Influence of myosin heavy chains on the Ca^{2+} -binding properties of light chain, LC_2

S. SRIVASTAVA,* A. MUHLRAD and J. WIKMAN-COFFELT†

University of California, San Francisco, Cardiovascular Research Institute, San Francisco, CA 94143, U.S.A.

(Received 12 May 1980/Accepted 30 October 1980)

The association of myosin light chains with heavy chains, i.e. the intact oligomeric structure, profoundly affects the Ca^{2+} -binding properties of the light chains. The Ca^{2+} -binding affinity of the light chains is more than two magnitudes higher in the presence of heavy chains than in its absence. Modification of the reactive SH_2 thiol of myosin results in an alteration in the conformation of heavy chains of the molecule that influences the Ca^{2+} -binding properties of light chains and generation of tension. When the SH_2 moiety is blocked with *N*-ethylmaleimide the influence of the heavy chains on the Ca^{2+} -binding properties of light chain LC_2 is lost; under these conditions the Ca^{2+} -binding affinity value of SH_2 -*N*-ethylmaleimide-blocked myosin ($3.3 \times 10^4 \text{ M}^{-1}$) decreases to near that expressed with the dissociated light chain LC_2 ($0.7 \times 10^4 \text{ M}^{-1}$). Conversely, the presence of actin, nucleotides or modification of either the reactive lysyl residue or SH_2 thiol does not affect Ca^{2+} binding. The native secondary and tertiary structure of myosin seem to be required for Ca^{2+} binding; binding does not occur in the presence of 6M-urea with either native myosin or the dissociated light chains. With SH_2 -*N*-ethylmaleimide-blocked myosin normal Ca^{2+} - and (Mg^{2+} + actin)-stimulated ATPase activities are expressed; however, there is a loss in K^{+} -stimulated ATPase activity and the synthetic actomyosin threads of such myosin express no isometric tension. There are also variances in the binding of Ca^{2+} with alterations in pH values. In the absence of Ca^{2+} /EGTA buffer the biphasic Ca^{2+} -binding affinity of myosin is twice as high at pH 7.4 (site one: $1.2 \times 10^6 \text{ M}^{-1}$ and site two: $0.4 \times 10^6 \text{ M}^{-1}$) as compared with values obtained at pH 6.5 (site one: $0.64 \times 10^6 \text{ M}^{-1}$ and site two: $0.2 \times 10^6 \text{ M}^{-1}$). The Ca^{2+} -binding affinity of light chain LC_2 and S_1 , where the (S-1)–(S-2) junction was absent, were not influenced by changes in pH values. Both expressed a low Ca^{2+} -binding affinity, approx. $0.7 \times 10^4 \text{ M}^{-1}$, whereas heavy meromyosin, where both (S-1) and (S-2) myosin subfragments were present, expressed a Ca^{2+} -binding affinity value similar to that of native myosin, but was not biphasic. However, it is important to point out that in preparation of S_1 myosin subfragment light chain LC_2 was lost and thus was added back to the purified S_1 fraction. Light chain LC_2 was not, however, added to the heavy meromyosin fraction because it was not lost during preparation of the heavy meromyosin subfragment. In conclusion, it appears that the (S-1)–(S-2) junction is needed for the positioning of light chain LC_2 and thus influences its essential conformation for Ca^{2+} binding.

Abbreviations used: HC, heavy chain; LC_1 , light chain-1; LC_2 , Ca^{2+} -binding light chain-2; LC_3 (second alkali light chain), light chain-3; S_1 , myosin subfragment head; SDS, sodium dodecyl sulphate; Tes, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

* Present address: Boston Medical Research Institute, Boston, MA, U.S.A.

† To whom reprint requests should be addressed.

The heavy chains of myosin influence the Ca^{2+} -binding properties of light chain LC_2 (Kuwayama & Yagi, 1979; Srivastava *et al.*, 1980; Bagshaw & Kendrick-Jones, 1979; Szent-Gyorgyi *et al.*, 1973; Higuchi *et al.*, 1978; Stafford *et al.*, 1979; Wikman-Coffelt, 1980; Wikman-Coffelt *et al.*, 1979); the region of the heavy chain that modifies light chain LC_2 is near the (S-1)–(S-2) junction in cardiac (Kuwayama & Yagi, 1979) and scallop (Stafford *et al.*, 1979) myosin.

Fragmentation of heavy meromyosin results in three S-1 peptides (Balint *et al.*, 1978; Cardinaud, 1979; Mornet *et al.*, 1979). The 27 K-mol.wt. tryptic peptide includes the N-terminal (Cardinaud, 1979), the reactive lysine residue critical for complete myosin ATPase activity (Takashi *et al.*, 1980) and the nucleotide binding site(s) (Yamamoto & Sekine, 1980; Szilagyi *et al.*, 1979); adjacent to this is the 50 K-mol.wt. peptide containing the actin-binding site (Mornet *et al.*, 1979) and then a 20 K-mol.wt. fragment containing the SH₁ and SH₂ reactive thiol groups (Balint *et al.*, 1978; Cardinaud, 1979). Adjacent to the (S-1) 20 K-mol.wt. fragment is an (S-2) (Balint *et al.*, 1975) 40 K-mol.wt. peptide (Cardinaud, 1979). The (S-1)–(S-2) junction, i.e. the chymotryptic-EDTA-susceptible region (Kuwayama & Yagi, 1979), appears to be located in this area (Cardinaud, 1979). The region between myosin subfragments S-1 and S-2 contains the universal joint (swivel region) that allows the myosin head to freely pivot around a flexible joint (Mendelson & Cheung, 1976). Light chain LC₂ interacts with the (S-1)–(S-2) junction and the 20 K-mol.wt. peptide containing the SH₁ and SH₂ thiol groups (Srivastava *et al.*, 1980; Cardinaud, 1979). The (S-1)–(S-2) junction may be necessary for the positioning of light chain LC₂ in rabbit skeletal muscle myosin, as (S-1)-containing LC₂ is unregulated in scallop myosin whereas single-headed myosin containing Ca²⁺ and LC₂ is regulated (Stafford *et al.*, 1979). The Ca²⁺-binding light chain of rabbit skeletal muscle myosin can be substituted for that of scallop (Kendrick-Jones *et al.*, 1970) to restore Ca²⁺-sensitivity to desensitized scallop myosin.

