (pH 7.9), and 0.001 M dithiothreitol in the reservoir. After concentration by 70 % $(NH_4)_2SO_4$ precipitation, the purified subfragment was dialyzed against 0.05 M KCl, 0.0025 M Tris · HCl (pH 7.4), 0.001 M dithiothreitol and then clarified in a Spinco ultracentrifuge at 100 000 $\times g$ for 1 h.

Human skeletal natural actomyosin was prepared by the method of Szent-Györgyi [19] with the addition of 0.001 M dithiothreitol at all stages of the preparation which were performed at 4 °C. Particulate matter and aggregated protein were removed by centrifugation at 100 000 $\times g$ for 1 h. The actomyosin was stored in a deep freeze in 50 % glycerin prior to use.

A complex of actin, tropomyosin and troponin was isolated from acetone-dried human muscle powder at 37 °C by the method of Katz [20] with the following modification: the extracting solution contained 0.001 M ATP (pH 7.0) and 0.001 M dithiothreitol. For the isolation of the actin-tropomyosin-troponin complex from acetone-dried rabbit skeletal fast muscle powder, the extracting solution contained 0.0001 M ATP (pH 7.0) but no dithiothreitol.

Human skeletal actin essentially free of the regulatory proteins was extracted from an acetone-dried powder [21] and it was purified as described earlier [22].

Reconstituted actomyosin was prepared by combining myosin and the actin-tropomyosin-troponin complex in a 3 : 1 ratio (myosin : actin complex, w/w). The reconstituted and natural actomyosins were then assayed for ATPase activity.

The ATPase activity of myosin and its proteolytic fragments in the presence of

Ca^{2+} or K^+ (EDTA) at high ionic strength (0.25 M KCl) or low ionic strength (0.04 M KCl) was determined as described previously [23]. The ATPase activity of an actomyosin was determined at 25 °C in 0.08 M KCl, 5 mM $MgCl_2$, 25 mM histidine, 2 mM ATP at pH 6.8. The reaction medium contained $1.5 \cdot 10^{-4}$ M $CaCl_2$ and various amounts of EGTA to attain specific ionized Ca^{2+} concentrations. A Ca-EGTA binding constant of $4.4 \cdot 10^5$ at pH 6.8 [24] was used to calculate the ionizable Ca^{2+} (free) from the Ca-EGTA buffers. The Ca^{2+} concentrations were varied from 10^{-7} to 10^{-4} M and for Ca^{2+} concentrations above 10^{-5} M, a $CaCl_2$ solution was used instead of a Ca-EGTA buffer. In the assay mixtures, the myosin concentration was 0.24 mg per ml; that of the actin-tropomyosin-troponin complex was 0.08 mg per ml. Reactions were initiated by the addition of ATP and stopped by the addition of 1 ml of 10 % trichloroacetic acid. Inorganic phosphate liberation was determined as described earlier [23].

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed with 10 % gels in 12.5 cm tubes at a current of 4 mA per tube for 18 h. A 0.1 M phosphate buffer (pH 7.1) containing 0.1 % sodium dodecyl sulfate [26] was used according to procedures described recently [27]. The protein samples were dialyzed overnight at room temperature against 0.1 M sodium phosphate buffer (pH 7.1), 1 % sodium dodecyl sulfate and 0.001 M dithiothreitol. Densitometer scanning was accomplished with an E-C Apparatus Transmission Densitometer equipped with an integrator and a 550–570 nm filter for Coomassie blue. Molecular weights were determined by co-electrophoresis of a mixture of standard proteins: cytochrome *c* (12 400); myoglobin (17 800); chymotrypsinogen (25 000); ovalbumin (45 000); bovine serum albumin (67 000); and phosphorylase *a* (90 000). The purified proteins were obtained from Schwarz-Mann with the exception of phosphorylase, which was obtained from Boehringer-Mannheim.

Amino acid analysis. The composition of human skeletal myosin and its proteolytic fragments was determined as described previously [23]. The acid hydrolyzed and washed samples were analyzed for their amino acid content. Tryptophan and tyrosine were determined by the spectrophotometric method of Goodwin and Morton [28]. Cysteine was determined as *S*-carboxy-methylcysteine after treatment of the proteins with [^{14}C]iodoacetate ($6.6 \cdot 10^5$ cpm/μmol) at pH 7.4 in the presence of urea. The method is essentially that of Tada et al. [18].

Protein concentration was determined by the biuret method, using bovine serum albumin as a standard [29].

Trypsin, soybean trypsin inhibitor, ribonuclease and papain were purchased from Worthington Biochemical Corp. Papain was obtained as a suspension in 0.05 M sodium acetate and it was activated by dialysis against 0.005 M dithiothreitol and 0.002 M EDTA (pH 5.9) with one change of the dialysis solution overnight [17, 18]. The assay of the proteolytic enzymes has been reported [18].

RESULTS

Protein components of human skeletal myosin and natural actomyosin

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed that human skeletal myosin and natural actomyosin contained high and low molecular weight components resembling those found in rabbit skeletal muscle (Fig. 1). There

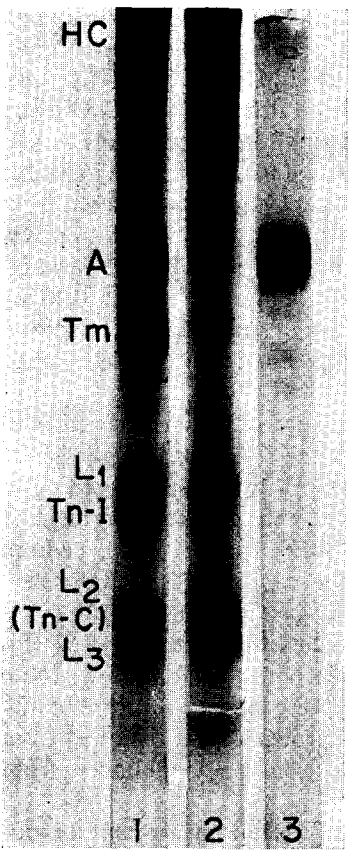

Fig. 1. Gel electrophoresis of human skeletal contractile proteins. Samples of natural actomyosin, myosin and actin were dialyzed against the electrophoresis buffer solution and 150 μg of actomyosin, myosin and 25 μg of actin were subjected to polyacrylamide gel electrophoresis. For further details, see Methods and Materials. (1), natural actomyosin; (2), myosin; (3), actin. HC, heavy chains; A, actin; Tm, tropomyosin; Tn-I and Tn-C, components of troponin; L_1, L_2 and L_3, light chains of myosin.

