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Regulatory Properties of Single-Headed Fragments of Scallop Myosin†

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ABSTRACT: Calcium control was studied in single-headed myosin and subfragment-1 (S1) preparations obtained by papain digestion of scallop myosin. Single-headed myosin, containing light chains in stoichiometric amounts, was calcium regulated; in contrast, the actin-activated Mg–ATPase of all S1 species lacked calcium sensitivity. Both regulatory and essential light chains were retained by S1 and single-headed myosin preparations provided divalent cations were present during papain digestion, although a peptide amounting to 10% of the mass was removed from regulatory light chains. The modified regulatory light chain retained its ability to confer calcium binding and restore calcium sensitivity to the ATPase of desensitized myofibrils. Regulatory light chains protected the essential light chains from fragmentation by papain. S1 bound regulatory light chains with a uniformly high affinity and appeared to consist of a single species. The results demonstrate that head to head interactions are not obligatory for calcium control, although they may occur in the intact myosin molecule, and suggest a role for the subfragment-2 region in calcium regulation of myosin.

Contractile activity in all muscles is regulated by intracellular levels of calcium ion [cf. Ebashi & Endo (1968), Weber & Murray (1973), and Szent-Györgyi (1975)]. Upon stimulation, calcium is released into the sarcoplasm of the muscle and is bound by regulatory proteins on the myofilaments. Binding of calcium allows the two types of myofilaments to interact with each other to produce contractile force. The regulatory proteins are located on either or both types of filaments. In many invertebrates, notably mollusca, the regulation is associated with myosin, while in vertebrate striated muscles the regulatory proteins are located on the actin containing thin filaments (Kendrick-Jones et al., 1970; Lehman & Szent-Györgyi, 1975). Actin-linked regulation is mediated through the troponin complex and tropomyosin which prevent interaction of actin and myosin at low levels of calcium (Ebashi & Ebashi, 1964). The myosin molecule is an enzyme composed of two high molecular weight subunits (M_r 200 000) and two pairs of low molecular weight subunits (M_r 16 000–30 000) (Lowey & Risby, 1971; Weeds & Lowey, 1971). In molluscan systems, notably scallops, one pair of low molecular weight subunits (regulatory light chains) has been shown to be directly involved in regulation while the role of the other pair in regulation, if any, remains obscure (Szent-Györgyi et al., 1973; Kendrick-Jones et al., 1976).

One mole of regulatory light chain (R-LC)[1] can be removed from myosin of scallop by treatment with 10 mM EDTA at 0 °C, resulting in complete loss of regulation with concomitant loss of 1 mol of calcium binding sites per myosin (Szent-Györgyi et al., 1973). The actin-activated ATPase activity

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[1] Abbreviations used: S1, myosin subfragment-1; R-LC, regulatory light chain; SH-LC, essential light chain; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; IAA, iodoacetic acid; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane.

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of such a myosin no longer requires calcium; i.e., the regulatory subunit that is retained by one of the myosin heads is unable to prevent its interaction with actin. This observation suggested that an interaction between the two myosin heads, mediated through the light chains, was a requirement for relaxation; however, the possibility that the "unregulated" head interfered with the otherwise functional remaining head could not be ruled out [cf. Kendrick-Jones & Jakes (1977)]. To decide the question of whether or not two heads were an absolute requirement for relaxation, an investigation of regulation in single-headed derivatives of scallop myosin was carried out. The regulatory properties of myosin subfragment-1 (S1) and single-headed myosin were studied. Part of the work described here has been presented previously (Stafford et al., 1977; Szent-Györgyi et al., 1978).

Materials and Methods

Scallops (*Aequipecten irradians*) were obtained from the Marine Biological Laboratory, Woods Hole, MA. The cross-striated adductor muscles were excised, separated from the opaque smooth muscle portion, and stored in 50% glycerol, 20 mM NaCl, 2.5 mM phosphate, pH 7.0, 0.5 mM $MgCl_2$, 0.05 mM EDTA, 1.5 mM NaN_3, 0.05 mM phenylmethanesulfonyl fluoride, and 0.005% sodium diazine.

Preparation of Myosin. Scallop myosin was prepared by the method of Focant & Huriaux (1976) as modified by J. Sellers (personal communication). The glycerinated muscles were blended 2 times for 10 s in washing solution (40 mM NaCl and 5 mM phosphate buffer, pH 7.0) and washed twice. The resuspended myofibrils were brought to 0.5 M NaCl and 5 mM ATP, and the insoluble residue was removed by centrifugation. To the supernatant in an ice bath, EGTA (0.1 mM), $MgSO_4$ (20 mM), and additional 5 mM ATP were added, the pH was adjusted to 7.0 with Na_2HPO_4 (0.5 M), and it was brought to 40% saturation with ice-cold $(NH_4)_2SO_4$. The pH was readjusted to 7.0 during addition of $(NH_4)_2SO_4$. The precipitate, containing varying amounts of myosin and all the actin present, was discarded; the supernatant was made 70% saturated $(NH_4)_2SO_4$ and centrifuged again. The 70% precipitate was dialyzed entensively against the washing solution containing 0.1 mM EDTA and 1 mM $MgCl_2$. The suspension was washed to remove tropomyosin contamination, redissolved in 0.5 M NaCl, cleared by high-speed centrifugation, and dialyzed against the digesting solution.