Factors that cause dissociation of light chain LC₂ may also cause alterations in the swivel region of myosin. Light chain LC₂ is more firmly attached to myosin in the presence of Ca²⁺ (Szent-Gyorgyi, 1975; Kasman & Kados, 1977). When in association with the heavy chains the binding of Ca²⁺ is weaker at pH 6.5 as compared with pH 7.4 (Wikman-Coffelt, 1980; Muhlrads *et al.*, 1962; Werber, 1978). H⁺ may induce conformational changes in the swivel region of the heavy chains (Mendelson & Cheung, 1976; Chiano & Harrington, 1979), whereas pH has no influence on the conformation of the dissociated light chains (Werber, 1978; Stafford & Szent-Gyorgyi, 1978). The light chains are not denatured with dissociation because reassociation again promotes a high Ca²⁺-binding affinity (Wikman-Coffelt *et al.*, 1979; Okamoto & Yagi, 1977). Likewise, increases in temperature that induce light chain dissociation in the presence of chelating agents (Stafford *et al.*, 1979; Wikman-Coffelt *et al.*, 1979; Higuchi *et al.*, 1978) also promote conformational changes in myosin heavy chains (Mendelson & Cheung, 1976; Chiano & Harrington, 1979; Ishigami & Morita,

1977), but not the light chains (Chantler & Szent-Gyorgyi, 1978; Stafford & Szent-Gyorgyi, 1978).

Experimental

Rabbit skeletal muscle myosin was purified by methods described earlier (Fabian *et al.*, 1977) and actin was prepared from rabbit skeletal muscle by the method of Spudich & Watt (1971). Purification of S-1 was as described by Weeds & Taylor (1975) and heavy meromyosin as described by Chiano & Harrington (1979).

Light chains were prepared with 8 M-urea as described in earlier reports (Long *et al.*, 1977), except that the light chains were concentrated by 80%-satd.-(NH₄)₂SO₄ precipitation instead of freeze-drying. Light chains were stored at 4°C and used within 1 week after preparation.

For gel electrophoresis the 5–20% polyacrylamide-gradient slabs were prepared as described in earlier reports (Long *et al.*, 1977); this type of high resolution electrophoresis contained SDS and thus allowed for identification of peptides by molecular weight. Use of polyacrylamide gels containing urea was by the method of Ritz-Gold *et al.* (1980), which was a modification of that by Pires & Perry (1977), and allowed for identification of myosin light chain LC₂ phosphorylation.

Ca²⁺-activated ATPase activity was measured in a medium containing 4 mM-ATP/0.5 M-KCl/10 mM-CaCl₂/0.02 M-Tris/HCl (pH 7.6) at 30°C. (Actin + Mg²⁺)-activated myosin ATPase activity was measured in a medium containing 0.05 M-Tes (pH 7.0)/0.05 M-KCl/4 mM-MgCl₂/1 mM-ATP with 0.1 mg of myosin/ml and 0.1 mg of actin/ml at 25°C. K⁺/EDTA-activated ATPase activity of myosin was measured in a mixture containing 0.05 M-Tris/HCl (pH 7.6)/5 mM-EDTA/0.5 M-KCl/5 mM-ATP at 30°C. The time was chosen so that <15% of ATP was hydrolysed during the course of the reaction. P_i was estimated by the method of Muhlrads & Eisenberg (1976).

The SH₂ thiol of myosin was selectively blocked as described by Reisler *et al.* (1974), with slight modifications. Myosin (0.2% in 0.5 M-KCl/0.01 M-Tris/histidine, pH 8.3) was incubated with a 4-fold excess of fluorodinitrobenzene for 30 min at 5°C and then precipitated with 45%-satd.-(NH₄)₂SO₄; (NH₄)₂SO₄ precipitation was used instead of lowering the ionic strength because of the difficulty of precipitating myosin at this higher pH. Myosin was centrifuged, the pellet washed in water, solubilized in 0.5 M-KCl/0.05 M-Tris/histidine (pH 7.9)/1 mM-MgADP and was incubated with a 4-fold excess of N-ethylmaleimide for 30 min at 5°C. The reaction was stopped with 45%-satd.-(NH₄)₂SO₄. The mixture was centrifuged and the pellet washed twice in

water; myosin was then dissolved in 0.5 M-KCl/0.05 M-Tris/histidine (pH 7.5). The dinitrophenylated groups were subsequently removed by treating the protein for 45–60 min with 0.05 M- β -mercaptoethanol in 0.5 M-KCl/0.01 M-Tris/histidine (pH 8.5)/1 mM-EDTA, under nitrogen at room temperature. The protein was subsequently precipitated with 10 vol. of water, then centrifuged and solubilized in 0.5 M-KCl/0.05 M-Tris/histidine (pH 7.0) and dialysed extensively against this buffer. Control samples were similarly treated except that the incubation step with *N*-ethylmaleimide was omitted.