were several proteins in human natural actomyosin which corresponded to the regulatory proteins of the thin filament of rabbit skeletal muscle. The proteins that resembled actin and tropomyosin had electrophoretic mobilities that corresponded to molecular weights of 45 000 and 35 000$\pm$2000, respectively (gels 1 and 3 of Fig. 1). A 22 000 dalton component resembling the inhibitory protein of the troponin complex from rabbit skeletal muscle [1, 3, 30] was found (gel 1 of Fig. 1). However, a more rapidly migrating component of molecular weight 18 000 corresponding to the calcium-binding component of rabbit skeletal troponin could not be resolved under these conditions (gel 1 of Fig. 1). A 33 000 dalton component was also present and its mobility was slightly greater than the protein found in the tropomyosin (Tm) region (gel 1 of Fig. 1).

When the gel pattern of human skeletal natural actomyosin was compared with that of purified human skeletal myosin alone, the heavy and light chains of human

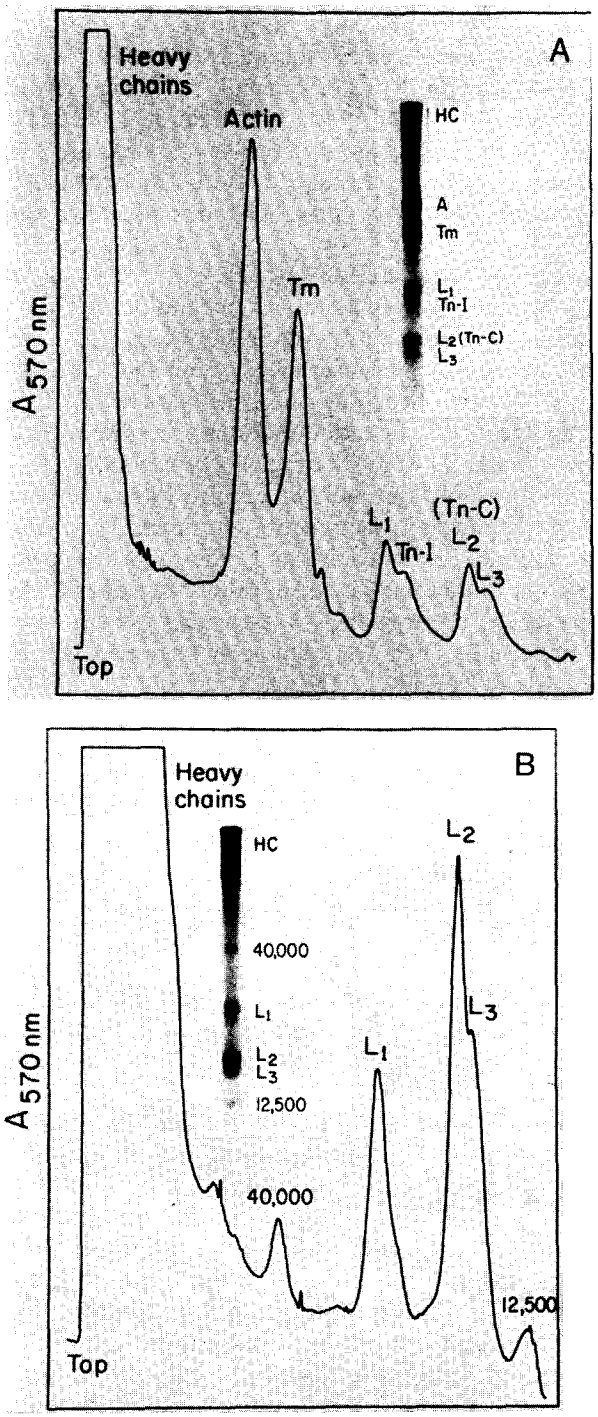

Fig. 2. Gel pattern and densitometric scan of human skeletal contractile proteins. A, natural actomyosin; B, myosin. Samples were prepared as described in the legend to Fig. 1. HC, heavy chains; A, actin Tm, tropomyosin; Tn-I and Tn-C, components of troponin; L_1, L_2 and L_3, light chains of myosin.

myosin became apparent (gels 1 and 2 of Fig. 1). The proteins near the top of the gels which stained intensely with Coomassie blue resemble the migration of the heavy chains of rabbit skeletal myosin [17, 31, 32]. In the myosin sample there were several rapidly moving components, most notably those of molecular weight 25 000, 18 000, and 15 000 (gel 2 of Fig. 1). Other components present in lesser amounts migrated with mobilities that corresponded to molecular weights of 85 000, 70 000, and 40 000. Moreover, a 22 000 dalton component appeared and a protein band at 12 500 daltons was also found (gel 2 of Fig. 1).

