Subunit Function in Cardiac Myosin: Effect of Removal of Lc_2 (18 000 Molecular Weight) on Enzymatic Properties†

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ABSTRACT: The 18 000-dalton subunit of dog cardiac myosin (Lc_2) was selectively removed by the treatment of myosin with a cardiac myofibrillar protease under mild experimental conditions. Removal of Lc_2 did not affect the Ca^{2+} ATPase activity of myosin. The basic Mg^{2+} ATPase and actin-activated Mg^{2+} ATPase activities (in 0.1 M KCl) were significantly altered as a consequence of removal of the Lc_2. A comparison of native myosin and Lc_2 deficient myosin showed (a) the actin-activated ATPase of Lc_2 deficient myosin was increased threefold over that of native myosin; identical results were obtained with respect to this measurement with pure actin or regulated actin as a cofactor; (b) the actin-activated ATPase of Lc_2 deficient myosin was relatively insensitive to increase in KCl concentration; (c) Mg^{2+} ATPase of actomyosin, reconstituted with Lc_2 deficient myosin, showed a fivefold increase in the optimum substrate (Mg^{2+} ATP) concentration, indicating a resistance of the rigor complexes to dissociation by ATP; (d) Ca^{2+} sensitivity of the actin-activated ATPase (in the presence of troponin–tropomyosin) was identical for both the Lc_2 deficient and native myosins; (e) 18 000 dalton light chain from canine cardiac and rabbit skeletal myosin was found to reassociate with the Lc_2 deficient myosin with the lowering of both Mg^{2+} ATPase and actin-activated ATPase activities to values close to those for native myosin. The results of our study suggest that removal of Lc_2 from cardiac myosin changes the conformation of myosin to a state that enhances its interaction with actin. Evidence is also presented for functional homology between the cardiac Lc_2 and 18 000-dalton light chain of skeletal muscle myosin.

The role of low molecular weight subunits (light chains) of myosin in regulating the ATPase activities of myosin and the properties of the integrated actomyosin system has been the subject of study by a number of investigators. Studies of Dreizen & Gershman (1970), and Dreizen & Richards (1972), showed a close relationship between ATPase inactivation and the dissociation of light chains of myosin under denaturing conditions. From the studies of Sarkar (1972) and Wagner & Weeds (1977), it is apparent that skeletal muscle myosin may exist as an isozyme with either alkali 1 (A_1) or alkali 2 (A_2) light chain. Most conclusive evidence about the occurrence of myosin isozymes has been provided in the studies of Holt & Lowey (1977) who showed that the two heads of a myosin molecule contain only a single species of the alkali light chain. The function of alkali light chains is not fully clear yet. Wagner & Yount (1976) demonstrated that the modification of alkali light chains by a purine disulfide analogue of ATP inhibited both ATPase activity and actin binding properties of myosin. Myosin subfragments characterized by the presence of either A_1 or A_2 do not differ with respect to Ca^{2+} or Mg^{2+} ATPase activities. However, the kinetic parameters for actin activated Mg^{2+} ATPase are significantly different between the two types of subfragments (Wagner & Weeds, 1977).

A common feature of myosins from mammalian muscle types is the existence of a light chain of approximate molecular weight of 18 000 which can be readily phosphorylated (Perrie et al., 1973). In skeletal myosin this subunit can be removed partially under relatively mild conditions by treatment with Nbs_2[1] (Gazith et al., 1970; Weeds & Lowey, 1971). Removal of this light chain does not significantly affect the Ca^{2+} and K^+–EDTA ATPase activities of rabbit skeletal myosin. In contrast, treatment of the cardiac myosin with DTNB does not lead to the release of the 18 000-dalton light chain. Cardiac myosin subfragments (Bhan & Malhotra, 1976; Weeds & Frank, 1972) and chymotryptic subfragments of skeletal myosin (Weeds & Taylor, 1975) are characterized by the lack of the 18 000 dalton subunit; yet the Ca^{2+} and K^+–EDTA ATPase activities are retained at normal levels. Thus, it would seem that the 18 000 dalton light chain from striated muscle myosin is not necessary for the expression of the ATPase activity. The fact that the 18 000 dalton subunit from cardiac or skeletal myosins can effectively substitute for the EDTA light chain of scallop myosin with the restoration of full Ca^{2+} sensitivity indicates a functional homology between the Nbs_2 light chain and cardiac Lc_2 (Kendrick-Jones et al., 1976). Nbs_2 light chain can bind Ca^{2+} reversibly under physiological conditions (Morimoto & Harrington, 1974), but a significant role in regulating the calcium sensitivity of the actomyosin has not been demonstrated. Recent studies of Pemerick (1977) show shifts in the Ca^{2+} level requirement of actomyosin reconstituted with Nbs_2 light chain deficient myosin, and effective reversal to normal values on addition of the purified light chain to the DTNB myosin. In contrast, Holt & Lowey (1975a,b), using antibodies directed against Nbs_2 light chain, showed that the Ca^{2+} regulation through troponin–tropomyosin system was unaffected; however, in the presence of antisera to light chains, the actin activated ATPase was lower. The studies dealing with the role of the 18 000-dalton light chains in skeletal myosin have not been conclusive. This may be due to the fact that there is never a complete removal of the light chain after Nbs_2 treatment. The residual light chains might be sufficient to maintain the conformation of myosin in its native state and thus mask changes which would be obvious if the removal of the light chain was complete.