The two reactive lysine residues involved in the active site of myosin were modified with 2,4,6-trinitrobenzenesulphonate as described in earlier reports by Muhlrads *et al.* (1976).

Isometric tension was measured with synthetic actomyosin threads as described earlier (Srivastava *et al.*, 1980). Threads were formed by extrusion of the concentrated solution of actomyosin through a syringe into a trough that contained buffer (20 mM-KCl/5 mM-MgCl₂/10⁻⁵ M-CaCl₂/20 mM-Tes, pH 7.0). The threads were removed from the trough, mounted on the tensiometer and glued to the mounting rod with a drop of acetone and Plexiglass. The tensiometer was as designed and described in detail by Crooks & Cooke (1977). The threads were then immersed in 2.5 ml of the above buffer maintained at 24°C containing 4 mM-phosphocreatine and 0.4 mg of creatine kinase/ml. The threads were allowed to equilibrate with the tensiometer in the isometric mode; ATP was added and the tension increased. After the tension reached its maximum, the tensiometer was switched momentarily into the isotonic mode and the position of the arm was recorded on an *x-y* recorder. The tensiometer was returned to the isometric mode and the thread was allowed to return to its initial length before subsequent velocity was recorded at a different tension.

Ca²⁺ binding was carried out in the absence of a Ca²⁺/EGTA buffer as described earlier (Wikman-Coffelt & Muhlrads, 1980) so as to avoid the disparate results of using ⁴⁵Ca along with a ⁴⁰Ca²⁺/EGTA system. Two binding sites were obtained for myosin in the absence of the metal buffer and the binding affinity was higher than if such a buffer was present (Wikman-Coffelt & Muhlrads, 1980). All buffers and water were rapidly passed through Chelex-100 immediately before use to remove bivalent cation contaminants. All treatment was continued rapidly to avoid an equilibrium of contaminants with Chelex. All containers, measuring flasks and pipettes were of a disposable plastic. Portions of myosin (7–8 mg/ml) or light chains (1 mg/ml) were added to individual bags (1 ml) and predialysed in 0.2 M-Tris/maleate (pH 7.4 or 6.5)/0.1 M-KCl/1 mM-EDTA/1 mM-dithiothreitol. The second and third dialysis contained the same

except that no EDTA was present. Stock solutions were prepared as described earlier (Wikman-Coffelt & Muhlrads, 1980). Under these conditions the contaminants, Ca²⁺ and Mg²⁺, were less than 10⁻⁷ M. The high buffer concentration retained a constant pH throughout the reaction. The Ca²⁺ concentration was varied from 10⁻⁷ to 10⁻⁵ M for all analyses; experimental and control samples were dialysed together in the same flask as described by Holroyde *et al.* (1979). To determine the number of sites and affinity values of the dissociated light chains, heavy meromyosin S-1 and SH₂-*N*-ethylmaleimide-blocked myosin Ca²⁺ concentrations of 10⁻⁵–10⁻³ M were also analysed. At this range of Ca²⁺ concentrations the two binding sites on the myosin oligomer were filled; however, all of the sites on the SH₂-*N*-ethylmaleimide-blocked myosin, S-1 and the light chains were not filled until the Ca²⁺ concentration was raised to 10⁻³ M. The dialysis medium consisted of 0.2 M-Tris/maleate (pH 6.5 or 7.4)/0.1 M-KCl and 5 μ Ci of ⁴⁵Ca in a total volume of 100 ml. After a dialysis time of 48 h (4°C), with continuous shaking, samples from both inside and outside the bag were removed (0.1 ml) in duplicates, an equal volume of H₂O₂ was added and the mixture heated in closed vials for 4 h at 55°C; 10 ml of scintillation fluid (PPO) was added and the samples were analysed in a Beckman scintillation counter for assessing radioactivity. Duplicate samples of protein solution inside the bags were taken for the determination of protein. Calculations were as those described earlier (Wikman-Coffelt & Muhlrads, 1980).

Results

After SH₂ modification, myosin expressed normal Ca²⁺- and (actin + Mg²⁺)-activated ATPase activity (Table 1), similar to that described by Schaub *et al.* (1979) for SH₂ modification of myosin. The SH₂-*N*-ethylmaleimide-blocked myosin expressed no isometric tension when analysed as synthetic actomyosin threads (Fig. 1). Similarly, blocking of the SH₂ thiol of myosin resulted in a large decrease in Ca²⁺-binding properties of the light chain (see Fig. 3). The Ca²⁺-binding affinity of SH₂-*N*-ethylmaleimide-blocked myosin (3.3×10^4 M⁻¹) (Figs. 2 and 3), the dissociated light chains (0.7×10^4 M⁻¹) (Fig. 4) and S-1 (0.8×10^4 M⁻¹) (Fig. 4) were considerably lower than that of native myosin oligomer (Figs. 3 and 5). The Ca²⁺-binding affinity values for the purified light chains were similar to that of earlier reports (Kuwayama & Yagi, 1979; Wikman-Coffelt, 1980; Wikman-Coffelt *et al.*, 1979). Native myosin consistently expressed a higher Ca²⁺-binding affinity when assayed at pH 7.4 as compared with pH 6.5 (Figs. 2 and 3), similar to reports where Ca²⁺/EGTA buffer was added (Werber, 1978; Wikman-