Quantification of the major protein bands at 25 000, 18 000 and 15 000 daltons was accomplished by densitometer scanning and simultaneous integration. A typical gel scan of human skeletal natural actomyosin and myosin is shown in Fig. 2. An approximate ratio of 1.5 : 1.0 : 1.6 : 1.0 was calculated for the protein bands designated as L_1, Tn-I, L_2 (Tn-C) and L_3. It was difficult to determine the mass ratios of the light chains of myosin and the components of troponin due to the presence of small peaks and the inability to resolve L_2 and Tn-C in the region corresponding to a molecular weight of 18 000. For this reason, a gel scan of purified human skeletal myosin was obtained (Fig. 2B). To facilitate densitometry, the intensely stained region at the top of the gel containing the heavy chains and other high molecular weight components was not selected for full scale adjustment. In this way, the scan of the low molecular weight proteins could be expanded so as to allow for a more accurate determination of their mass ratios (Fig. 2B). Although there was still an overlap of the protein bands designated L_2 and L_3, the mass ratio of the 25 000, 18 000 and 15 000 dalton components was 1.1 : 2.3 : 1.0 respectively (L_1, L_2 and L_3 bands in Fig. 2B). The 22 000 dalton protein band, which could be seen in the gel pattern, was not resolved in the gel scan (compare gel pattern and scan of Fig. 2B). The protein peaks of the gel scan with molecular weights of 70 000, 40 000, and 12 500 comprise less than 6 % of the total mass calculated from the integrated areas under all the peaks. Higher molecular weight proteins which may be contaminants of the preparation could not be quantified due to a broadening effect observed in the heavy chain region (see "Top" of gel scan in Fig. 2B).

Comparison of actin complexes extracted from human and rabbit skeletal muscles

To establish the presence and identity of the components of troponin and tropomyosin in human skeletal muscle, actin complexes obtained from human and rabbit skeletal muscle were compared.

The protein complex extracted from the acetone-dried powder of human or rabbit skeletal muscle at 37 °C consisted of actin, tropomyosin and the troponin components as judged by gel electrophoresis in sodium dodecyl sulfate (Fig. 3). A 45 000 dalton component had the same mobility as the major protein band found in purified human skeletal actin. A similar component was also found in the rabbit skeletal actin-tropomyosin-troponin complex (compare gels 1, 2 and 3 in Fig. 3). The protein component of the rabbit skeletal actin complex which migrated with a molecular weight of 35 000 is tropomyosin and a similar protein was found in the human skeletal actin complex although it was not clearly visible due to overloading of the gel (compare Tm protein region in gels 2 and 3 of Fig. 3). It is noteworthy that a slightly faster-moving component of molecular weight 33 000 was also found in the tropomyosin (Tm) region of the human skeletal and rabbit skeletal actin complexes. There

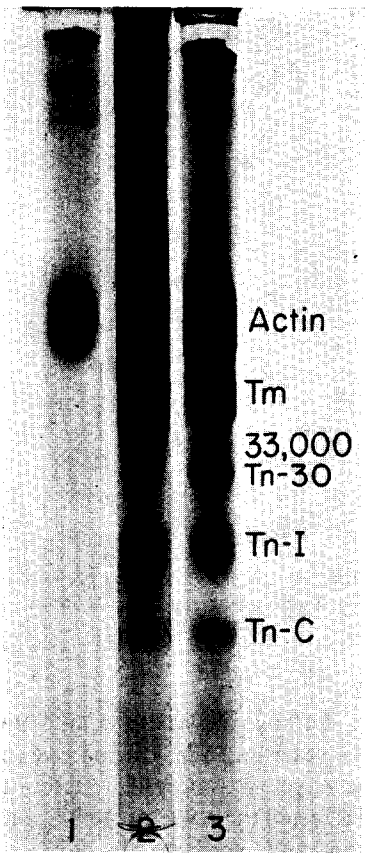

Fig. 3. Gel electrophoresis of an actin-tropomyosin-troponin complex. The protein complex was obtained by extracting an acetone-dried muscle powder at 37 °C. Samples of the actin complex were dialyzed against the electrophoresis buffer solution and 25–50 μg were subjected to polyacrylamide gel electrophoresis. For further details see Methods and Materials. (1), purified human skeletal actin; (2), human skeletal actin complex; (3), rabbit skeletal actin complex. Tm, tropomyosin; Tn-I and Tn-C, components of troponin; Tn-30, proteolytic digestion product of Tn-T.

were at least two additional proteins present in the actin-tropomyosin-troponin complexes. A 22 000 dalton protein in the human skeletal actin complex resembling the inhibitory component of troponin, Tn-I, migrated with the same mobility as the Tn-I component of the rabbit skeletal actin-tropomyosin-troponin complex (compare Tn-I protein bands of gels 2 and 3 in Fig. 3). A protein component of molecular weight 18 000 also found in the human skeletal actin complex resembles the calcium binding component of rabbit skeletal troponin, Tn-C (compare Tn-C protein bands of gels 2 and 3 in Fig. 3). The rabbit skeletal actin-tropomyosin-troponin complex also contained a protein with a molecular weight of 30 000 which was also found in the human skeletal actin complex (Tn-30 band in gels 2 and 3). Furthermore, the tropomyosin binding component of troponin, termed Tn-T, was not clearly seen in either of the actin complexes.

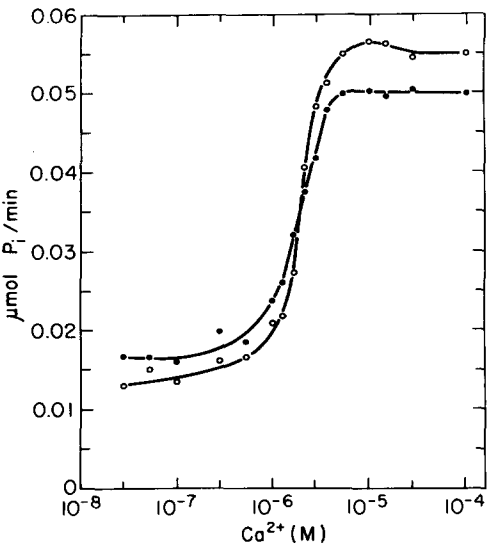

Fig. 4. Effect of Ca^{2+} on the hydrolysis of ATP by human skeletal actomyosin. The assay procedure and determination of ionized Ca^{2+} concentration are described under "Materials and Methods". ○—○, natural actomyosin; ●—●, reconstituted actomyosin.