Phosphorylation of the 18 000-dalton subunit of smooth muscle and platelet myosins profoundly influences their actin activated ATPases (Gorecka et al., 1976; Adelstein & Conti,

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Abbreviations used: Nbs_2, 5,5-dithiobis(2-nitrobenzoic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; M-Lc_2, light chain deficient myosin.

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1975). Although the 18 000-dalton subunit from cardiac and skeletal myosins can be phosphorylated by specific "light chain kinases" (Frearson & Perry, 1975; Perrie et al., 1973; Pires et al., 1974), no correlations are known to exist between the actin-activated ATPase and the state of phosphorylation of this subunit. In perfused rabbit hearts a decrease in phosphorylation of the 18 000-dalton subunit was observed to occur in conditions characterized by increased contractility (Frearson et al., 1976a), suggesting a possible physiological significance for the phosphorylation, dephosporylation of this subunit. In contrast recent studies of Barany & Barany (1977) showed that phosphorylation of myosin light chain in frog skeletal muscle was associated with increased contractile force. To date no studies are available in the literature on the function of the cardiac Lc_2 in regulating the properties of cardiac myosin. This is primarily due to the fact that it has been impossible to selectively remove this subunit under conditions which do not denature the myosin active site.

In this paper we will describe (a) a method for the complete removal of the 18 000-dalton light chain (Lc_2) from cardiac myosin and (b) the effects of removal of this light chain on the enzymatic properties of myosin. We will also present evidence for functional homology between Lc_2 and Nbs_2 light chain of skeletal myosin.

Material and Methods

ATP, Nbs_2, and EGTA were purchased from Sigma, St. Louis, MO; soybean trypsin inhibitor was from Nutritional Biochemicals, Cleveland, Ohio.

Myosin was prepared from left ventricle of dog hearts and leg and back muscles of rabbits as described earlier (Bhan & Malhotra, 1976). Actin was extracted from the rabbit skeletal muscle acetone powder and purified by a combination of procedures of Bailin & Barany (1972) and Spudich & Watt (1971). Troponin and tropomyosin were purified from the rabbit skeletal muscle as detailed by Greaser & Gergley (1971). Regulated actin was reconstituted by combining purified actin, troponin, and tropomyosin at 1:0.5:0.5 weight ratios in 0.1 M KCl–2 mM $MgCl_2$ and 10 mM Tris-Cl, pH 7.6 (Daniel & Hartshorne, 1972). Nbs_2 light chain was prepared by treatment of the rabbit skeletal myosin with DTNB and EDTA by the method of Weeds & Lowey (1971) and purified by DEAE-cellulose chromatography. Canine cardiac myosin light chains (phosphorylated and unphosphorylated) were a gift from Dr. R. S. Adelstein.

Preparation of Lc_2 deficient myosin. We have recently isolated a protease fraction from the hearts of genetically dystrophic hamsters which can specifically destroy the 18 000-dalton subunit of cardiac myosin (Bhan et al., 1978a,b). Dog cardiac myosin (~50 mg) in 0.4 M KCl, 0.001 M EDTA, and 0.01 M imidazole buffer, pH 6.9, was incubated with 1.0 mg of the purified myofibrillar protease for 20 h at 4 °C. The reaction was terminated by the addition of 3 mg of soybean trypsin inhibitor. Myosin was precipitated by tenfold dilution with cold deionized water. The precipitated myosin was collected by centrifugation at 100 000*g* for 1 h and dissolved in 0.4 M KCl, pH 7.0.

Reassociation of the Lc_2 deficient cardiac myosin with the cardiac Lc_2 or Nbs_2 light chain was performed by equilibrating Lc_2 deficient myosin with the cardiac Lc_2 or Nbs_2 light chain (molar ratio of 1:10) in 0.6 M KCl, 0.002 M dithiothreitol 0.002 M EDTA, 0.001 M $MgCl_2$, and 0.01 M imidazole, pH 6.9, for 24 h at 0 °C. The mixture was diluted tenfold with glass-distilled water to precipitate myosin. Myosin was dissolved in a minimum volume of the equilibration buffer and chromatographed on an analytical column of Sephacryl S-200 (0.9 × 40 cm) to separate the excess of the light chains. The column was equilibrated and eluted with 0.4 M KCl, 0.002 M EDTA, and 0.01 M imidazole, pH 6.9. Myosin peak (void volume) was used for the enzymatic measurements and electrophoresis.