Table 1. Activities of myosin ATPase after SH₂-modification

Myosin (0.2% in 0.5 M-KCl/0.01 M-Tris/histidine, pH 8.3) was incubated with a 4-fold excess of fluoro-dinitrobenzene for 30 min at 5°C and then precipitated with 45%-satd.-(NH₄)₂SO₄. After centrifugation the pellet was washed in water and solubilized with a 4-fold excess of *N*-ethylmaleimide for 30 min at 5°C. The reaction was stopped, the mixture centrifuged and the pellet washed twice in water. Myosin was then dissolved in 0.5 M-KCl/0.05 M-Tris/histidine (pH 7.5). After removal of the dinitrophenylated groups, the protein was precipitated with 10 vol. of water, centrifuged and solubilized in 0.5 M-KCl/0.05 M-Tris/histidine (pH 7.0) and dialysed against this buffer. Such SH₂-modified myosin was then assayed for K⁺/EDTA-stimulated, (actin + Mg²⁺)-stimulated and Ca²⁺-stimulated ATPase activities as described in the Experimental section.

	ATPase activity ($\mu\text{mol of P}_i \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of myosin)		
	K ⁺ -EDTA-stimulated	(Actin + Mg ²⁺)-stimulated	Ca ²⁺ -stimulated
Control	2.90	0.41	0.91
SH ₂ -blocked	0.63	0.40	0.88

Fig. 1. Force velocity curve of actomyosin threads. Threads were formed from the solution of myosin and actin (55 mg/ml) in a ratio of 1:2. Native myosin (●—●) and SH₂-*N*-ethylmaleimide-blocked myosin, shown by the empty circle at the origin, were analysed. The process was initiated with 125 μg of ATP, similar to conditions reported earlier (Srivastava *et al.*, 1980).

Coffelt, 1980); likewise comparable results were obtained by Muhlrad *et al.* (1962), where analyses were carried out in the absence of a Ca²⁺/EGTA

Fig. 2. Scatchard plots of Ca²⁺ binding at pH 7.4 for rabbit skeletal muscle myosin

Analyses were as described in the Experimental section. Data are shown for native myosin (●—●). Site one: $K = 1.2 \times 10^6 \text{ M}^{-1}$, $n = 1.08$. (Although contaminants Ca²⁺ and Mg²⁺ were less than 10^{-7} M , free Ca²⁺ values $< 10^{-7} \text{ M}$ could not be studied with the present technique.) Site two: $K = 3.8 \times 10^4 \text{ M}^{-1}$, $n = 2.1$. The open circles (○—○) show the binding of trinitrophenylated myosin (two lysine residues blocked). Included in the same dialysis flask as native myosin (●—●) and the myosin treated with 2,4,6-trinitrobenzenesulphonate (Muhlrad *et al.*, 1976) (○—○) was SH₂-*N*-ethylmaleimide-blocked myosin (□—□). The K value for the latter was $3.3 \times 10^4 \text{ M}^{-1}$, $n = 2.0$. Also shown are the values for

Fig. 3. Scatchard plots of Ca²⁺ binding at pH 6.5 for rabbit skeletal muscle myosin

Analyses were as described in the Experimental section. For native myosin site one (●—●): $K = 0.64 \times 10^6 \text{ M}^{-1}$, $n = 1.1$. Site two: $K = 2.4 \times 10^5 \text{ M}^{-1}$, $n = 2.3$. Myosin treated with 2,4,6-trinitrobenzene-sulphonate (○—○) gave similar values. For SH₂-N-ethylmaleimide-blocked myosin (□—□) $K = 3.3 \times 10^4 \text{ M}^{-1}$, $n = 2.0$. All symbols are the same as in Fig. 2. A high concentration of buffer was used in the dialysis to retain a constant pH throughout the reaction. Control and experimental samples were analysed in the same flask. Five individual experiments were carried out analysing pH differences; all analyses gave similar results. Shown also are the values for myosin binding in 6M-urea (△—△).

buffer. Conversely, H⁺ had no effect on the Ca²⁺-binding properties of the purified light chains (Fig. 4), S-1 (Fig. 4) or SH₂-N-ethylmaleimide-blocked myosin (Figs. 2 and 3).

myosin binding in 6M-urea (△—△). The urea was first treated with Chelex-100 to remove contaminants as described in the Experimental section.

S-1 was cleaved from S-2 with the chymotryptic digestion procedures described here (Taylor & Weeds, 1975) and thus S-1 lacked an important (S-1)–(S-2) junction that may be required for Ca²⁺ binding, as in the case of cardiac myosin (Kuwayama & Yagi, 1979). Light chains were added (mol/mol) to the S-1 fraction during equilibrium Ca²⁺-binding studies, since light chain LC₂ was hydrolysed during the preparation of S-1. The low Ca²⁺-binding affinity value of S-1 (Fig. 4) is either the result of the Ca²⁺-binding light chain not reassociating with the S-1 subfragment lacking the (S-1)–(S-2) junction or partial reassociation occurred but the Ca²⁺-binding properties were no longer influenced by the heavy chains. Native myosin, conversely reassociated under these conditions (Wikman-Coffelt *et al.*, 1979; Higuchi *et al.*, 1978; Wikman-Coffelt, 1980; Hollosi *et al.*, 1980) and the high Ca²⁺-binding affinity value was restored. The Ca²⁺-binding light chain has a different affinity for Ca²⁺ when complexed to the heavy chains as compared with when it is in the dissociated state (Wikman-Coffelt *et al.*, 1979) and only binds at Ca²⁺ concentrations required to induce a conformational change in free light chains (Ca²⁺ 10^{-5} – 10^{-3} M) (Morakovic *et al.*, 1979; Yamamoto *et al.*, 1980).