Adenosine triphosphatase activity of human actomyosin

Hydrolysis of ATP by human skeletal natural actomyosin was activated by free Ca^{2+} concentration in the micromolar range when reactions were carried out as described in Methods and Materials. The activation response followed a sigmoid pattern (Fig. 4). When human skeletal myosin alone was tested there was no activation of the ATPase activity in the free Ca^{2+} concentration range studied. The ATPase activity of human skeletal natural actomyosin was activated by Ca^{2+} with half maximal activation occurring at 3.5 ± 0.21 μM (S.E.M., $n = 4$) Ca^{2+} concentration (Fig. 4).

A reconstituted human skeletal actomyosin made from human skeletal myosin and the actin-tropomyosin-troponin complex was also activated by Ca^{2+} (Fig. 4). The reconstituted human actomyosin showed a comparable sigmoid response with half maximal activation at 3.2 ± 0.07 μM Ca^{2+} (S.E.M., $n = 4$) (Fig. 4). There was no significant difference in the values obtained for the natural and reconstituted acto-

TABLE I

EFFECT OF Ca^{2+} ON THE ATPase ACTIVITY OF HUMAN ACTOMYOSIN

Actomyosin	ATPase activity (μmol of Pi·min^{-1}·mg^{-1})	Half maximal Ca^{2+} activation (μM)	Inhibition[a] (%)
Natural	0.054 ± 0.005[b]	3.5 ± 0.21	73
Reconstituted	0.053 ± 0.005	3.2 ± 0.07	67

[a] Percentage inhibition values are calculated from the following equation: 100−(AM-ATPase at 10^{-7} M Ca^{2+}/AM-ATPase at 10^{-5} M Ca^{2+})×100.

[b] Standard error of the mean ($n = 4$).

myosins (Table I). This was also the case for the specific ATPase activity measured at 10^{-4} M Ca^{2+} concentration, which was 0.054 ± 0.005 μmol of P_i/min per mg of protein (S.E.M., $n = 4$) (Table I). In addition, the percentage inhibition of the actomyosin ATPase activity, expressed as the Ca^{2+}-sensitivity by Kendrick-Jones et al. [33], was approximately 70 % for both types of actomyosins (Table I).

Proteolytic digestion of human skeletal myosin

An attempt was made to localize the enzymic activity of human skeletal myosin by limited proteolysis with trypsin and papain to obtain smaller active fragments as shown previously by Lowey et al. [16, 17] for rabbit skeletal myosin.

Trypsin digestion of human skeletal myosin at pH 6.2 for 30 min at a ratio of 100 : 1 (myosin : trypsin, w/w) liberated human heavy meromyosin in a 57 % yield. When the proteolytic digest was applied to a Sephadex G-200 column, a protein was obtained that was eluted rapidly and characteristically, just after the void volume. No other active components were eluted as the ATPase activity was only associated with the major peak. No further purification of the heavy meromyosin was undertaken, since it was digested with papain and the final product, subfragment 1, was purified.

Human skeletal subfragment 1

Papain digestion of human skeletal heavy meromyosin at a ratio of 25 : 1 (heavy meromyosin : papain, w/w) pH 7.85 for 30 min, at 25 °C was found to be a most suitable method for proteolysis and subsequent formation of a smaller active

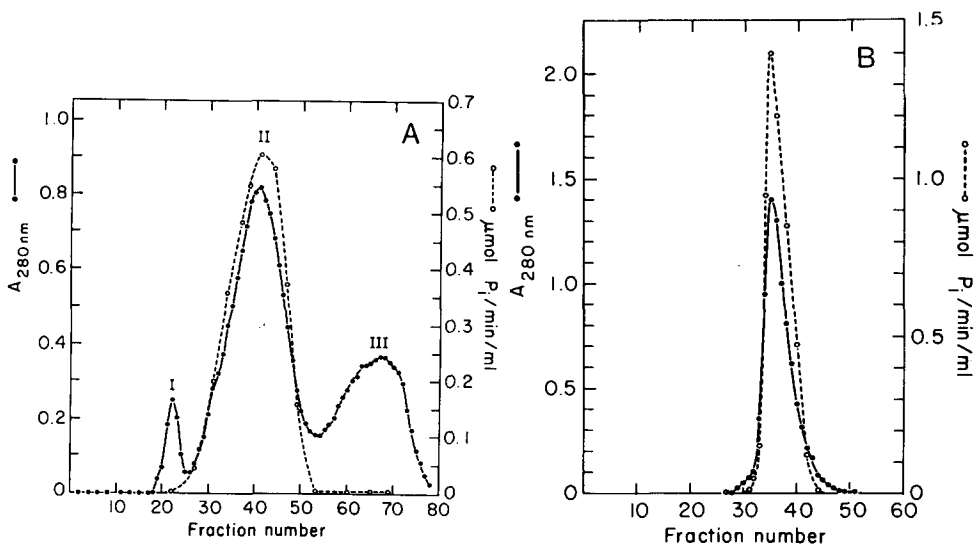

Fig. 5. Column chromatography of papain-treated human skeletal heavy meromyosin. (A) Sephadex G-200 chromatography of a papain digest of heavy meromyosin (110 mg). The final volume was 11 ml before chromatography and 3-ml fractions were collected. See Methods and Materials for the preparation of the papain digest of heavy meromyosin. Solid line, absorbance at 280 nm; broken line, Ca^{2+}-ATPase activity (μmol of Pi/min per ml). (B) DEAE-cellulose (Whatman DE-52) chromatography of human skeletal subfragment 1. The major fractions of peak II in Fig. 5A were pooled, concentrated and placed on a 1.5×30 cm column and 3-ml fractions were collected. For further details see Methods and Materials. Solid line, absorbance at 280 nm; broken line, Ca^{2+}-ATPase activity (μmol of Pi/min per ml).

fragment resembling subfragment 1 [14, 18]. When the papain digestion was followed by Sephadex G-200 chromatography, the resulting chromatogram showed only one protein peak which had ATPase activity (Fig. 5A, Peak II). The major fractions of Peak II were rechromatographed on a small DEAE-cellulose column and one major peak with ATPase activity emerged (Fig. 5B).

Initial characterization of this proteolytic fragment from human skeletal myosin which resembles subfragment 1, indicated that it had a molecular weight of 110 000 as judged by results obtained from Sephadex G-200 chromatography.