ATPase activity measurements were performed in a final volume of 2 mL at pH 7.6, 30 °C. For the Ca^{2+}-dependent ATPase, the reaction mixture consisted of KCl (0.3–0.6 M), 0.05 M Tris-Cl, pH 7.6, 0.01 M $CaCl_2$, 0.005 M Tris–ATP, and 100–150 μg of myosin. K^+–EDTA ATPase activity was measured in 0.6 M KCl and 0.01 M EDTA. Actin-activated Mg^{2+} ATPase activity was measured in 0.1 M KCl, 0.009 M Tris-Cl, 0.002 M Mg–ATP, 180 μg of myosin, and varying amounts of actin. In studies with regulated actin, Ca^{2+}, 10^{-4} M, was also present. Any deviations in experimental conditions are described in the legends to the figures. The reaction was initiated by the addition of the substrate and terminated after 20 min by the addition of 1.0 mL of cold 10% Cl_3CCOOH. Inorganic phosphate was generally determined by the method of Fiske & Subbarow (1925). However, in experiments dealing with the effect of substrate concentration on actin-activated ATPase, the method of Martin & Doty (1949) was used to measure the inorganic phosphate. Protein concentration was determined by the biuret technique using bovine serum albumin (BSA) as standard. Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis was performed in a discontinuous system according to Maizel (1971). Protein samples in 1% $NaDodSO_4$, 1% β-mercaptoethanol, and 0.05 M Tris–glycine, pH 8.0, were heated in a boiling water bath for 2 min. Samples, containing 10% glycerol and traces of bromophenol blue, were layered directly on top of the resolving gel (10–15% cross-linked). Tris–glycine, 0.05 M, pH 8.6, containing 0.1% $NaDodSO_4$ was used as the reservoir buffer. Electrophoresis on 5% cross-linked gels was performed in the $NaDodSO_4$–phosphate system of Weber & Osborn (1969). The electrophoresis was carried out at 2 mA per tube until the dye entered the gel and then at 10 mA per tube for 4 h. Staining and destaining of the gels was done according to Weber & Osborn (1969).

Results

Figure 1 shows the $NaDodSO_4$ gel electrophoretic pattern of native dog cardiac myosin and protease treated myosin on 5% (scan) and 10% cross-linked gels (inset). The myosin was essentially free of actin, tropomyosin, and troponin. M line and C protein were present. However, we did not make any attempt to further purify myosin because the presence of these proteins did not interfere with the enzymatic measurements. The preparation of actin was free of tropomyosin and troponin. Protease treated myosin showed a complete lack of the 18 000-dalton subunit. It is also apparent from the gel scans that the heavy chains of myosin were virtually intact after protease treatment. The ATPase activities of native and Lc_2 deficient (M-Lc_2) myosin are shown in Table I. The Ca^{2+} ATPase activity (in 0.3 or 0.6 M KCl) was not altered by the removal of Lc_2. However, the K^+–EDTA ATPase of the Lc_2 deficient myosin was 25% lower ($p = 0.001$, 9 studies) and the Mg^{2+} ATPase in 0.1 M KCl was 75% greater ($p = 0.001$, 17 studies) as compared with these activities in the native myosin. Ca^{2+} ATPase activity of native myosin and M-Lc_2 as a function of increasing KCl concentration showed identical profile of inhibition.

Figure 2 shows the actin-activated ATPase activities of native and Lc_2 deficient myosin as a function of increasing actin concentration. The ATPase activity of M-Lc_2 was significantly enhanced over that of the native myosin. The

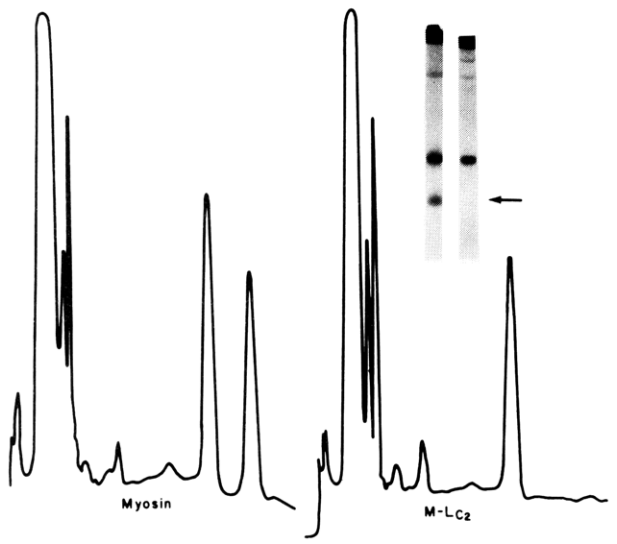

FIGURE 1: $NaDodSO_4$ gel electrophoretic patterns of native and protease treated dog cardiac myosin. Scans show the profile on 5% gels in $NaDodSO_4$ phosphate system. Inset: Electrophoretic pattern on 10% gels in the discontinuous system of Maizel (1971). Sample loading was approximately 40 μg.

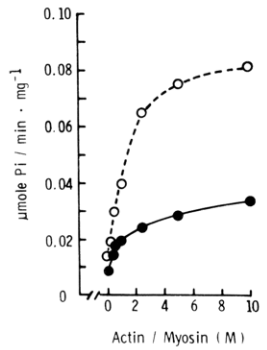

FIGURE 2: Mg^{2+} ATPase of native (●—●) and Lc_2 deficient (O---O) myosin as a function of increasing F-actin concentration.

actomyosin–ATPase rates with pure actin as cofactor, obtained from the Lineweaver–Burk plots, were 0.037 μmol of P_i mg^{-1} min^{-1} for native myosin and 0.1 μmol mg^{-1} min^{-1} for the Lc_2 deficient myosin. The K_{app} for pure actin was approximately 40% lower in M-Lc_2.