Although myosin light chains were prepared in the presence of 8 M-urea, such procedures did not affect the Ca²⁺-binding properties of the dissociated light chains as long as the denaturing agent was removed before equilibrium dialysis (Wikman-Coffelt, 1980; Wikman-Coffelt *et al.*, 1979); however, the presence of 6 M-urea in the dialysis medium prevented all Ca²⁺ binding, both for native myosin (Figs. 2 and 3) and the dissociated light chains (Fig. 4).

When Ca²⁺ binding was analysed in the absence of the Ca²⁺/EGTA buffer as described here the binding profiles for the two native myosin sites were biphasic (Figs. 2 and 3), similar to the results obtained by Watterson *et al.* (1979); in this latter study binding was analysed with techniques other than the addition of ⁴⁵Ca to a Ca²⁺/EGTA system, where labelling effects often obscure data (Wikman-Coffelt & Muhrad, 1980). Such biphasic binding was not due to phosphorylation of myosin light chain LC₂, since the purified myosin of rabbit skeletal muscle contained no phosphate after purification (Fig. 5). Conversely, although purified sheep cardiac myosin demonstrated as 30% light chain LC₂ phosphorylation (Fig. 6), it nevertheless gave a similar type of biphasic binding (Fig. 7). (It is unlikely that the minor contaminant protein present in cardiac myosin (Fig. 6) is a Ca²⁺-binding protein, e.g. troponin or calmodulin, because it dissociates with the light chains (Fabian *et al.*, 1977) and the dissociated light chains have a low 'non-biphasic' Ca²⁺-binding affinity, as shown here; also the same

Fig. 4. Scatchard plots of (a) myosin light chains and (b) purified S-1 with additional light chains added. Light chains were dissociated with 8 M-urea and concentrated with 80%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation as described in the Experimental section. Calculation of light chain LC_2 (mol) was carried out as described earlier (Wikman-Coffelt *et al.*, 1979): (a) $K = 0.7 \times 10^4 \text{ M}^{-1}$, $n = 1.1$. Analyses were at pH 7.4 (●—●) and pH 6.5 (○—○). Also shown are data for analyses of myosin light chains with 6 M-urea present in the dialysate (Δ — Δ). (b) $K = 0.8 \times 10^4 \text{ M}^{-1}$, $n = 1.0$ (mol/mol). Analyses were at pH 7.4 (●—●) and pH 6.5 (○—○). As in the studies of Kuwayama & Yagi (1979) S-1 was prepared in the presence of EDTA, thus the swivel region was not present.

degree of contamination is present after SH_2 -modification (see Fig. 9); SH_2 -modified myosin also has a low Ca^{2+} -binding affinity.] Blocking of the SH_2 thiol of the heavy chains with *N*-ethylmaleimide reduced the binding affinity of the oligomer to that of the dissociated light chains. There was no oligomeric dissociation with such thiol modification (Fig. 8); furthermore, pH did not affect the binding affinity of SH_2 -*N*-ethylmaleimide-blocked myosin (Figs. 2 and 3), the dissociated light chains (Fig. 4) nor S-1 (Fig. 4). It is assumed from the ATPase activity and the gel-electrophoresis patterns (Fig. 9) that the alkali light chains were functional as LC_3 after preparation of S-1 with chymotryptic digestion; the actin-activated ATPase activity of S-1 was $0.6 \mu\text{mol of P}_i \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of myosin. Conversely, the Ca^{2+} -binding properties of heavy meromyosin (Fig. 9b), where both S-1 and S-2 were present, were similar to that of native myosin (Fig. 10).

Modification of the two reactive lysine residues of myosin similar to SH_1 -*N*-ethylmaleimide blocked myosin (Srivastava *et al.*, 1980) did not affect Ca^{2+} binding of rabbit skeletal muscle myosin (Figs. 2 and 3).

Discussion

Although the reactive SH_1 and SH_2 moieties of myosin are located on the 20 K-mol.wt. peptide of

S-1 (Balin *et al.*, 1978) and are only separated by nine amino acids (Flink *et al.*, 1977). SH_1 -*N*-ethylmaleimide-blocked myosin still has normal Ca^{2+} -binding properties and a normal expression of isometric tension as actomyosin threads (Srivastava *et al.*, 1980), whereas SH_2 -*N*-ethylmaleimide-blocked myosin does not. Conversely, when the reactive lysine residue in the 27 K-mol.wt. peptide is blocked with 2,4,6-trinitrobenzenesulphonate there are normal Ca^{2+} -binding properties. Such an exclusive effect of the SH_2 region of myosin heavy chains on the Ca^{2+} -binding properties of the light chains is either owing to the small conformational change that occurs in this region of the molecule when it is blocked (Kameyama, 1980) or the SH_2 thiol directly participates in the heavy chain's influence on the Ca^{2+} -binding properties of light chain LC_2 .