Preliminary amino acid analysis of human skeletal myosin and of the protein resembling subfragment 1 did reveal major differences in their amino acid composition (Table II). The glutamic acid, alanine, lysine and arginine contents of myosin were higher than those of subfragment 1. When compared to rabbit skeletal myosin and its proteolytic fragments, the cysteine and tryptophan contents of human skeletal myosin and the protein resembling subfragment 1 were lower. For instance, the cysteine contents of human skeletal myosin and subfragment 1 were: 6.8 and 8.6 mol per 10^5 g of protein, respectively. These values are less than those of rabbit skeletal myosin and its subfragment 1 viz., 8.8 and 11.0 mol per 10^5 g of protein [17, 23, 34]. Furthermore, the tryptophan content of human skeletal myosin was 1.3 mol per 10^5 g of protein compared with 6.7 mol per 10^5 g of protein for rabbit skeletal myosin [23].

TABLE II

AMINO ACID COMPOSITION OF HUMAN SKELETAL MYOSIN AND SUBFRAGMENT 1

Values are given in mol per 10^5 g of protein.

Amino acid	Myosin	Subfragment 1
Cysteine	6.8	8.9
Aspartic acid	81	64
Threonine	39	27
Serine	31	21
Glutamic acid	127	84
Proline	25	17
Glycine	42	32
Valine	62	39
Methionine	42	29
Isoleucine	28	21
Leucine	36	31
Tyrosine	16	14
Phenylalanine	27	28
Lysine	72	43
Histidine	11	8
Arginine	40	19
Tryptophan	1.3	4.6

The Ca^{2+} or K^+ (EDTA) activated ATPase activities of human skeletal myosin, heavy meromyosin, and a protein resembling subfragment 1 increased from myosin to the smallest active fragment at low or high ionic strength (Table III). The enzymic activity of the myosin and its proteolytic fragments was low. For example, the Ca^{2+} and K^+ (EDTA)-ATPase activities of human skeletal myosin were 0.07 and

TABLE III

THE ATPase ACTIVITY OF MYOSIN AND ITS PROTEOLYTIC FRAGMENTS AT pH 7.5
Values are given in μmol Pi · min^{-1} · mg^{-1}.

Protein	Ca^{2+}-ATPase		K^+(EDTA)-ATPase
	Low ionic strength	High ionic strength	
Myosin	0.061	0.07	0.171
Heavy meromyosin	0.122	0.135	0.346
Subfragment 1	0.203	0.237	0.467

0.171 μmol of P_i/min per mg of protein, respectively, at high ionic strength (0.25 M KCl). Despite this finding, the ATPase activity of human skeletal myosin and its proteolytic fragments was pH dependent in the pH 6–9 range (Fig. 6). In this pH range, the protein resembling subfragment 1 had a 4–4.6 fold higher ATPase activity than myosin and a 1.8-fold higher ATPase activity than heavy meromyosin at low or high ionic strength. The Ca^{2+}-ATPase activity of human skeletal myosin and its proteolytic fragments passed through a minimum value in the vicinity of pH 7.5 at low or high ionic strength (Fig. 6). As the pH approached 9.0, the Ca^{2+}-ATPase activity of human skeletal myosin and its proteolytic fragments decreased markedly (Fig. 6).

The effect of higher pH on the stability of human skeletal myosin was examined more closely when the myosin was incubated at pH 9.0 and samples were removed for enzymic assay. The Ca^{2+} or K^+(EDTA)-ATPase activity of human skeletal myosin

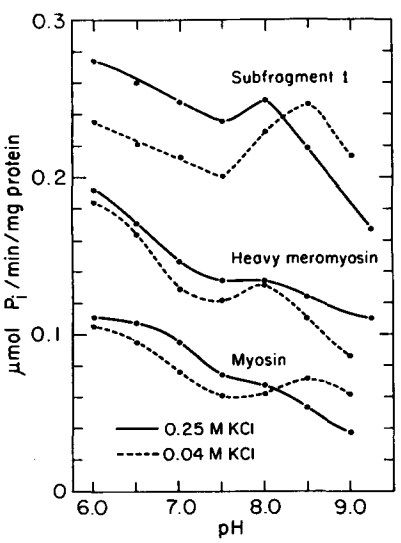

Fig. 6. Effect of pH on the Ca^{2+}-ATPase activity of human skeletal myosin and its proteolytic fragments. The assay of the ATPase activity was performed with solutions containing 0.01 M $CaCl_2$, at various pH values in 0.04 M KCl (low ionic strength) or 0.25 M KCl (high ionic strength) and 0.02 M Tris · HCl buffer. The assay was started by the addition of ATP and the reaction time was 10 min at 25 °C. Solid line, 0.25 M KCl; broken line, 0.04 M KCl.

TABLE IV

THE ATPase ACTIVITY OF MYOSIN INCUBATED AT pH 9.0

Human skeletal myosin at 5 mg per ml (14 ml) in 0.6 M KCl, 0.001 M dithiothreitol was adjusted to pH 9.0 by dropwise addition of 0.1 M KOH. It was incubated at this pH value with stirring at 25 °C. Myosin (5 mg/ml) adjusted to pH 7.0 served as a control. At specified intervals, aliquots (3 ml) were removed and the myosins were precipitated in 30 ml of cold distilled water. The myosin was reprecipitated three times from 0.6 M KCl prior to assay and dialysis in the electrophoresis buffer. The ATPase activity is expressed as percentage of control. The Ca^{2+}-ATPase 100 % value is 0.07 μmol of Pi/min per mg of myosin. The K^+(EDTA)-ATPase 100 % value is 0.20 μmol of Pi/min per mg of myosin.