Figure 3 shows the Lineweaver–Burk plots of the Mg^{2+} ATPase of native myosin and M-Lc_2 vs. increasing concentration of regulated actin. The results presented here show mean values of eight different experiments. With regulated

Table I: ATPase[a] Activities of Native, M-Lc_2, and Reconstituted Myosins

	Ca^{2+} ATPase		K^+ ATPase	Mg^{2+} ATPase	actin-activated ATPase
	0.3[b] (3)	0.6[b] (9)	0.6[b] (9)	0.1[b] (17)	0.1[b] (3)
native myosin	0.36 ±0.01	0.28 ±0.01	0.71 ±0.04	0.0074 ±0.002	0.040
M-Lc_2	0.38 ±0.02	0.29 ±0.02	0.53 ±0.04	0.0128 ±0.002	0.090
M-Lc_2 + cardiac Lc_2				0.008	0.041
M-Lc_2 + Nbs_2 light chain				0.0085	0.0551

[a] Micromoles of P_i per milligram of protein per minute; results are mean ± SD. Values in parentheses show the number of studies. Myosin was 160 μg and actin, 460 μg, in the assay for actin-activated ATPase. [b] M KCl.

Table II: KCl Concentration vs. Actin-Activated ATPase[a] of Native and Lc_2 Deficient Myosin

actin/myosin (M)		KCl (M) 0.027	0.050	0.100
5	myosin	0.079	0.050 (37)	0.028 (65)
	M-Lc_2	0.129	0.113 (13)	0.090 (30)
10	myosin	0.095	0.068 (38)	0.032 (67)
	M-Lc_2	0.145	0.120 (12)	0.094 (35)

[a] $(V - V_0)$, μmol of P_i per mg per min; myosin (M-Lc_2) concentration was fixed at 180 μg. Values in parentheses indicate percentage inhibition.

actin there was only marginal potentiation of the ATPase activity over that observed with pure actin. As with pure actin, V_{max} was threefold greater in case of M-Lc_2. The K_{app} for regulated actin was sixfold lower in M-Lc_2 (0.8 μmol/L for M-Lc_2 and 5.0 μmol/L for native myosin). Table II shows the effect of KCl concentration on the actin-activated ATPase of M-Lc_2 and native myosin. With increasing KCl concentration, inhibition of the ATPase activity was apparent in both myosin and M-Lc_2. However, the extent of inhibition at higher KCl concentration was greater in native myosin. Thus increasing the KCl concentration from 0.027 to 0.1 M caused 65% inhibition of the ATPase of the native myosin as opposed to 30% in the case of M-Lc_2. From the data in Table II it is also apparent that at lower KCl concentrations the differences in the ATPase activities of the native myosin and M-Lc_2 are narrowed.

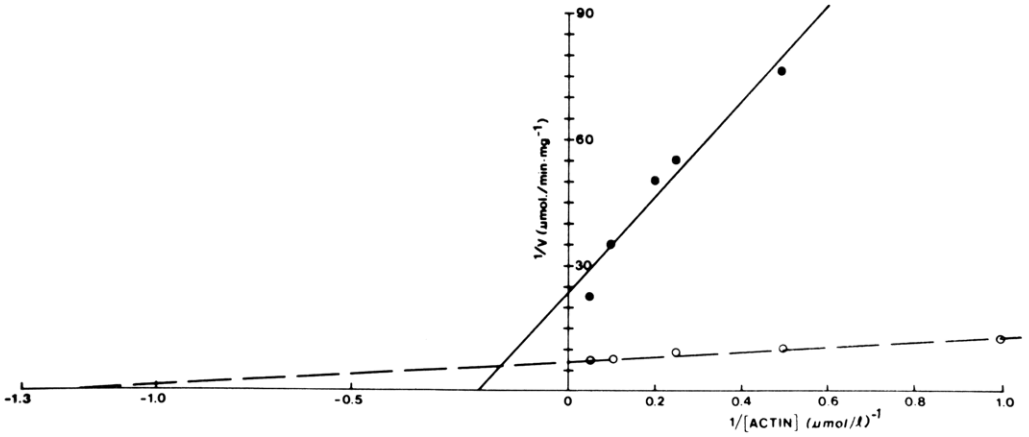

FIGURE 3: Lineweaver–Burk plots of actin-activated Mg^{2+} ATPase activities of native and Lc_2 deficient myosin with regulated actin as cofactor. (●—●) native myosin; (O---O) Lc_2 deficient myosin. Best fit linear regression line is shown.