The heavy meromyosin (S-1)–(S-2) junction can act as a fulcrum in generating force to push the thin filaments towards the centre of the sarcomere. Any change in molecular interaction and conformation in this region appears to affect Ca^{2+} -binding, the rate of K^+ -stimulated ATPase activity and isometric tension of the synthetic actomyosin threads. This region may be important for positioning of the Ca^{2+} -binding light chain so that its affinity for Ca^{2+} is increased; however, Ca^{2+} binding does not appear to be a factor involved in tension as 70% of the Ca^{2+} -binding light chain can be dissociated from

Fig. 5. Electrophoresis of rabbit skeletal muscle myosin analysed in 8 M-urea

Light chain LC₂ gave satellite bands for both the phosphorylated and non-phosphorylated components of light chain LC₂ as identified by Pires *et al.* (1974). Such satellite bands are apparently some type of oxidation product (Perrie *et al.*, 1973). Phosphorylation was carried out by Ritz-Gold as described in previous reports (Ritz-Gold *et al.*, 1980). (a) Myosin as used in the Ca²⁺-binding studies described here and (b) phosphorylated myosin standard.

Fig. 6. Electrophoresis of cardiac myosin in 8 M-urea. Light chain LC₂ gave four bands with this system as described by Ritz-Gold *et al.* (1980). (a) Cardiac myosin purified as described earlier (Long *et al.*, 1977) and as used here for Ca²⁺ binding and (b) phosphorylated cardiac myosin standard.

myosin with no change in isometric tension (Srivastava *et al.*, 1980). The SH₂ thiol is near the (S-1)–(S-2) junction (Cardinaud, 1979), perhaps in a type of tertiary folding, and blocking of this thiol causes a shift in regional charge distribution (Kameyama, 1980). This alteration in molecular interaction does not affect Ca²⁺-stimulated ATPase activity in the head region nor complexing of S-1 with actin as shown in these studies; however, the alterations are sufficient to inhibit Ca²⁺ binding,

K⁺-stimulated ATPase activity and isometric tension. The intact S-1 and S-2 regions are needed for influencing the Ca²⁺-binding properties of light chain LC₂ in rabbit skeletal muscle myosin; this influence of the heavy chains on the Ca²⁺-binding properties of the light chains occurs with native and heavy meromyosin, but not with S-1.

With the susceptibility of the (S-1)–(S-2) junction to molecular interaction, conformational changes and/or proximity of charge distribution (Kameyama, 1980) H⁺ also modify this region (Chiano & Harrington, 1979; Mendelson & Cheung, 1976); such modification results in a decrease in the

Fig. 7. Scatchard plot of sheep ventricular myosin of Ca^{2+} binding

Binding was carried out at pH 7.4 in the absence of a Ca^{2+} /EGTA buffer as described in the Experimental section: $K = 1.25 \times 10^6 \text{ M}^{-1}$ for site one and $K = 3.0 \times 10^5 \text{ M}^{-1}$ for site two. The Ca^{2+} binding of cardiac myosin was influenced by changes in pH but not by SH_2 -modification, as will be discussed in later studies.

Ca^{2+} -binding affinity of myosin at pH 6.5 as compared with pH 7.4, as shown here. A decrease in pH may relax the positioning of light chain LC_2 . Conversely, the dissociated light chain LC_2 , like troponin (Stull & Buss, 1978), was not susceptible to changes in pH relative to Ca^{2+} binding, thus confirming that the decrease in Ca^{2+} binding of myosin with a decrease in pH is owing to the effect of H^+ on the conformation of the heavy chains and thereby influencing its interaction with light chain LC_2 . This was further supported by the fact that the

Fig. 8. Gel electrophoresis patterns (5–20% polyacrylamide gradient containing SDS) of (a) rabbit skeletal and (b) sheep cardiac myosins

I and II before and after treatment with *N*-ethylmaleimide respectively. The dye, Coomassie Blue, was eluted from the stained protein bands and quantified as described in earlier reports (Higuchi *et al.*, 1978). The percentage of protein present was: (a) (I) 88.2% HC; 3.8% LC_1 ; 6.1% LC_2 ; 1.9% LC_3 . (a) (II) 87.9% HC, 4.2% LC_1 ; 5.9% LC_2 ; 2.0% LC_3 . (b) (I) 89.0% HC; 6.6% LC_1 ; 4.4% LC_2 . (b) (II) 88.6% HC; 6.7% LC_1 ; 4.7% LC_2 .

Ca^{2+} binding of SH_2 -modified myosin, where the effect of heavy chains on Ca^{2+} binding was diminished, did not show pH-susceptibility. Since there was no pH-susceptible change in Ca^{2+} binding with S-1, but there was with heavy meromyosin, it appears that the intact (S-1)–(S-2) region is needed for influencing Ca^{2+} binding. It is not known at present how much of S-2 is needed for this influence; however, in cardiac myosin it appears that only a small peptide of the (S-1)–(S-2) junction is required (Kuwayama & Yagi, 1979).