Preparation of Myosin Subfragment-1 (S1). Papain digestion was carried out under two sets of conditions according to a modified version of the procedure reported by Cooke (1973). Washed myofibrils were digested in the presence either of excess calcium and magnesium or of EDTA. Myofibrils were either washed and digested in 0.1 M NaCl, 10 mM phosphate, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 1 mM EDTA, pH 7.0, to produce (Ca,Mg)S1 or washed with 40 mM NaCl and 5 mM phosphate, pH 7.0, and digested in the presence of 10 mM EDTA to produce (EDTA)S1. Digestion of washed myofibrils (10 mg/mL) with papain (Worthington Biochemical; twice recrystallized) was performed at 23 °C for 10 min, unless otherwise stated, by adding activated papain (Lowey et al., 1969) at a ratio of 0.03 unit/mg of myofibrils and stopped by adding iodoacetic acid (IAA), pH 7.3, to a final concentration of 5 mM. After further incubation for 5 min at 23 °C, unwanted digestion products, IAA, and papain were removed by washing several times at 4 °C. In most experiments, (Ca,Mg)S1 was isolated from digested myofibrils by the pyrophosphate extraction procedure of Cooke (1973) in the presence of 0.2 M NaCl and 1 mM magnesium pyrophosphate, pH 7.5. The extract was dialyzed against DEAE column buffer (10 mM imidazole, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 1 mM EDTA, pH 7.5), and insoluble material was removed by centrifugation for 15 min at 48000*g*. The supernatant, containing mainly S1 with some actin and tropomyosin, was applied to a Bio-Rad DEAE-Bio-Gel-A column (2.5 × 20 cm), and the S1 was eluted with a gradient of 0–0.15 M NaCl in column buffer.

The isolation of later (Ca,Mg)S1 and all (EDTA)S1 preparations followed the procedure described for myosin. The precipitate at 37% ammonium sulfate saturation, which contained mainly actin, was removed by low-speed contrifugation, and the supernatant which contained S1, myosin, single-headed myosin, myosin rod, and tropomyosin was made 70% in ammonium sulfate. The resulting precipitate was dissolved in 0.6 M NaCl and dialyzed overnight against column buffer. Insoluble material was removed by centrifugation, and the supernatant which contained predominantly S1 with some tropomyosin was applied to the DEAE-Bio-Gel-A column and eluted as described above.

Single-Headed Myosin. Washed myofibrils or myosin were routinely digested in a solution of 20 mM NaCl, 10 mM phosphate, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 1 mM EDTA, pH 7.0, at a ratio of 0.03 unit of papain per mg of myofibrils. The suspension was continuously stirred on a water bath at 23 °C. Digestion was terminated by adding IAA (cf. S1 preparation). Digestion of myosin heavy chain was carried out to 75% completion to give a high yield of single-headed myosin relative to double-headed myosin. The papain-digested myofibrils were diluted with a large volume of ice-cold washing solution, centrifuged, and washed twice more with the same buffer. Digested actomyosin and S1 were extracted with 0.5 M NaCl, 5 mM phosphate, and 2 mM ATP, pH 7.0. After centrifugation at 30000*g* for 15 min to remove insoluble residue, actomyosin together with actin-bound S1 was precipitated by a 10-fold dilution with ice-cold water, collected by centrifugation, and redissolved in 0.5 M NaCl. Ammonium sulfate was added to 37% saturation (now in the absence of ATP), precipitating both the digested myosin and S1 together with actin, leaving the free rod in the supernatant. The precipitate was redissolved in 0.6 M NaCl, 0.1 mM EGTA, and 20 mM $MgCl_2$. ATP (10 mM) was added to the solution and made 37% saturated in ammonium sulfate as described above for the preparation of myosin. The supernatant contained double- and single-headed myosin and S1 free of actin. The S1 could be removed easily by precipitation of myosin at low ionic strength. Attempts to remove more rod by a second ammonium sulfate fractionation of actomyosin in the absence of ATP led to considerable loss of calcium regulation, and, therefore, it was not performed routinely.

Preparation of Modified R-LC. Modified R-LC (mR-LC) was obtained from an extensively digested crude (Ca,Mg)S1 preparation (3 mg/mL), by using dithionitrobenzoic acid (DTNB) as described for the preparation of scallop R-LC (Kendrick-Jones et al., 1976). After 10-min incubation with DTNB, the pH was brought first to 5.1 and then to 7.0. The denaturated, dissociated S1 heavy chain was removed by centrifugation, and the supernatant was dialyzed against 25 mM phosphate, pH 6.0, and applied to a DEAE-Bio-Gel-A column in the same buffer. The regulatory light chains were eluted with a 25–400 mM phosphate gradient.

Exchange of R-LC. Exchange of mR-LC for intact R-LC in (Ca,Mg)S1 was studied under two conditions. Intact R-LC was added to S1 solutions (1) in 0.1 M NaCl, 10 mM imidazole, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 1.0 mM EDTA, pH 7.0, and (2) in the same buffer which had been made 4.0

mM in EDTA. After incubation in the EDTA buffer for 45 min, excess calcium and magnesium each were added to 5 mM final concentration. Samples were applied to a Sephadex G-150 column to remove excess light chain. Exchange was determined quantitatively by densitometry of urea gels.

$NaDodSO_4$ Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was carried out essentially according to the procedure of Shapiro et al. (1967), except that 0.05 M Tris–acetate, pH 8.1, was used instead of 0.1 M phosphate, and according to Weber & Osborn (1969). Gels were stained in either Coomassie Brilliant Blue R-250 or Acid Fast Green, depending on the loads and whether or not densitometry was to be performed.

Urea Gel Electrophoresis. Urea gel electrophoresis was carried out according to the procedure of Perrie & Perry (1970) as described previously (Kendrick-Jones et al., 1976).

Native Gel Electrophoresis. Gels were composed of 3% acrylamide, 0.08% *N,N'*-methylenebis(acrylamide), 5 mM sodium pyrophosphate, 0.33% *N,N,N',N'*-tetramethylethylenediamine, 10% glycerol, and a final ammonium persulfate concentration of 2.2%. The gel mixture was adjusted to pH 9.0, deaerated, and cooled to 4 °C before addition of ammonium persulfate. Gels (0.6 × 7 cm) were poured in the cold room. Polymerization occurred in less than 1 h. Samples (0.1 g/L) were dialyzed against running buffer (5 mM sodium pyrophosphate, pH 9.0) and made 10% in glycerol with bromophenol blue as the tracking dye. Electrophoresis was carried out in the cold room (4 °C) for 4 h at 140 V at a power consumption of about 0.08 W/(cm gel tube). Running buffer was recirculated from the lower to the upper buffer reservoir.