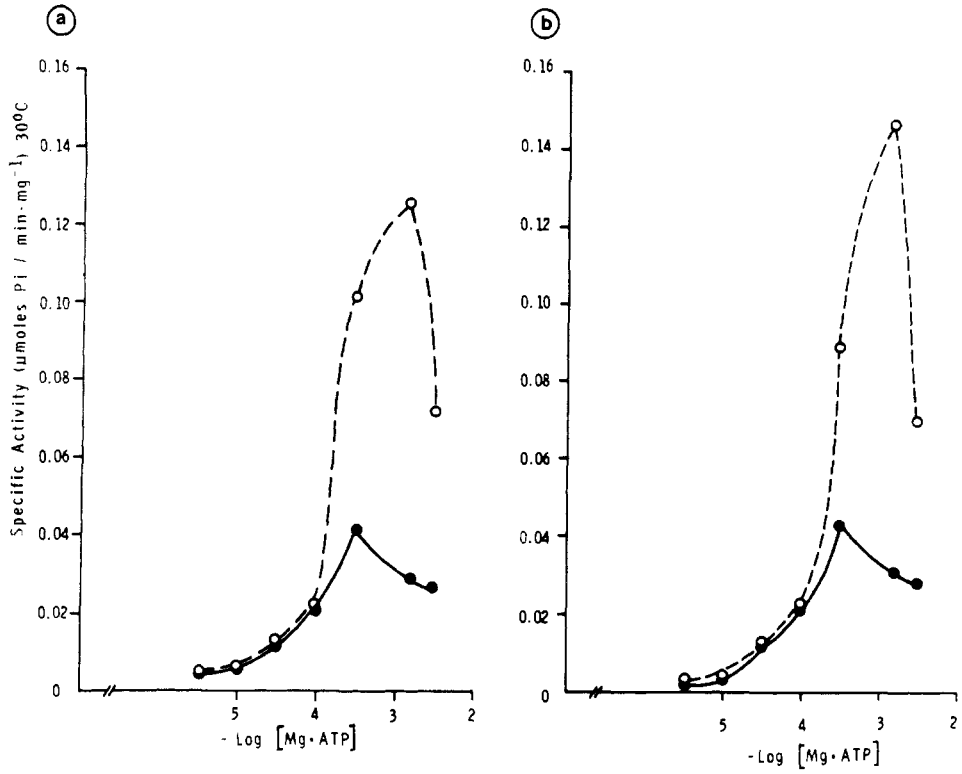

FIGURE 4: Actin-activated Mg^{2+} ATPase of native (●—●) and Lc_2 deficient (O- - -O) myosin as a function of Mg^{2+} ATP concentration in the presence of pure actin (a) and regulated actin (b). (Actin) = 460 μg; (myosin) and (M-Lc_2) = 180 μg.

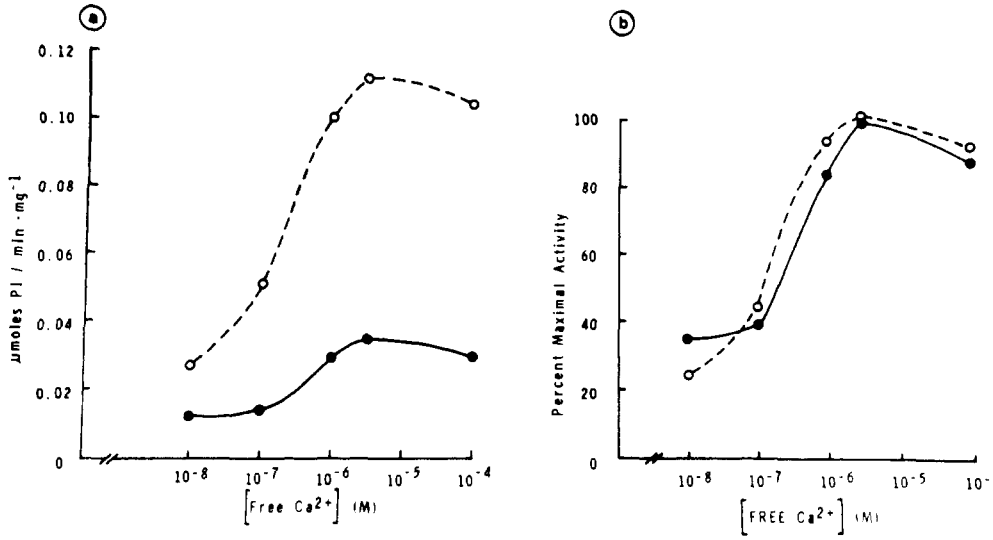

FIGURE 5: Actin-activated ATPase of native (●—●) and Lc_2 deficient (O- - -O) myosin as a function of free Ca^{2+} concentration. (a) Absolute activity; (b) data normalized to highest ATPase activity.

Figure 4 compares the effect of increasing MgATP concentration on the actin activated ATPase activity of native myosin and M-Lc_2 with pure actin in the absence of Ca^{2+} (Figure 4a) and with regulated actin, in the presence of Ca^{2+} (Figure 4b). The ATPase activity showed a biphasic response to increase in the concentration of the substrate. The activation, inhibition profiles were identical for both types of myosin in studies with pure actin and the regulated actin. However, the optimum increase in the ATPase of native myosin was obtained at 0.5×10^{-3} M ATP in contrast to 2.5×10^{-3} M ATP for M-Lc_2.

Actin-activated ATPase of native myosin and M-Lc_2 (in presence of troponin and tropomyosin) as a function of the Ca^{2+} ion concentration is shown in Figure 5. No significant shifts were observed in the Ca^{2+} ion requirements as a consequence of removal of the Lc_2 from myosin; however, the absolute values for the ATPase activity at any Ca^{2+} concentration were higher with M-Lc_2 (Figure 5a).