The Ca^{2+} binding of myosin, unlike the binding of the Ca^{2+} -specific site of troponin, appears to depend on the secondary and tertiary structure of the molecule, such that binding does not take place in the presence of 6M-urea, as in the case of the Ca^{2+} -binding-specific site of troponin as was first shown by Head & Perry (1974) and later by Nagy & Gergely (1979). There are several disparities in the Ca^{2+} -binding properties of myosin as compared with troponin; the Ca^{2+} -binding loops of troponin and the corresponding site on light chain LC_2 are similar (Kendrick-Jones & Jakes, 1977), but sufficiently different for us to believe that the overall three-dimensional structure of these regions are not the same and thus differences, as described here for

Fig. 9. Polyacrylamide-gradient slab gel (5–20%) after electrophoresis in SDS

(a) S-1 fraction before addition of light chains; (b) heavy meromyosin; (c) native myosin. Heavy meromyosin was further purified by column chromatography by using Sepharose 4B equilibrated and eluted with 50 mM-Tris/HCl (pH 7.0)/0.05 M-KCl/0.2 mM-dithiothreitol; S-1 was purified by column chromatography with Sephacryl equilibrated in the same buffer. HMM, heavy meromyosin.

light chain LC₂, relative to pH and oligomer influence, would be expected. Also, the Ca²⁺-binding sites on myosin are non-specific in that they can be displaced with Mg²⁺ (Wikman-Coffelt, 1980; Holroyde *et al.*, 1979), whereas there are both specific and non-specific Ca²⁺-binding sites with troponin (Potter & Gergely, 1975).

Although modification of the reactive SH₂ thiol produces a moderate conformational change in myosin (Kameyama, 1980), which in turn alters the influence of the heavy chains on the Ca²⁺-binding properties of the light chains, still other added factors such as nucleotides, which also cause a small conformational change in myosin (Seidel & Gergely, 1971; Yamamoto & Sekine, 1980; Szilagyi *et al.*, 1979), as well as actin (Mornet *et al.*, 1979; Yamamoto & Sekine, 1980), did not alter the Ca²⁺-binding properties of myosin with analyses as described here.

Rabbit skeletal muscle myosin as an oligomer, but not as dissociated subunits, binds Ca²⁺ at physiological Ca²⁺ concentrations, whereas with frog

Fig. 10. Scatchard plots of rabbit skeletal heavy meromyosin

Ca²⁺ binding at pH 7.4 (○—○) and pH 6.5 (□—□) in the presence of Ca²⁺/EGTA buffer as described in the Experimental section.

both skeletal muscle myosin and the dissociated light chain LC₂ bind Ca²⁺ at physiological Ca²⁺ concentrations (Wikman-Coffelt & Srivastava, 1979). However, in all cases Mg²⁺ competes for these sites at a lower but significant affinity and thus negates any type of myosin regulation (Holroyde *et al.*, 1979; Wikman-Coffelt & Srivastava, 1979; Wikman-Coffelt, 1980). Furthermore the rate of Mg²⁺ dissociation is too slow to assume a Mg²⁺—Ca²⁺ exchange during the contraction cycle owing to Ca²⁺ transients (Bagshaw & Reed, 1977).

We express gratitude to Professor M. Morales and Professor R. Cooke for their helpful discussions and also R. Cooke for use of the tensiometer. We also thank Professor D. Mason for the myosin used in these studies, and Dr. C. Ritz-Gold and Dr. G. Hollosi for carrying out

the gel electrophoresis. This research was supported by grant NIH R02 HL 23518 and a grant from the University of California, San Francisco, and Career Development Award HL 00282 to J. W.-C. and a grant by the Muscular Dystrophy Association awarded to A. M. who is on leave from the Hebrew University of Hadassah School of Medicine, Department of Oral Biology, Jerusalem, Israel.