Sample	Time (min)	ATPase activity	
		Ca^{2+} (%)	K^+(EDTA) (%)
Control myosin at pH 7.0	0	100	100
	30	100	93
	60	92	103
Myosin at pH 9.0	15	68	71
	30	34	42
	60	23	26
	90	17	18

incubated for 30 min at pH 9.0 was only 34–42 % of a control myosin at pH 7.0 (Table IV). After 60 min incubation, human skeletal myosin had only 23–27 % ATPase activity compared to 92–103 % activity for a control myosin. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed that all the myosin samples at pH 9.0 contained the heavy and light chain components compared to a control myosin at pH 7.0.

DISCUSSION

Composition of natural actomyosin, myosin and an actin complex from human skeletal muscle

Analysis of the gel electrophoresis of natural actomyosin from human skeletal muscle indicated that the protein bands corresponded to those of rabbit skeletal actin, tropomyosin, troponin components and the high and low molecular weight proteins of myosin (gel 1 of Fig. 1). Actin, tropomyosin and troponin were also identified in electrophoresis studies on human skeletal actomyosins [7, 12] and human muscle (quadriceps femoris) homogenates [10]. The data reported here suggest that these proteins of human skeletal natural actomyosin are composed of subunits that are similar to those found in rabbit skeletal natural actomyosin or myofibrils [1, 2, 35–37].

The light chain components of human skeletal myosin with molecular weights of 25 000, 18 000, and 15 000 in a mass ratio of 1.1 : 2.3 : 1.0, respectively, (gel 2 of Fig. 1) resemble those designated L_1, L_2 and L_3 in rabbit fast skeletal myosin [31, 32, 37, 38]. The similarity between the light chains of the two kinds of myosin is, however,

not unequivocal, as a 22 000 dalton component was also found (gel 2 of Fig. 1). This protein may be some Tn-I or a part of a doublet (including the 25 000 dalton protein) of the L_1 light chain region. Furthermore, the L_2 region may contain a mixture of fast and slow myosin light chains [39, 40] which would account for its higher content. Thus, it appears that human skeletal myosin contains light chains in the L_1 and L_2 region of fast and slow muscle myosins. Recent gel electrophoresis studies on human skeletal myosin [13] and histochemical studies [7, 11, 13, 41] on human skeletal muscle (vastus lateralis) suggest that fast and slow twitch fibers are present. The results of this paper do not allow an exact number of light chains to be stated, but at least three major components (representing light chains of fast and slow myosin) are present in the myosin molecule in agreement with the findings of Sreter et al. [13]. The L_3 component of 15 000 daltons may be a unique protein constituent of the myosin or a proteolytic digestion product of the 25 000 dalton protein L_1. Recent evidence concerning the amino acid sequence of L_1 and L_3 of rabbit skeletal myosin [42] makes the latter possibility unlikely.

In addition to the major low molecular weight components, proteins with molecular weights of 40 000 and 12 500 were found in human skeletal myosin (gel 2 of Fig. 1 and Fig. 2B). The 40 000 dalton protein could be an actin contaminant or possibly a subunit of a light chain kinase [43, 44] which has been identified in human skeletal myosin [44]. The 12 500 dalton protein could be a muscle parvalbumin [45, 46] or cytochrome *c*, but it is more likely that it is a contaminant of the myosin preparation made from tissue which contains endogenous proteolytic activity. These components account for less than 4 % of the total protein (Fig. 2). The intensely stained and broad region corresponding to band HC of the gel indicates that the heavy chains are heterogeneous and that proteins such as C-protein [47, 48] or those of the M-line [49, 50] may be present (band HC in gel pattern and scan of Fig. 2B). Moreover, α-actinin has been described as a possible contaminant in this region [10].

The gel electrophoresis pattern of a protein complex isolated from human or rabbit skeletal muscle showed that actin, tropomyosin and the subunit components of troponin were present (Fig. 3). The migration of an 18 000 dalton component was the same in both cases, resembling the calcium binding component of troponin, Tn-C, in rabbit skeletal muscle [1, 25]. The 22 000 dalton protein was also the same in both actin complexes and corresponds to the inhibitory component of troponin, Tn-I [1, 25]. These proteins are not light chain contaminants of myosin, as the extraction of the actin complexes from the acetone-dried powders were performed under mild conditions. The removal of the light chains of rabbit skeletal myosin, for instance, requires alkali at high pH (ref. 32 and references therein) or denaturing reagents [51]. The Tn-I and Tn-C components of troponin did not stain intensely with Coomassie blue when compared to actin or tropomyosin (Fig. 3). The relative differences in the staining intensities of these proteins makes a determination of their content in troponin [35] or in the actin complex difficult. Nevertheless, a mass ratio of 1 : 1 was estimated for the Tn-I and Tn-C components of the human skeletal actin complex in agreement with that found for rabbit skeletal troponin [35].

The tropomyosin binding protein of troponin, Tn-T, was not resolved in the gels of the human or rabbit skeletal actin complexes (gels 2 and 3 of Fig. 3). This may be due to overloading the gels. However, the actin complexes contained a protein component of molecular weight 33 000 which is probably a proteolytic digestion

product of Tn-T [52] rather than a subunit or digestion product of tropomyosin. The human and rabbit skeletal actin complexes also contained a protein component with a molecular weight of 30 000 (Tn-30 band in gels 2 and 3 of Fig. 3). In all probability, it is also a proteolytic digestion product of Tn-T [52] which could explain, in part, why this component was not seen in the actin complex.

The molecular weights of all the proteins estimated by gel electrophoresis in sodium dodecyl sulfate remain to be confirmed by amino acid analysis and sedimentation studies. The mobility of proteins with a high proportion of basic amino acid residues such as the light chains of rabbit skeletal myosin [42] and cardiac Tn-I [53], is anomalous on the gels which gives rise to high estimates of their molecular weights [42, 53, 54].