The results of equilibration of M-Lc_2 with the cardiac Lc_2 and Nbs_2 light chains are shown in Figure 6. Shown are the $NaDodSO_4$ gel patterns of light chains in native myosin (panel A), M-Lc_2 (panel B) and M-Lc_2 after equilibration with cardiac Lc_2 (panel C), and Nbs_2 light chain (panel D). The binding of externally added light chains to M-Lc_2 is evident. The ratio of Lc_2 to Lc_1 in native myosin was approximately 0.8. Approximately 50% of the cardiac Lc_2 and 30% of the Nbs_2 light chain were incorporated back into the molecule.

Table I shows the effects of binding of cardiac Lc_2 and Nbs_2 light chain on the Mg^{2+} ATPase and actin-activated ATPase activities of Lc_2 deficient myosin. The Mg^{2+} ATPase and

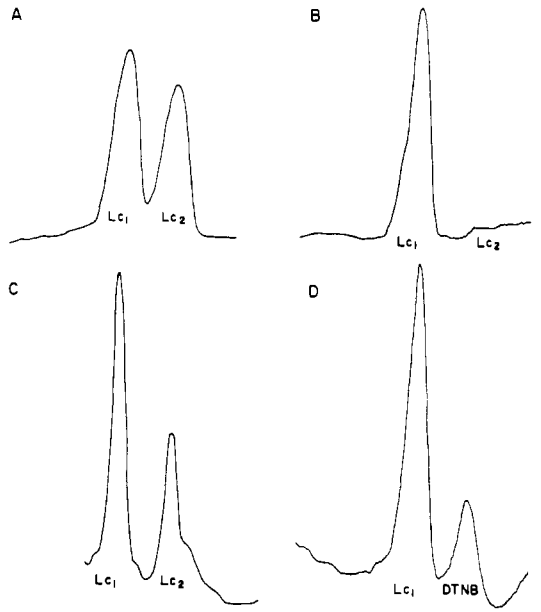

FIGURE 6: Densitometric scans of $NaDodSO_4$ gels (7.5% cross-linked) in the light chain region. (A) Native myosin; (B) Lc_2 deficient myosin; (C) M-Lc_2 recombined with cardiac Lc_2; and (D) M-Lc_2 recombined with DTNB light chain. Gels were scanned at 660 nm.

actin-activated ATPase, which were increased in M-Lc_2, reverted back to values close to those observed in native myosin. With the cardiac Lc_2 the reversal in these two measurements was essentially complete. ATPase activities of the M-Lc after equilibration with the Nbs_2 light chain were only 16 and 29% higher than in the native myosin.

Discussion

In this paper we have shown that the Lc_2 can be removed from cardiac myosin under very mild experimental conditions. Unlike the Nbs_2 treatment of skeletal myosin, which at best removes only 50% or 60% of the 18 000-dalton subunit, the present method appears to have achieved a 100% removal of the Lc_2. Incubation of myosin with the protease did not result in the breakdown of the heavy chains or the 29 000-dalton light chain (Lc_1) as visualized by $NaDodSO_4$ gel electrophoresis. Ultraviolet circular dichroism (CD) spectra of native myosin and M-Lc_2 were identical indicating no major conformational changes after treatment of myosin with the protease. Elution profiles of native myosin and M-Lc_2 on analytical column of Sepharose 4B were also found to be virtually identical, with no evidence of aggregate formation in the protease treated myosin.

The removal of Lc_2 did not affect the Ca^{2+} ATPase activity of myosin. In this respect, the cardiac myosin resembles skeletal myosin, where the partial removal of Nbs_2 light chain does not alter the Ca^{2+} ATPase. However, the removal of the Lc_2 of cardiac myosin caused a 25–30% reduction of the K^+–EDTA ATPase and a 50–75% increase in the Mg^{2+} ATPase activity. In contrast, the K^+–EDTA ATPase of skeletal myosin remains unaltered after the partial removal of the Nbs_2 light chain (Pemerick, 1977). An increase in the Mg^{2+} ATPase and a decrease in K^+–EDTA ATPase raise the question about the integrity of the regulatory sulfhydryl (–SH) groups, in particular the S_2 of myosin (Reisler et al., 1974) after protease treatment. Presence of β-mercaptoethanol or 2 mM dithiothreitol during protease treatment and subsequent manipulations did not change our experimental results which rules out the oxidation of –SH group as a probable cause of the imbalance of the ATPase activity. It would be reasonable to assume that the removal of the Lc_2 causes a conformational change in the active site of myosin which mimics the change due to S_2 modification. That the modification of S_2 was not responsible for the decrease of K^+–EDTA ATPase and increase of Mg^{2+} ATPase is strongly suggested by the results of the experiments where the addition of the cardiac Lc_2 to the deficient myosin reversed completely the changes in the ATPase activities.

