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Dissociation of Light Chains from Cardiac Myosin

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The substrate, ATP, protects the active site of cardiac myosin during a 10-min treatment at 37 °C and neutral pH in the absence of divalent cations; under these conditions there is an approximate 20% dissociation of light chain C_1 and 60% loss of light chain C_2 with no corresponding decrease in myosin ATPase activity. Higher temperatures, the absence of divalent cations, increased treatment time, and low protein concentration were conducive to light chain dissociation; loss of light chains did not appear to be influenced by increments of pH from 7.0 to 9.0. On the other hand, a decrease in protein concentration and an increase in both duration of exposure at 37 °C and an alkaline pH caused a decrease in myosin ATPase activity. Concomitant with the dissociation of cardiac myosin light chains, particularly light chain C_2, there was a corresponding loss in the number of high-affinity calcium binding sites. When the dissociated light chains were recombined with light-chain-deficient myosin, reassociation took place and the number of high-affinity calcium binding sites were regained. Reassociation of light chains with light-chain-deficient myosin did not restore diminished light-chain-deficient myosin ATPase activity. There is a shift in the pH optimum of myosin at 37 °C in the absence of divalent cations, where conditions used for light chain dissociation are employed. The presence of calcium prevents light chain dissociation, helps retain myosin ATPase activity at 37 °C and prevents a shift in the pH optimum.

The calcium-binding light chain protects the subfragment-1 site of myosin from proteolytic digestion in the presence of calcium [1]; it is more firmly attached to the myosin heavy chains in the presence of calcium [2,3]. The C-terminal half of the calcium-binding light chain binds calcium, and is in permanent association with the myosin heads [4,5]. Partial sequence analysis of bovine cardiac myosin light chain C_2, the Ca^{2+}-binding 18000-M_r [6] light chain [7], indicates that it is homologous to the calcium-binding light chain of skeletal muscle myosin, the 5,5′-dithiobis(2-nitrobenzoate) light chain [8,9]. Cardiac myosin light chain C_1 (M_r = 28000 [6]) is comparable to alkali light chains A_1 and A_2 of rabbit skeletal muscle myosin [8,9]. The functions of the two alkali light chains, A_1 and A_2, are not known but their separation from myosin is accompanied by a loss of ATPase activities [10–12]. The studies described here were undertaken to determine how essential the light chains are to the structure of myosin and to determine whether light chain dissociation may be associated with a shift in the pH activity curve of K^+-stimulated cardiac myosin when myosin is assayed in the absence of divalent cations at 37 °C relative to 25 °C. These studies were also made to ascertain whether the absence of divalent cations and the low protein concentration used in the K^+-stimulated enzymatic reaction mixture promote light chain dissociation.

Enzyme. Myosin ATPase (EC 3.6.1.3).

EXPERIMENTAL PROCEDURE

Myosin Purification

Myosin was purified from both ventricles of the rat as well as right ventricles of the dog, sheep, and beef [13,14]. After the tissue was minced, it was sheared, washed three times in a low salt buffer (0.05 M KH_2PO_4, pH 6.8, 0.001 M EDTA, 0.1 M $NaPP_i$, 0.001 M dithiothreitol), and centrifuged (9000 × *g*, 10 min), after each wash. Myosin was then extracted from the pellet in a buffer with a high salt concentration (0.1 M KH_2PO_4, 0.3 M KCl, 0.01 M $NaPP_i$, 0.001 M dithiothreitol, 0.001 M EDTA, and 0.05 M K_2HPO_4, pH 7.5). After stirring the homogenate for 15 min [13] the solution was filtered and centrifuged (9000 × *g*, 15 min). Myosin was precipitated with a 9-fold dilution of water containing 0.002 M EDTA. Myosin, after pelleting at 9000 × *g* for 10 min, was homogenized in 0.05 M $NaPP_i$, pH 7.5, 0.001 M dithiothreitol, 0.001 M EDTA, and 0.002 M ATP, stirred for 10 min and centrifuged

(40000 × *g*) for 20 min. Myosin was collected from the supernatant by a salt fractionation using the 35–45% $(NH_4)_2SO_4$ fraction, dialyzed, and centrifuged as described earlier [13]. Myosin was then precipitated twice with a 9-fold dilution of water at pH 6.5.

Dissociation of Myosin Light Chains

Myosin light chains were dissociated from myosin by making the solution of myosin 0.1 mg/ml in 0.1 M Tris · HCl buffer, pH 7.8 containing 1 mM EDTA, 0.65 M KCl and 5 mM ATP. Myosin was incubated at 37 °C for 10 min. At the end of the reaction time, light-chain-deficient myosin was precipitated by bringing the solution to 45% $(NH_4)_2SO_4$ saturation. The light chains remaining in the supernatant were precipitated by bringing the supernatant to 80% $(NH_4)_2SO_4$ saturation. After centrifugation myosin was solubilized in 0.05 M Tris buffer, pH 7.5, containing 0.5 M KCl and 1 mM dithiothreitol, and dialyzed overnight against the same buffer. The solubilized myosin was again centrifuged to remove remaining contaminants.

In other studies a jacketed column retained at 37 °C was used. The column (1.0 × 30 cm) containing Sephadex G-100 was equilibrated with 0.05 M Tris, pH 7.8, 5 mM ATP, 1 mM EDTA, and 0.50 M KCl. The void volume was analyzed for myosin and myosin heavy chains and the inclusion volume for dissociated light chains.

Reassociation of Myosin

Conditions for reassociation of dissociated light chains with light-chain-deficient myosin are described in Table 3.