References

- Bagshaw, C. R. & Kendrick-Jones, J. (1979) *J. Mol. Biol.* **130**, 317–336
- Bagshaw, C. R. & Reed, G. H. (1977) *FEBS Lett.* **81**, 386–390
- Balint, M., Sreter, F. A., Wolf, I., Nagy, B. & Gergely, J. (1975) *J. Biol. Chem.* **260**, 6168–6177
- Balint, M., Wolf, I., Tarcsfalvi, A., Gergely, J. & Sreter, F. A. (1978) *Arch. Biochem. Biophys.* **190**, 793–799
- Cardinaud, R. (1979) *Biochimie* **61**, 807–821
- Chantler, P. D. & Szent-Gyorgyi, A. G. (1978) *Biochemistry* **17**, 5440–5448
- Chiano, Y. C. & Harrington, W. F. (1979) *Biochemistry* **77**, 361–366
- Crooks, R. & Cooke, R. (1977) *J. Gen. Physiol.* **69**, 37–55
- Fabian, F., Mason, D. T. & Wikman-Coffelt, J. (1977) *FEBS Lett.* **81**, 381–385
- Flink, I. L., Morkin, E. & Elzinga, M. (1977) *FEBS Lett.* **84**, 261–265
- Gornall, A. G., Bradawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–756
- Head, J. F. & Perry, S. V. (1974) *Biochem. J.* **137**, 145–154
- Higuchi, M., Fabian, F., Mason, D. T. & Wikman-Coffelt, J. (1978) *Eur. J. Biochem.* **92**, 317–323
- Hollosi, G., Srivastava, S. & Wikman-Coffelt, J. (1980) *FEBS Lett.* in the press
- Holroyde, G., Potter, J. D. & Solaro, R. J. (1979) *J. Biol. Chem.* **254**, 6478–6482
- Ishigami, F. & Morita, F. (1977) *J. Biochem. (Tokyo)* **80**, 297–303
- Kameyama, T. (1980) *J. Biochem. (Tokyo)* **87**, 297–303
- Kasman, K. & Kadol, O. (1977) *Biochim. Biophys. Acta* **491**, 509–514
- Kendrick-Jones, J. & Jakes, R. (1977) Myosin-linked regulation: a chemical approach, in *Myocardial Failure* (Riecker, G., Weber, A. & Goodman, J., Jr., eds.), pp. 28–44, Springer-Verlag, Berlin, Heidelberg and New York
- Kendrick-Jones, J., Lehman, W. & Szent-Gyorgyi, A. G. (1970) *J. Mol. Biol.* **54**, 313–326
- Kuwayama, H. & Yagi, K. (1979) *J. Biochem. (Tokyo)* **85**, 1245–1255
- Long, L., Fabian, F., Mason, D. T. & Wikman-Coffelt, J. (1977) *Biochem. Biophys. Res. Commun.* **76**, 625–635
- Mendelson, R. A. & Cheung, P. (1976) *Science* **194**, 190–192
- Morakovic, A., Oda, S. & Reisler, E. (1979) *Biochemistry* **19**, 5960–5966
- Mornet, R. A., Pantel, P., Audermard, E. & Kassab, R. (1979) *Biochem. Biophys. Res. Commun.* **89**, 925–932
- Muhlrads, A., Biro, N. A. & Vertes, K. (1962) *Acta Physiol. Acad. Sci. Hung.* **21**, 15–28
- Muhlrads, A., Oplatka, A. & Lamed, R. (1976) *Biochim. Biophys. Acta* **452**, 227–239
- Muhlen, S. A. & Eisenberg, E. (1976) *Biochemistry* **15**, 5702–5708
- Nagy, B. & Gergely, J. (1979) *J. Biol. Chem.* **254**, 12732–12737
- Okamoto, Y. & Yagi, K. (1977) *Biochem. J.* **167**, 137–146
- Perrie, W. T., Smillie, L. B. & Perry, S. V. (1973) *Biochem. J.* **135**, 151–164
- Pires, J. D. & Gergely, J. (1975) *Biochem. J.* **167**, 137–146
- Pires, E. M. V., Perry, S. V. & Thomas, M. A. W. (1974) *FEBS Lett.* **41**, 292–296
- Potter, J. D. & Gergely, J. (1975) *J. Biol. Chem.* **250**, 4628–4633
- Reisler, E., Burke, M. & Harrington, W. F. (1974) *Biochemistry* **13**, 2014–2023
- Ritz-Gold, C. J., Cooke, R., Blumenthal, D. K. & Stull, J. (1980) *Biochem. Biophys. Res. Commun.* **93**, 209–214
- Schaub, M. C., Watterson, J. G. & Washer, P. G. (1979) *Biochimie* **61**, 791–801
- Seidel, J. C. & Gergely, J. (1971) *Biochem. Biophys. Res. Commun.* **44**, 826–829
- Spudich, F. A. & Watt, S. (1971) *J. Biol. Chem.* **246**, 4866–4871
- Srivastava, S., Cooke, R. & Wikman-Coffelt, J. (1980) *Biochem. Biophys. Res. Commun.* **92**, 1–7
- Stafford, W. F. & Szent-Gyorgyi, A. G. (1978) *Biochemistry* **17**, 607–614
- Stafford, W. F., Szentkiralyi, E. M. & Szent-Gyorgyi, A. G. (1979) *Biochemistry* **18**, 5273–5280
- Stone, D. & Perry, S. V. (1973) *Biochem. J.* **131**, 127–137
- Stull, J. T. & Buss, J. E. (1978) *J. Biol. Chem.* **253**, 5932–5938
- Szent-Gyorgyi, A. G. (1975) *J. Supramol. Struct.* **3**, 348–353
- Szent-Gyorgyi, A. G., Szentkiralyi, E. M. & Kendrick-Jones, J. (1973) *J. Mol. Biol.* **74**, 179–203
- Szilagy, L., Balint, M., Sreter, F. A. & Gergely, J. (1979) *Biochem. Biophys. Res. Commun.* **89**, 936–945
- Takashi, R., Muhlrads, A., Hozumi, T. & Botts, J. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1936
- Taylor, R. S. & Weeds, A. G. (1975) *Nature (London)* **257**, 54–56
- Watterson, J. G., Kohler, L. & Schaub, M. C. (1979) *J. Biol. Chem.* **254**, 6470–6479
- Weeds, A. G. & Pope, B. (1977) *J. Mol. Biol.* **111**, 129–157
- Weeds, A. G. & Taylor, R. S. (1975) *Nature (London)* **257**, 54–56
- Werber, M. M. (1978) *Experientia* **34**, 575–576
- Wikman-Coffelt, J. (1980) *Biochem. J.* **185**, 265–268
- Wikman-Coffelt, J. & Srivastava, S. (1979) *J. Biochem. (Tokyo)* **86**, 829–832
- Wikman-Coffelt, J. & Muhlrads, A. (1980) *FEBS Lett.* **114**, 39–44
- Wikman-Coffelt, J., Srivastava, S. & Mason, D. T. (1979) *Biochimie* **61**, 1309–1314
- Yamamoto, K. & Sekine, T. (1980) *J. Biochem. (Tokyo)* **87**, 593–599
- Yamamoto, K., Honjo, R. & Sekine, T. (1980) *J. Biochem. (Tokyo)* **87**, 213–217