The most striking changes as a consequence of Lc_2 removal were observed in the actin-activated Mg^{2+} ATPase activity of myosin. The V_{max} of ATPase extrapolated at infinite actin concentration (with pure or regulated actin) was increased at least three times the value in the control after the removal of the Lc_2. In the presence of troponin, tropomyosin, and Ca^{2+} only a marginal potentiation of the ATPase activity occurred. This lack of potentiation due to the Tn–Tm complex seems to be due to higher KCl concentrations we have used in our studies. The apparent dissociation constant for actin was reduced approximately 40% in M-Lc_2 with pure actin as a cofactor. With regulated actin as a cofactor, in the presence of Ca^{2+}, the K_{app} was increased at least fivefold in native myosin, whereas it was virtually unaltered in M-Lc_2. This decrease in the affinity of native myosin for regulated actin (in the presence of Ca^{2+}) suggests a role for Lc_2 in actin–myosin interaction. It is possible that the binding of Ca^{2+} to the Lc_2 is responsible for this decrease. It is pertinent to point out that Margossian et al. (1975) showed a decreased binding of myosin subfragment to actin in response to Ca^{2+}, when the 18 000-dalton subunit was present. The results indicate an overall increase in the actin–myosin interaction after the removal of light chain and this increase seems to be relatively independent of the presence of troponin–tropomyosin. It is also evident that actin-activated ATPase of Lc_2 deficient myosin is insensitive to changes in the ionic strength which again is a reflection of the resistance of actomyosin links to increase in the salt concentration.

The biphasic behavior of the actin-activated ATPase of cardiac myosin under the conditions of our experiment (10 μM actin monomers and 0.4 μM myosin) both with pure and regulated actin is unlike that reported for skeletal myosin subfragment (S_1) by Bremel et al. (1972). In their studies the actin-activated ATPase was a simple hyperbolic function of ATP concentration when the actin concentration was far in excess of the S_1 concentration. Presumably the differences between our results and those reported by Bremel et al. (1972) are due to our use of the double-headed species (myosin in an insoluble assay system) as against a single headed species (S_1) used in a soluble system. The focal point of interest here is not the shape of the substrate concentrations curves but rather the shift of the optimal ATP concentration to the right in case of the acto-M-Lc_2. The inhibition of ATPase of actomyosin (native) was apparent at concentration of ATP greater than 0.5×10^{-3} M, whereas with M-Lc_2 the inhibition point was reached at fivefold greater concentration of ATP (2.5×10^{-3} M). A similar shift of the ATP optimum (of actin-activated ATPase of S_1) to right and potentiation of the ATPase have been observed in studies of Bremel et al. (1972) when the concentration of myosin head was increased at a constant actin concentration. This shift has been explained in terms of cooperative effects of rigor complexes. It would be reasonable to explain the results of Figure 4 in terms of increased rigor complex formation between M-Lc_2 and actin and that the rigor complexes are relatively resistant to increase in ATP concentration. However, our results differ from those of Bremel et al. (1972) in that this increase in ATPase activity does not

depend on the presence of the regulatory proteins and therefore cannot be accounted for by cooperative behavior on thin filament. These results may reflect inherent differences in cardiac and skeletal myosin.

Ever since the discovery of the myosin linked regulation in molluscs (Kendrick-Jones, 1974; Kendrick-Jones et al., 1976), various investigators have looked for similar myosin-linked regulatory function in the mammalian myosin types. An obvious choice has been the 18 000-dalton subunit of skeletal myosin because it can bind to EDTA treated scallop myosin with the restoration of Ca^{2+} sensitivity. Other than the fact that the Nbs_2 light chain can bind Ca^{2+} at physiological concentration, no direct evidence exists for a role for this light chain in the Ca^{2+} sensitivity of actomyosin. Our results do not show any differences or shifts in the Ca^{2+} requirements of the actin activated ATPase of M-Lc_2 in the presence of troponin–tropomyosin complex compared with the actin-activated ATPase of native myosin. The differences between our data and those of Pemerick (1977) with respect to Ca^{2+} sensitivity of actomyosin after the removal of the 18 000-dalton subunit may be due to the fact that this subunit has different functional roles in cardiac and skeletal myosin. In this respect the cardiac Lc_2 may function more like the 18 000-dalton subunit of smooth muscle myosin (Gorecka et al., 1976) where the actin activated ATPase activity is known to be modulated by the phosphorylation–dephosphorylation of this subunit.