Effect of Ca^{2+} on the actomyosin ATPase activity

The ATPase activity of human skeletal natural actomyosin or a reconstituted actomyosin made from human skeletal myosin and the actin-tropomyosin-troponin complex was activated by free Ca^{2+} (Fig. 4) and half maximal activation occurred at 3.5 μM Ca^{2+} concentration. This value, which corresponds to 0.75 μM Ca^{2+} concentration when the Schwarzenbach binding constant of 10^{11} for $CaEGTA^{4-}$ is used [55, 56], is in accord with the half maximal value at 1 μM Ca^{2+} concentration reported for rabbit skeletal actomyosin [55–57]. As mentioned earlier, human skeletal muscle tissue appears to be composed of mixed fiber types and predominantly those of a slow twitch variety [7, 11, 13, 41]. The low ATPase activity of human skeletal actomyosin (Table I) or myosin (Table III) which has been reported previously [6, 7, 11–13] is characteristic of a slow muscle [58, 59]. The ATPase activity of human skeletal myosin [11, 60] and other skeletal myosins [40, 61, 62] correlates well with fiber composition. The lower ATPase values found in this study could also be due to the auto-oxidation of -SH groups in the myosin as suggested by the relatively low cysteine content of human skeletal myosin (Table II). For example, 6.8 mol of cysteine per 10^5 g of protein were found in human skeletal myosin, compared to an average value of 8.8 and 8.2 mol of cysteine per 10^5 g of protein for rabbit skeletal fast [17, 23, 34] and rabbit skeletal slow [63] myosin, respectively. Based on the results obtained from gel electrophoresis studies and ATPase activity measurements, it is most probable that the myosin was partially oxidized despite the precautions taken.

Human skeletal subfragment 1

Trypsin digestion of human skeletal myosin released a protein resembling heavy meromyosin in agreement with the findings of Gröschel-Stewart [7]. Papain digestion of human skeletal heavy meromyosin at pH 7.85, 25 °C liberated subfragment 1 as shown by column chromatography with Sephadex G-200 and DEAE-cellulose (Fig. 5). Subfragment 1 from human skeletal myosin had an estimated molecular weight of 110 000 which is similar to values reported for rabbit skeletal [17], chicken gizzard [14] or bovine cardiac [18] subfragment 1. The amino acid composition of subfragment 1 differed markedly from that of the original myosin (Table II). The amino acid content of human skeletal myosin was comparable to data obtained by Gröschel-Stewart (7) and resembled the amino acid composition of human [64] or bovine [18] cardiac myosin. The human skeletal subfragment 1 purified by column chromatography (Fig. 5) was heterogeneous on polyacrylamide gels in sodium dodecyl

sulfate, due probably to prolonged digestion of the heavy meromyosin and subfragment 1. Usually, shorter intervals are chosen for the formation of subfragment 1 from rabbit skeletal muscle [65].

The Ca^{2+}-ATPase activity of human skeletal subfragment 1, although higher than the original myosin (Table III and Fig. 6), was much lower than that of rabbit skeletal subfragment 1. At low ionic strength (0.04 M KCl) the Ca^{2+}-ATPase activity (pH 7.0) of rabbit fast skeletal myosin, heavy meromyosin and subfragment 1 [66] was nearly 10-fold higher than the corresponding human skeletal myosin and its proteolytic fragments. At high ionic strength (0.25 M KCl) the Ca^{2+}-ATPase activity of rabbit fast skeletal myosin and its fragments was 6-fold higher than the human skeletal myosin and its proteolytic fragments. The chemical nature of these differences may be explained, in part, by differences in the -SH content and extent of oxidation of these groups in the myosins. However, differences in the enzymic activity must also indicate that the ATPase sites of human and rabbit skeletal myosin are not the same. This has been shown for bovine cardiac [18] and chicken gizzard [14] myosins.

Finally, human skeletal myosin and its proteolytic fragments showed a significant decrease in ATPase activity at pH 9.0 (Fig. 6). Incubation of the myosin at pH 9.0 resulted in a loss of 60 % of the Ca^{2+} or K^{+}(EDTA)-ATPase activity (Table IV). This alkali lability is consistent with the data reported for human skeletal myosin [6, 8, 60] and myosin from rabbit slow skeletal muscle [39, 58, 59]. The loss of activity was not associated with any changes in the distribution or number of light chains in the human skeletal myosin at pH 9.0. Therefore, it would appear that a non-specific denaturation effect of conformational origin had occurred in the myosin molecule.

ACKNOWLEDGEMENTS

The author greatly appreciates the expert assistance of Mu-Ju Shen. We are indebted to Dr. E. Rubin of the Pathology Department for providing the muscle tissue. This work was supported in part by a grant from the Muscular Dystrophy Association and by grant No. AA-00316 from the National Institute on Alcohol Abuse and Alcoholism.

REFERENCES

1 Ebashi, S., Endo, M. and Ohtsuki, I. (1969) Quar. Rev. Biophys. 2, 361–384
2 Taylor, E. W. (1972) Annu. Rev. Biochem. 41, 577–616
3 Weber, A. and Murray, J. M. (1973) Physiol. Rev. 50, 63–158
4 Squire, J. M. (1975) Annu. Rev. Biophys. Bioeng. 4, 137–163
5 Szent-Györgyi, A. G. (1975) Biophys. J. 15, 707–723
6 Samaha, F. J. and Gergely, J. (1969) Arch. Neurol. 21, 200–207
7 Gröschel-Stewart, U. (1971) Biochim. Biophys. Acta 229, 322–334
8 Samaha, F. J. (1972) Exp. Neurol. 35, 30–38
9 Penn, A. S., Cloak, R. A. and Rowland, L. P. (1972) Arch. Neur. 27, 159–173
10 Sugita, H., Katagiri, T., Shimizu, T. and Toyokura, Y. (1973) in Basic Research in Myology, Pt. 1, Proc. 2nd Intl. Congr. Muscle Dis., 1971, pp. 291–297, Excerpta Medica, Amsterdam
11 Taylor, A. W., Essen, B. and Saltin, B. (1974) Acta Physiol. Scand. 91, 568–570
12 Puszkin, S. and Rubin, E. (1975) Science 188, 1319–1320
13 Sreter, F. A., Aström, K.-E., Romanul, F. C. A., Young, R. R. and Jones, H. R. (1976) J. Neurol. Sci. 27, 99–116
14 Bailin, G. and Bárány, M. (1971) Biochim. Biophys. Acta 236, 292–302
15 Schneider, W. C. (1957) in Methods in Enzymology III, (Colowick, S. P. and Kaplan, N. O., eds.), p. 680, Academic Press, New York