ATPase Measurements. ATPase measurements were carried out with a pH stat as described previously (Kendrick-Jones et al., 1970).

Protein Determination. Protein determinations were done either by the biuret method, by the Lowry method, or by measurement of absorbance at 280 nm. The extinction coefficient of scallop S1 was found to be 0.8 cm^2/mg and of scallop myosin, 0.53 cm^2/mg at 280 nm.

Calcium Binding. Calcium binding measurements were carried out by equilibrium dialysis of protein solutions or by double-labeling techniques in suspension as described previously (Kendrick-Jones et al., 1970; Szent-Györgyi et al., 1973) in the presence of 1 mM $MgCl_2$.

Analytical Ultracentrifugation. Sedimentation velocity runs were performed on a Beckman Instruments Model-E analytical ultracentrifuge, equipped with a UV photoelectric scanner. Equilibrium runs using the meniscus depletion method (Yphantis, 1964) were carried out on a Model-E equipped with an externally adjustable Rayleigh slit assembly attached to the collimating lens mount (Stafford, 1978). The camera lens was focused at the 2/3 plane and sapphire cell windows were used. Improved cell components and techniques described by Ansevin et al. (1970) were used. Protein samples were dialyzed for at least 24 h against their respective buffers (Cassasa & Eisenberg, 1964). A value of 0.728 cm^3/g was used for the partial specific volume. The speed was 16 000 rpm, and the temperature was 5 °C.

Results

Characterization of S1 Preparations. The light chain content of S1 preparations depended on the presence of divalent cations during digestion. When washed myofibrils were digested in the presence of millimolar calcium and magnesium ion, an S1 containing a stoichiometric complement of both light-chain types could be obtained [(Ca,Mg)S1] (Figure 1a,b). On the other hand, when digestion was carried out in the absence of divalent cations, an S1 which was depleted in light chains was obtained [(EDTA)S1] (Figure 1c,d). Both (Ca,Mg)S1 and (EDTA)S1 contained a major heavy-chain component of apparent chain weight of about 89 000 (Figure 2 and Table I). In the presence of calcium and magnesium, papain partially digested the R-LC, reducing its apparent molecule weight to approximately 16 000. Essential light chains (SH-LC) were not noticeably digested by papain in the presence of divalent cations. However, in excess EDTA (10 mM), both types of light chains were digested very rapidly. The R-LC was digested completely after the first few minutes while the SH-LC was converted first to one and then another fragmented form which resisted further digestion and had an apparent chain weight of 8000 determined by Laemmli slab $NaDodSO_4$–polyacrylamide gel electrophoresis with a gradient from 15 to 25% polyacrylamide. Identity of the SH-LC fragment was established by Ouchterlony double-immunodiffusion

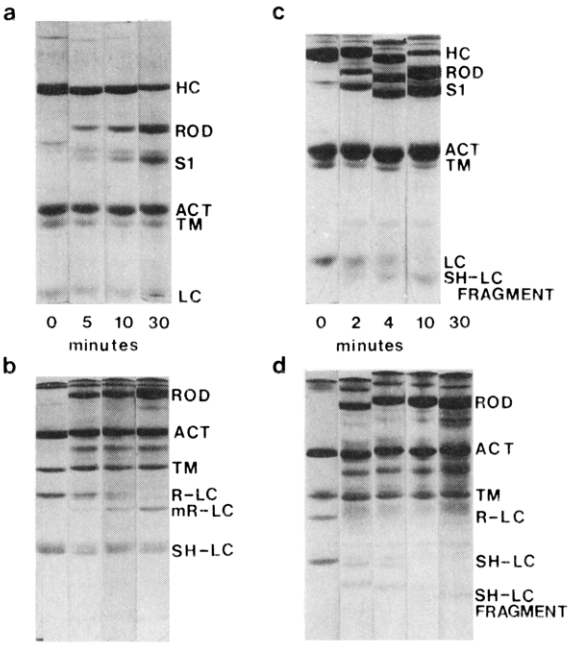

FIGURE 1: Time course of papain digestion of washed myofibrils. (a and b) Digestion in the presence of excess calcium and magnesium ions. (c and d) Digestion in the presence of excess EDTA. (a) and (c) are 4 and 8% $NaDodSO_4$–polyacrylamide gels, respectively, showing the digestion of the myosin heavy chain and the appearance of myosin rod and S1. (c) also shows digestion of the light chains in EDTA. (b) and (d) are 8% urea–polyacrylamide gels showing the digestion of the light chains (S1 heavy chain does not enter urea gels). In (b) note conversion of R-LC to mR-LC and that the SH-LC is not digested. In (d) note the rapid complete digestion of R-LC and conversion of SH-LC to SH-LC fragments. Abbreviations used: HC, heavy chain; ACT, actin; TM, tropomyosin; LC, total light chains.