The above studies show that the Lc_2 of cardiac myosin can be removed selectively by protease treatment and that after protease treatment the actin activated ATPase of myosin is altered in a significant manner. It can be argued, however, that protease could also degrade the heavy chains, possibly a very small segment in the vicinity of the active site which may not be apparent on $NaDodSO_4$ gel electrophoresis, and that the changes observed may not be due to the loss of Lc_2. The best evidence in favor of a role for Lc_2 in these changes would be an effective reversal of the observed changes on addition of the purified cardiac Lc_2 to M-Lc_2. Incubation of M-Lc_2 with the purified cardiac Lc_2 or Nbs_2 light chain from the rabbit skeletal myosin reversed the changes in the Mg^{2+} and actin-activated ATPase activities to values very close to those observed in native myosin. That this effect was not nonspecific was clearly demonstrated by the gel electrophoretic patterns of chromatographically purified M-Lc_2 subsequent to incubation with cardiac Lc_2 or the Nbs_2 light chain. A significant amount of the added light chain (at least 50% of the original Lc_2) was bound to the M-Lc_2. Binding of less than stoichiometric amount of the added light chains raises the possibility that the light chain is merely clipped at a number of places, but not removed completely from the molecule. The clipped light chain would fall apart in the $NaDodSO_4$ system and thus not be apparent on the gels. In a series of experiments M-Lc_2 was incubated with increasing amounts of phosphorylated ^{32}P cardiac Lc_2 for 10 h at 0 °C. The mixture was chromatographed on a column of Sephadex G-100 (see Materials and Methods for experimental conditions) and the myosin peak monitored for radioactivity. Two moles of the ^{32}P-labeled light chain were incorporated per mol of M-Lc_2 when M-Lc_2 was incubated with two- to ninefold molar excess of the phorphorylated light chain. The results indicate that the Lc_2 was in fact completely removed by the protease treatment of myosin (no exchange of the externally added ^{32}P-labeled light chain can be demonstrated with the Lc_2 of native myosin under nondenaturing conditions). We do not have any explanation for the less than stoichiometric binding of unphosphorylated light chains to M-Lc_2. What is interesting is the fact that only 50% of the light chain was required for almost complete reversal of the Mg^{2+} and actin-activated ATPase activities. This suggests a critical need for the complete removal of Nbs_2 light chain from skeletal myosin (as opposed to only partial removal) in studies pertaining to its function in the skeletal myosin. The binding of Nbs_2 light chain to M-Lc_2 with a partial reversal of the ATPase activities (Mg^{2+} and actin-activated) was not surprising in view of the fact that cardiac Lc_2 and the Nbs_2 light chain show very strong sequence similarities (Leger & Elzinga, 1977). The results of our experiments clearly rule out the degradation of heavy chains as being responsible for the observed changes in the enzymatic properties of myosin after treatment with the protease.

The results of the present study show that the removal of Lc_2 from cardiac myosin changes the conformation of myosin to a state which strongly enhances interaction of myosin with actin. In the native cardiac myosin, the actin-activated ATPase is very low at near physiological KCl concentrations, suggesting an inhibitory function of this subunit in the preparation isolated by conventional techniques. We do not yet know the mechanism which can overcome this inhibition in vivo. Phosphorylation of the 18 000-dalton subunit could be a likely regulating mechanism. It is pertinent to point out here that in cardiac muscle sarcoplasm the phosphatase activity is at least 3.5-fold greater than the light chain kinase activity in contrast to skeletal muscle where the kinase activity is far in excess of the phosphatase activity (Frearson et al., 1976b). It is likely that, during the extraction of myosin from cardiac muscle, the light chain phosphatase completely dephosphorylates the 18 000-dalton subunit leading to low levels of actin activated ATPase activity.

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Complete Amino Acid Sequence of the γ Chain from the Major Fetal Hemoglobin of the Pig-Tailed Macaque, *Macaca nemestrina*†

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ABSTRACT: The complete primary structure of the γ chain of the major fetal hemoglobin from the pig-tailed macaque, *Macaca nemestrina*, was obtained by the automated sequencing of fragments produced by three nonenzymatic cleavage reactions. About two-thirds of the sequence was established from the amino terminus of the intact chain and two of the three fragments produced by cleavage at methionyl residues by cyanogen bromide. Acid cleavage at the single aspartyl-prolyl linkage and cleavage at tryptophanyl residues in intact chains yielded the two fragments necessary to complete the sequence. This γ chain, the first from a nonhuman primate to be sequenced, differs from the human $^{G}\gamma$ and $^{A}\gamma$ chains at but 4 and 5 positions, respectively. All substitutions are conservative and unlikely to produce alterations in the oxygen-binding properties of the tetrameric fetal hemoglobin. Consideration of the data presented herein, together with published observations made on portions of other primate γ chains, provides some insight into the evolutionary history of the multiple γ-globin chains observed in several anthropoid primates.

All of 109 fetal and neonatal pig-tailed macaques (*Macaca nemestrina*) examined by us have possessed two structurally distinct γ chains in their fetal hemoglobins (Nute & Stamatoyannopoulos, 1971a,b; P. E. Nute, unpublished data). Further heterogeneity of their fetal hemoglobins, traceable to α-chain variation, has also been noted, and the presence of either two or four fetal hemoglobins per individual is ascribed to the combination of products of genes at duplicate γ-chain loci with one or both of the structurally distinct α chains commonly found in members of this species (Nute & Pataryas, 1974; Nute, 1974; W. C. Mahoney & P. E. Nute, unpublished data). The two γ chains, designated γ_{slow} and γ_{fast} according to the relative electrophoretic mobilities at basic pH of the hemoglobins in which they appear, are present in a $2\gamma_{slow}:1\gamma_{fast}$ ratio in circulating erythrocytes during gestation and throughout the period of replacement of fetal by adult hemoglobin (Nute & Stamatoyannopoulos, 1971b).

The existence of at least two (and perhaps as many as four) γ-chain loci (reviewed by Wood et al., 1977; Schroeder & Huisman, 1979) may be characteristic of many of the higher primates. Both $^{A}\gamma$ (with alanine in position 136) and $^{G}\gamma$ (with glycine in position 136) chains have been found in all of several hundred normal humans from widely separated geographical locations, indicating that there are at least two γ-chain loci in *Homo sapiens* (Schroeder et al., 1968, 1972). Nonallelic γ-chain genes may also exist in gorillas (*Gorilla gorilla*), orangutans (*Pongo pygmaeus*), rhesus monkeys (*Macaca*

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