16 Lowey, S. and Cohen, C. (1962) J. Mol. Biol. 4, 293–308
17 Lowey, S., Slayter, H. S., Weeds, A. G. and Baker, H. (1969) J. Mol. Biol. 42, 1–29
18 Tada, M. Bailin, G., Bárány, K. and Bárány, M. (1969) Biochemistry 8, 4842–4850
19 Szent-Györgyi, A. (1951) Chemistry of Muscular Contraction, Academic Press, Inc., New York
20 Katz, A. M. (1966) J. Biol. Chem. 241, 1522–1529
21 Bárány, M. and Bárány, K. (1959) Biochim. Biophys. Acta 35, 293–308
22 Bailin, G. and Bárány, M. (1972) J. Mechanochem. Cell Motility 1, 189–190
23 Bárány, M., Gaetjens, E., Bárány, K. and Karp, E. (1964) Arch. Biochem. Biophys. 106, 280–293
24 Ogawa, Y. (1968) J. Biochem. (Tokyo) 64, 255–263
25 Greaser, M. L. and Gergely, J. (1973) J. Biol. Chem. 248, 2125–2133
26 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
27 Bailin, G. (1975) Biochim. Biophys. Acta 400, 167–177
28 Goodwin, T. W. and Morton, R. A. (1946) Biochem. J. 40, 628–632
29 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–766
30 Perry, S. V. (1973) Soc. Exp. Biol. Symp. 27, 531–550
31 Gazith, J., Himmelfarb, S. and Harrington, W. F. (1970) J. Biol. Chem. 245, 15–22
32 Dreizen, P. and Gershman, L. C. (1970) Biochemistry 9, 1688–1693
33 Kendrick-Jones, J., Lehman, W. and Szent-Györgyi, A. G. (1970) J. Mol. Biol. 54, 313–326
34 Jones, J. M. and Perry, S. V. (1966) Biochem. J. 100, 120–130
35 Potter, J. D. (1974) Arch. Biochem. Biophys. 162, 436–441
36 Sobieszek, A. and Bremel, R. D. (1975) Eur. J. Biochem. 55, 49–60
37 Weeds, A. G. and Lowey, S. (1971) J. Mol. Biol. 61, 701–725
38 Sarkar, S. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 14–17
39 Sreter, F. A., Gergely, J., Salmons, S. and Romanul, F. (1973) Nature New Biol. 241, 17–19
40 Sreter, F. A., Luff, A. R. and Gergely, J. (1975) J. Gen. Physiol. 66, 811–821
41 Askanas, V. and Engel, W. K. (1975) Neurology 25, 879–887
42 Frank, G. and Weeds, A. G. (1974) Eur. J. Biochem. 44, 317–334
43 Pires, E., Perry, S. V. and Thomas, M. A. W. (1974) FEBS Lett. 41, 292–296
44 Scordilis, S. P. and Adelstein, R. S. (1976) Biophys. J. 16, 118a
45 Pechère, J. F., Demaille, J., Capony, J. P., Dutruge, E., Baron, G. and Pina, C. (1975) in Calcium Transport in Contraction and Secretion (Carafoli, W., Clementi, F., Drabikowski, W. and Margreth, A., eds.), pp. 459–468, North-Holland, Amsterdam
46 Kretzinger, R. H. (1975) in Calcium Transport in Contraction and Secretion (Carafoli, E., Clementi, F., Drabikowski, W. and Margreth, A., eds.), pp. 469–478, North-Holland, Amsterdam
47 Offer, G. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 87–95
48 Morimoto, K. and Harrington, W. F. (1974) J. Mol. Biol. 83, 83–97
49 Morimoto, K. and Harrington, W. F. (1972) J. Biol. Chem. 247, 3052–3061
50 Pepe, F. A. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 97–108
51 Gaetjens, E., Bárány, K., Bailin, G., Oppenheimer, H. and Bárány, M. (1968) Arch. Biochem. Biophys. 123, 82–96
52 Dabrowska, R., Barylko, F., Nowak, W. and Drabikowski, W. (1973) FEBS Lett. 29, 239–242
53 Burtnick, L. D., McCubbin, W. D. and Kay, C. M. (1975) Can. J. Biochem. 53, 1207–1213
54 Weber, K. and Osborn, M. (1975) in The Proteins (Neurath, H. and Hill, R. L., eds.), 3rd edn., Vol. I, pp. 179–229, Academic Press, New York
55 Katz, A. M., Repke, D. I. and Cohen, B. R. (1966) Circ. Res. 19, 1062–1070
56 Murphy, R. A. and Hasselbach, W. (1968) J. Biol. Chem. 243, 5656–5662
57 Weber, A., Herz, R. and Reiss, I. (1964) Proc. Roy. Soc. (London) Ser. B. 160, 489–501
58 Sreter, F. A., Seidel, J. C. and Gergely, J. (1966) J. Biol. Chem. 241, 5772–5776
59 Samaha, F. J., Guth, L. and Albers, R. W. (1970) J. Biol. Chem. 245, 219–224
60 Gollnick, P. D., Armstrong, R. B., Saubert, C. W., Piehl, K. and Saltin, B. (1972) J. Appl. Physiol. 33, 312–319
61 Bárány, M. (1967) J. Gen. Physiol. 50, 197–218
62 Bárány, M. and Close, R. I. (1971) J. Physiol. 213, 455–474
63 Stracher, A. and Dow, J. (1971) Biochemistry 10, 1316–1321
64 Kritcher, E. M., Thyrum, P. T. and Luchi, R. J. (1970) Biochim. Biophys. Acta 221, 264–271
65 Holt, J. C. and Lowey, S. (1975) Biochemistry 14, 4609–4620
66 Bailin, G. and Bárány, M. (1968) Biochim. Biophys. Acta 168, 282–297