Table I: Physical Properties

	(Ca,Mg)S1	(EDTA)S1
chain weights of $NaDodSO_4$ gels		
HC	~89 000	~89 000
modified R-LC	~16 000	
intact SH-LC	18 000	
SH-LC fragment		~8 000
M_r by sedimentation equilibrium (main component)	126 000	94 000
V_{max} (μmol of H^+ min^{-1} mg^{-1}) of actin-activated Mg-ATPase		
EGTA	3.5	4.4
EGTA + Ca	3.5	
Ca^{2+} binding	0.8 M Ca^{2+}/M S1 (at 5–8 × 10^{-6} M free Ca, pH 6.4)	traces

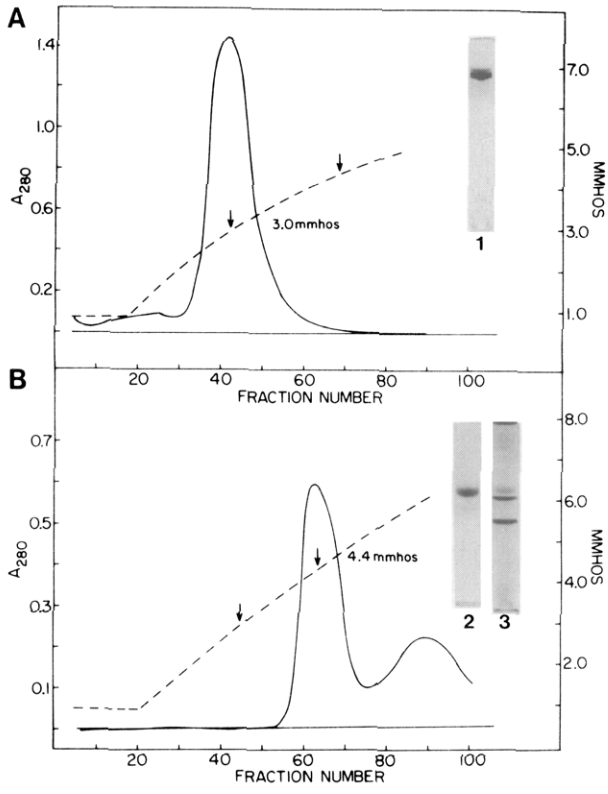

FIGURE 2: DEAE elution profiles of (EDTA)S1 and (Ca,Mg)S1. (A) (EDTA)S1 eluted at 0.07 M NaCl (3.0 mmho) and (B) (Ca,Mg)S1 eluted at 0.09 M NaCl (4.4 mmho; cell constant was 0.68 cm). (The second smaller peak in (B) is actin and is observed only in those preparations made by the pyrophosphate extraction procedure.) Arrows indicate relative elution positions of (EDTA)S1 and (Ca,Mg)S1. Inserts: (1) 8% $NaDodSO_4$–polyacrylamide gel of peak tube, (2) 5% $NaDodSO_4$, and (3) 10% urea–polyacrylamide gel of peak tube.

tests with antibodies prepared in this laboratory by Dr. T. Wallimann. (EDTA)S1 reacted with anti-SH-LC antibody but not with anti-R-LC antibody, while (Ca,Mg)S1 reacted with both antibodies.

Divalent cations did not protect SH-LC from digestion by papain in the absence of R-LC. When myofibrils, from which all R-LC had been removed by EDTA treatment at 35 °C (Chantler & Szent-Györgyi, 1978), were digested with papain in the presence of either excess magnesium or excess of both magnesium and calcium, an S1 with fragmented SH-LC was obtained. This material was indistinguishable from (EDTA)-S1.

Magnesium alone slows down the degradation of mR-LC but in the absence of calcium cannot prevent it. Attempts to achieve complete removal of the SH light chain fragment by lengthy digestion of purified myosin in the presence of EDTA were unsuccessful. An S1 preparation completely devoid of light chains could not be obtained.

Although S1 heavy chains moved as single bands on $NaDodSO_4$ gels, the preparations showed a time-dependent aggregation in sedimentation velocity and equilibrium ultracentrifugation studies. In fresh preparations a main component was seen with molecular weights of 94 000 ± 3000 for (EDTA)S1 and 126 000 ± 4000 for (Ca,Mg)S1, in good agreement with the values calculated from $NaDodSO_4$–polyacrylamide gel electrophoresis of the respective preparations (Table I).

Both types of S1 had high actin-activated Mg–ATPase activities but neither exhibited calcium sensitivity, although (Ca,Mg)S1 retained its specific calcium binding site (Table I). Lack of calcium sensitivity of ATPase activity could not

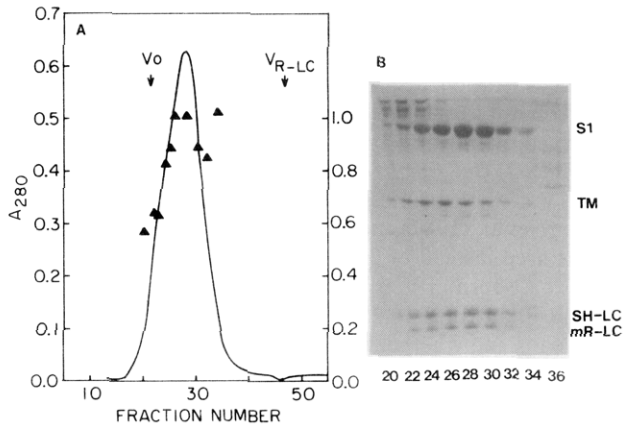

FIGURE 3: Chromatography of (Ca,Mg)S1 in 10 mM EDTA. Scallop myofibrils were digested with papain to about 70% completion. IAA was removed by precipitating the actin-bound proteins with 37% $(NH_4)_2SO_4$ to avoid losses in acto-S1 during washing. The low-salt soluble fraction was isolated by the procedure described for single-headed myosin. Protein (18 mg) was applied to a 1.5 × 100 cm Sephadex G-150 column which has been equilibrated with a solution of 0.5 M NaCl, 10 mM phosphate, and 10 mM EDTA at pH 7.0 and eluted (2.3-mL fractions) by using the same solvent. Eighty percent of the total OD units applied was recovered. (A) (—) A_{280} and (▲) molar ratio of total R-LC to SH-LC measured by densitometry of 10% acrylamide gels in 8 M urea. Over 90% of the R-LC was present as mR-LC. Note that the ratio mR-LC/SH-LC remains between 0.8 and 1.0 over the peak with lower values associated with the void-volume material. (B) 13% Laemmli microslab polyacrylamide gel electrophoresis of the fractions shown in (A). Fraction numbers of the samples are indicated below each sample slot.

be attributed to modification of the regulatory light chain since replacement of mR-LC with intact R-LC did not restore calcium sensitivity in S1. In addition, the mR-LC isolated from (Ca,Mg)S1 bound stoichiometrically to desensitized myofibrils containing 1 mol of R-LC per mol of myosin and restored calcium sensitivity. The question of whether or not mR-LC is capable of restoring Ca sensitivity in desensitized myofibrils from which both R-LC subunits have been removed is currently under investigation.

Calcium binding ability could be correlated with light-chain content. (Ca,Mg)S1 bound 1 mol of calcium while (EDTA)S1 bound little (<0.15 mol/mol), at 6.5×10^{-6} M Ca^{2+}, indicating that Ca binding depends on the presence of R-LC (Table I).

Light Chain–Heavy Chain Interactions. Generally, treatment of myosin with EDTA at 0 °C results in the removal of only 1 mol of R-LC per mol of myosin (Kendrick-Jones et al., 1976). However, treatment of (Ca,Mg)S1 with EDTA (Figure 3) did not remove significant amounts of mR-LC, suggesting that it binds to all the S1 heads with high affinity; that is, no evidence was found for two populations of S1 molecules, one with tightly bound and another with weakly bound mR-LC. The relatively low yields (38% of the theoretical S1) raised the question of whether or not the procedure was selecting for only a single type of S1 which had high affinity for mR-LC. However, an experiment in which desensitized myofibrils were digested indicated that both myosin heads were present in the final product. Papain digestion of myofibrils that had lost approximately 50% R-LC (by 0 °C EDTA treatment) in the presence of calcium and magnesium yielded two species of S1, one with and one without mR-LC. DEAE chromatography of the preparation showed S1 distributed over two peaks of approximately equal area and a major peak consisting of actin and tropomyosin. The first peak contained S1 lacking mR-LC and having an SH-LC fragment; the S1 of the second peak contained both mR-LC and intact SH-LC (Figure 4). This result indicates that the existence of single

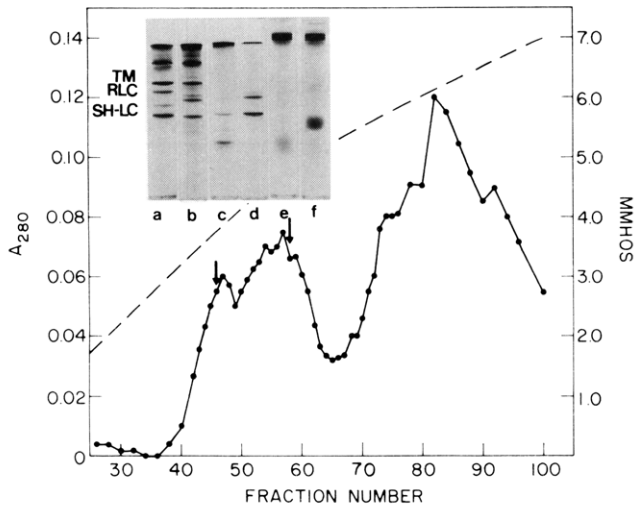

FIGURE 4: Formation of two S1 species from myosin with a reduced R-LC content. Myofibrils were treated with 10 mM EDTA for 10 min, washed twice (insert a), digested with papain, washed, extracted with pyrophosphate (insert b), and chromatographed on DEAE-Bio-Gel-A as described under Materials and Methods. Peaks between tubes 40 and 60 contain S1 and between 70 and 100 contain actin and tropomyosin. Insert: urea (a–d) and $NaDodSO_4$ (e and f) polyacrylamide gel electrophoresis; (c and e) tube 46 (arrow); (d and f) tube 57 (arrow).

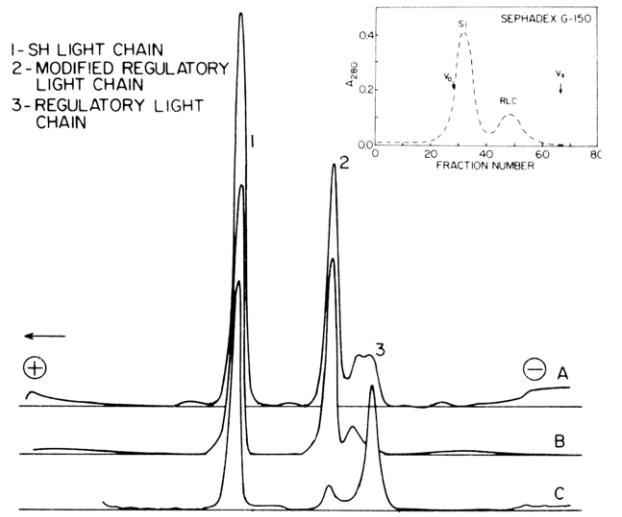

FIGURE 5: Exchange of intact R-LC for mR-LC on (Ca,Mg)S1. Densitometry tracings of 10% polyacrylamide gels in 8 M urea from the S1 peak fraction from the Sephadex G-150 column after the exchange experiment (see Materials and Methods for details). Peaks are 1, SH-LC, 2, mR-LC, and 3, intact R-LC. (The small peak between 2 and 3 (see Figure 1b) seems to be an intermediate digestion product in the conversion of R-LC to mR-LC.) (A) S1 peak fraction after incubation with intact R-LC in the presence of excess calcium and magnesium. (B) Control (Ca,Mg)S1 before exchange. (C) S1 peak fraction incubated with intact R-LC in the presence of EDTA. Insert: Sephadex G-150 elution profile showing the separation of excess unbound light chains from S1.

population of S1 is not an artifact of the purification procedure.

Modified R-LC could be replaced by intact R-LC in the presence of excess EDTA but not in the presence of calcium and magnesium (Figure 5). It seems that binding of one or both of these cations increases the affinity of R-LC for the S1 heavy chain inhibiting exchange. Some apparent exchange (<10%) of R-LC in the presence of divalent cations may be due to slow rate of exchange or some S1 lacking R-LC initially. It appears that the affinities of mR-LC and R-LC to S1 do not differ greatly. Intact R-LC was added in excess to S1 containing mainly mR-LC (Figure 5B) under exchange conditions, and the distribution of the bound light chains was measured after removing the excess light chains by chromatography on Sephadex G-150. Densitometry of urea gels showed that the ratio of R-LC to mR-LC on isolated S1's approximated their ratio in the original mixture.

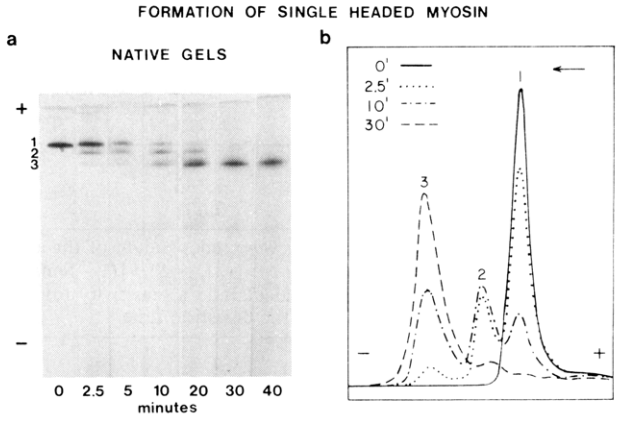

FIGURE 6: Native gels of papain digestion of myosin showing formation of single-headed myosin. (a) Bands are, from top to bottom, (1) double-headed myosin, (2) single-headed myosin, and (3) double-chained rod, respectively (cf. 10-min sample). (b) Densitometric tracing of gels at 0, 2.5, 10, and 30 min.

Single-Headed Myosin. Formation of a single-headed rabbit myosin intermediate during papain digestion was shown originally by Margossian & Lowey (1973a,b) and Lowey & Margossian (1974), who also isolated it on a preparative scale. In our experiments single-headed myosin could be demonstrated on native gels which showed an intermediate migrating between intact myosin and free rod (Figure 6). $NaDodSO_4$ gels showed no bands with a chain weight between myosin heavy chain and rod, indicating that the band seen on native gels was composed of both a heavy chain and a rod chain and was most likely single-headed myosin. A similar intermediate was seen on native gels during digestion of rabbit myosin by Cooke & Franks (1978) and was shown to be single-headed myosin. Our detailed kinetic analysis (see Figure 8A) of the time course of digestion of precipitated myosin supports this interpretation. The disappearance of double-headed myosin from native gels followed a first-order exponential decay (Figures 7 and 8A). The disappearance of heavy chain obtained from the analysis of $NaDodSO_4$ gels from the same experiment is shown also in Figure 7. The difference in the slope of the curve for intact myosin and for myosin heavy chain indicates that an appreciable amount of single-headed myosin was formed as an intermediate and that the myosin heads are removed randomly with about the same rate constants (Figure 8A). If both heads were cleaved simultaneously, the kinetics of disappearance of double-headed myosin and of myosin heavy chain would have been the same.

Rod, single-headed, and double-headed myosins, in contrast to S1, precipitate at low ionic strength. The ATPase activity of such precipitates thus represents only the contribution from single- and double-headed myosin. The actin-activated Mg–ATPase in the presence of calcium is proportional to the amount of heavy chain at each point during digestion (Figure 7), indicating that ATPase activity per myosin head is the same for double-headed and single-headed species as found previously for rabbit myosin (Margossian & Lowey, 1973b; Cooke & Franks, 1978). Therefore, it is possible to calculate the calcium sensitivity of single-headed myosin at each point during digestion.

The sensitivity of single-headed myosin at each time point was estimated from the experimentally determined calcium

Table II: Ca Sensitivity of Single-Headed Myosin Preparations from Myofibrils after Removal of Free Rod and S1

preparation, digestion time	% myosin heavy-chain digested	Mg-ATPase (μmol of H^+ min^{-1} mg^{-1})[a]		Ca sensitivity	ratio of single- to double-headed myosin, estimated	Ca sensitivity of single-headed myosin, estimated[b]
		EGTA	EGTA + Ca			
I, 15 min	76	0.019	0.124	0.85	3.7	0.80
II, 15 min	75	0.035	0.155	0.77	3.6	0.67
III, 15 min	76	0.031	0.132	0.77	3.7	0.67

[a] Specific activity and Ca sensitivity were independent of the actin to myosin weight ratio in a range of 0.13–0.71. [b] The following formula was used for the calculation: $Sens_S = 1 - [(2D + S)/S](1 - Sens)_t + (2D/S)(1 - Sens)_{t=0}$ for $S \neq 0$, where Sens is the observed total Ca sensitivity (defined in text), $Sens_S$ is the calculated Ca sensitivity for single-headed myosin, D and S are the mole fraction of double- and single-headed myosin, respectively, and t is the digestion time.

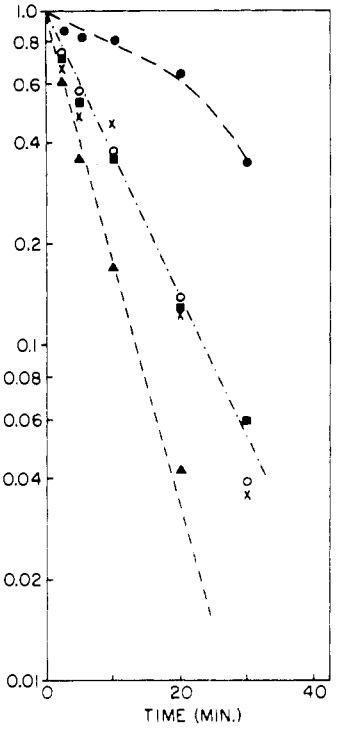

FIGURE 7: Time course of papain digestion of precipitated myosin. (▲) Double-headed myosin estimated by densitometry of native gels. (■) Total myosin heavy chain estimated by densitometry of $NaDodSO_4$ gels. (○) Mole fraction of undigested S1 heads (total S1 is assumed to represent 50% of myosin by weight) estimated by measuring the concentration of S1 released into the water-soluble fraction by the biuret method, expressed as $(S1_\infty - S1_t)/S1_\infty$. (×) Actin-activated Mg–ATPase in the presence of Ca^{2+}. (●) Calcium sensitivity. (All curves are normalized to 1.0.) (····) Calculated curve for myosin heavy chain on $NaDodSO_4$ gels assuming a 0.7 ratio for the rate constants of the digestion of the first and second myosin head.

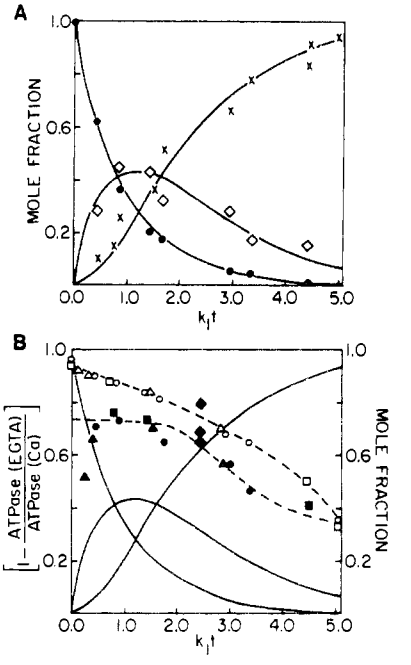

FIGURE 8: (A) Analysis of the time course of digestion of precipitated myosin on native gels. (●) Double-headed myosin. (◇) Single-headed myosin. (×) Double-chained rod. Two experiments were normalized on the time axis by plotting the data as a function of k_1t, where k_1 is the first-order rate constant measured for the disappearance of double-headed myosin (Figure 7). Solid lines are calculated curves assuming a ratio of 0.7 for the rate constants of the digestion of the first and second myosin head. (B) Calcium-sensitivity data plotted as a function of k_1t. Open symbols are total calcium sensitivity for three experiments. Circles and squares refer to the two experiments represented in (A). Closed symbols are the calculated sensitivity for single-headed myosin as explained in the text. (◆) Calculated sensitivity for the single-headed myosin component in the digested myosin preparations isolated from myofibrils shown in Table II for which the value of k_1t was 2.5. Broken lines show the trend of the data. Solid lines are the same as in (A).

sensitivity [1 − ATPase(EGTA)/ATPase(Ca)] of double-headed myosin (0.95), the observed sensitivity for the digested myosin after removal of S1, and the mole fractions of double- and single-headed myosin estimated from native gels (see Table II for the equations used to calculate sensitivity). The sensitivity of double-headed myosin was found in a separate control experiment to remain constant under the conditions of the digestion. Estimated values of the sensitivity of single-headed myosin were about 0.75 over the greater part of the reaction and decreased to a value of about 0.35 by the end. This decrease may be due to denaturation at lower concentrations. The results of three experiments are shown in Figure 8B.

In three large-scale preparations from myofibrils, digestion was carried out to about 76% completion as judged from $NaDodSO_4$ gels. This degree of digestion should yield a ratio of single- to double-headed myosin of about 3.7. After removal of S1, these digested myosin preparations had values of calcium sensitivity ranging from 0.76 to 0.85. Assuming that double-headed myosin had a sensitivity of 0.95, the estimated value for the calcium sensitivity of single-headed myosin ranged between about 0.67 and 0.80. These results agree well with those obtained in the more detailed kinetic studies of precipitated myosin and indicate that the digestion mechanism is probably the same for both precipitated myosin and myofibrils. Rod chains present in these preparations have been observed to copellet with actin, a behavior expected from single-headed myosin consisting of a myosin heavy chain and a rod chain (Figure 9). This independent observation further supports our contention that single-headed myosin is formed by our digestion procedure. The mixture of double- and single-headed myosin bound 1.07 mol of calcium per myosin head with an association constant of about 5×10^5 M, suggesting that single-headed myosin retains a high-affinity, specific calcium binding site.

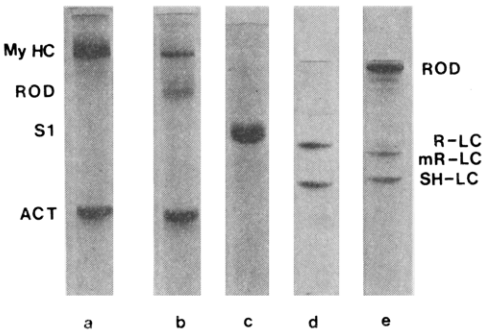

FIGURE 9: Subunit structure of single-headed myosin. A low-salt insoluble fraction of digested myosin was pelleted in the presence of actin to demonstrate that rod chains are associated with myosin heavy chains, an indication for the presence of single-headed myosin (see text). (a–c) 5% $NaDodSO_4$–polyacrylamide gel electrophoresis; (d and e) 10% urea–polyacrylamide gel electrophoresis. (a and d) Actin pellet before digestion with papain. (b and e) Actin pellet after 30-min digestion. (c) Water-soluble fraction after digestion.

The cleavage of R-LC to mR-LC proceeds at approximately the same rate as the disappearance of double-headed myosin. Therefore, preparations consisting mostly of single-headed myosin contain mostly mR-LC (Figures 8 and 9). The calcium sensitivity of single-headed myosin indicates that mR-LC, present in stoichiometric amounts (Figure 9), is competent to confer regulation. The lack of sensitivity of (Ca,Mg)S1 that contains mR-LC thus is unlikely to be due to light-chain modification.

Discussion

Divalent cations protect the regulatory light chains from extensive fragmentation during papain digestion [cf. Szent-Györgyi et al. (1973) in the case of scallop myosin and Margossian et al. (1975) and Bagshaw (1977) in the case of rabbit myosin]. S1 preparations formed in the presence of calcium and magnesium contain both R-LC and SH-LC in stoichiometric amounts with heavy chain. R-LC obtained in the presence of calcium and magnesium loses a peptide of approximately 1500 molecular weight while the SH-LC remains apparently intact. The protection of the R-LC by divalent cations may be the result of the role of these ions in holding the R-LC onto the myosin heads. The susceptibility of R-LC to digestion in the absence of divalent cations is likely to be due to the dissociation of R-LC at room temperature. In the absence of divalent cations, the SH-LC also is digested by papain, and a fragment of about 8000 molecular weight is formed and retained by the heavy chain. It appears, however, that SH-LC is protected by R-LC and not directly by divalent cations, since digestion of myosin preparations from which both R-LC subunits have been removed by 35 °C EDTA treatment yielded fragmented SH-LC even in the presence of divalent cations.

Loss of the 1500 molecular weight piece from R-LC does not significantly alter its functions. mR-LC binds to heavy chains with an affinity comparable to that of intact R-LC, contributes to calcium binding, confers calcium sensitivity to myosin, and protects SH-LC from fragmentation by papain. The missing piece has excess basic residues as judged from the change in mobility on urea gels and probably represents the N terminus of the chain (Jakes et al., 1976; Kendrick-Jones & Jakes, 1977). One notes that the 1500 molecular weight fragment may or may not be retained after digestion and could possibly contribute to the sensitivity of single-headed myosin.

R-LC is not readily dissociated from S1 preparations at 4 °C by EDTA in contrast to myosin which loses 1 of its 2 mol of R-LC by similar treatment. S1 preparations appear to have a uniformly high affinity for R-LC. Therefore, no evidence for two populations of S1 was obtained although such a finding could have accounted for the behavior of myosin in EDTA. The simplest interpretation of these results is to assume that the binding of the second light chain on the myosin molecule is weakened because of an interaction between the myosin halves. This assumption is supported by ATPase studies indicating that R-LC is bound to myosin with a negative cooperativity (Chantler and Szent-Györgyi, unpublished experiments). However, we cannot exclude the possibility that the relatively high affinity of R-LC for S1 may be due to the proteolysis in the S1–S2 hinge region. It is of some interest that DTNB treatment also removes only about 1 of the 2 mol of R-LC from rabbit myosin (Gazith et al., 1970; Weeds & Lowey, 1971).

Lack of calcium sensitivity in S1 is due to proteolysis of the heavy chain and not R-LC. (Ca,Mg)S1 containing intact R-LC is unregulated while single-headed myosin containing mR-LC is regulated.

Calcium sensitivity of single-headed myosin preparations proves that calcium regulation does not require direct interaction between two myosin heads. The difference in sensitivity between S1 preparations on the one hand and myosin, HMM, or single-headed myosin on the other cannot be explained on the basis that S1 is single headed but rather may be due to the absence of the S2 region from S1 preparations. Somehow, the S2 region, possibly the S1–S2 hinge, may be required for positioning the R-LC in the "off" position. On the basis of experiments showing protection of rabbit heavy chain by DTNB light chain during chymotrypic digestion in the presence of divalent cations (Weeds & Taylor, 1975; Weeds & Pope, 1977; Bagshaw, 1977), it was suggested that the DTNB light chain, which is analogous to R-LC, may extend to the S1–S2 hinge region. One notes that the scallop R-LC is an elongated molecule with length approaching 100 Å (Stafford & Szent-Györgyi, 1978; Hartt & Mendelson, 1979; Alexis & Gratzer, 1978), and it is perhaps not surprising if one end would extend to the region where S1 and S2 join.

Removal of 1 of the 2 mol of R-LC leads to a loss of regulation of scallop myosin (Szent-Györgyi et al., 1973). These findings were interpreted to mean that regulation requires a light chain mediated interaction between the two myosin heads. Since single-headed myosin was found to be Ca sensitive, the loss of regulation with EDTA treatment at 0 °C could not simply be attributed to loss of communication between the myosin halves. Possibly the remaining R-LC or the heavy chains undergo conformational changes which interfere with regulation but do not occur in the intact myosin [cf. Kendrick-Jones & Jakes (1977)]. Scallop myofibrils containing 2 mol of foreign R-LC, prepared from myosins which are not regulated, lack calcium regulation (Chantler et al., 1979), while mixed hybrids, retaining 1 mol of scallop R-LC in addition to 1 mol of foreign light chain, are fully calcium sensitive (Kendrick-Jones, 1974; Kendrick-Jones et al., 1976). This latter observation is difficult to explain without postulating an interaction between the two myosin halves.

In summary, even though calcium regulation is possible without interaction between the myosin heads, experiments reported here do not contradict the possibility of an interaction between the halves of an intact molecule. The consequences of this interaction are different from the original interpretation. The calcium sensitivity of single-headed myosin excludes the previous model in which light chain mediated cooperativity between the two myosin heads leads to regulation (Szent-Györgyi et al., 1973). The S2 region of myosin, possibly the

S1–S2 hinge, may be involved in calcium control.

Acknowledgments

We thank Dr. Theo Wallimann for preparing specific light-chain antibody and performing Ouchterlony immunodiffusion tests for us and Dr. Elaine Lai for performing polyacrylamide gradient slab electrophoresis. We thank Regina Niebieski for expert technical assistance. We also thank Drs. Peter Chantler, Carolyn Cohen, Susan Lowey, James Sellers, Peter Vibert, and Theo Wallimann for reading the manuscript and for helpful discussions. We thank Dr. Susan Lowey for use of her Model-E ultracentrifuge. We thank Dr. David DeRosier for the use of his flatbed scanner and computing facilities.

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