ATPase Activity of Myosin

Potassium/EDTA-activated ATPase activity of myosin and light-chain-deficient myosin were measured in a mixture containing 0.1 M Tris · HCl, pH 7.8, 1 mM EDTA, 0.65 M KCl and 5 mM ATP. Calcium-activated ATPase activity was measured in 0.2 M Tris · maleate, pH 5.7, 10 mM $CaCl_2$ and 5 mM ATP. Protein concentration and incubation time were chosen such that less than 20% of the ATP was hydrolyzed at any one time, thus giving a period of ATP hydrolysis linear with time. In most cases myosin (0.1 mg · ml) was assayed at 25 °C for 10 min. Following a 3-min preincubation period the reaction was initiated by addition of myosin. Hydrolyses of ATP in a 2-ml reaction mixture was stopped by addition of 1 ml of 20% trichloroacetic acid; 1-ml aliquots were assayed for inorganic phosphate by the method of Fiske and SubbaRow [15]. All glassware was acid-washed. Protein concentrations were determined according to Lowry [16]. All other techniques used for analyses of myosin ATPase activity were described earlier [6, 17]. Average values for canine right-ventricle myosin ATPase activity at 25 °C were 0.90 μmol P_i (mg protein)$^{-1}$ min^{-1} for K^+-stimulated myosin and 0.60 μmol P_i (mg protein)$^{-1}$ min^{-1} for Ca^{2+}-activated myosin.

Polyacrylamide Gel Electrophoresis and Determination of Protein Concentration of Stained Bands in Polyacrylamide Gels

Polyacrylamide gradient slab gels containing sodium dodecylsulfate were prepared as described earlier [14]. For determination of protein concentration present in Coomassie-blue-stained polyacrylamide gels, the dye was removed with 25% pyridine as described in earlier reports, and protein concentration determined [18].

Calcium Binding

Techniques for calcium binding were the same as those used earlier [19], including buffer conditions, protein concentrations, and calculations using best-fit programs.

RESULTS

In the absence of divalent cations there was a shift in the pH optimum of canine ventricular K^+-stimulated myosin ATPase when the enzymatic incubation temperature was increased from 25 °C to 37 °C (Fig. 1 A). When 2 mM $CaCl_2$ was added to the incubation mixture, this shift did not take place; the pH optimum was 8.5 at both 25 °C and 37 °C (Fig. 1 B). Rat cardiac myosin did not show a similar pH optimum shift in the absence of calcium with increases in temperature (Fig. 1 A).

The K^+/EDTA-stimulated myosin ATPase system [17] was used to promote light chain dissociation. The percentage of light chains complexed to the heavy chains and the rate of myosin ATPase activity were assessed for light-chain-deficient myosin. Myosin was precipitated twice with a 9-fold dilution of water at pH 6.5 to remove contaminants [19]. The starting myosin then had two high-affinity Ca^{2+} binding sites per myosin molecule indicating one Ca^{2+} binding light chain per head. Myosin was solubilized in 0.1 M Tris buffer at various pH values containing 0.65 M KCl, 5 mM ATP and 1 mM EDTA and then incubated at 37 °C for 10 min. At the end of the treatment light-chain-deficient myosin was precipitated with 45% $(NH_4)_2SO_4$ saturation and analyzed on polyacrylamide gradient slab gels in the presence of sodium dodecylsulfate (Fig. 2); since it was noted that there was dissociation of light chains, the 45% $(NH_4)_2SO_4$ supernatant was brought to 80% $(NH_4)_2SO_4$ saturation thus allowing for precipitation of the dissociated light chains (Fig. 2).

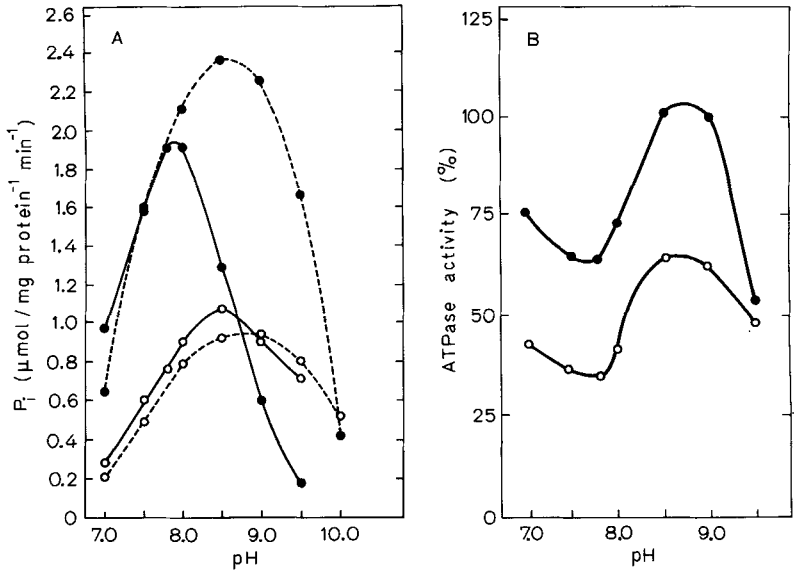

Fig. 1. *pH optimum of ATPase of canine ventricular (— —) and rat cardiac (– – – –) myosin.* K^+/EDTA-activated ATPase was measured in 0.1 M Tris · HCl, 1 mM EDTA, 0.65 M KCl, 5 mM ATP at the indicated pH. Incubation was carried out at 37 °C (●) and 25 °C (○) for 10 min in (A) the absence of Ca^{2+} and (B) the presence of 2 mM Ca^{2+}. In (A) ATPase activity is measured as the amount of P_i produced; in (B) it is measured as a percentage of the activity at 37 °C, pH 8.5

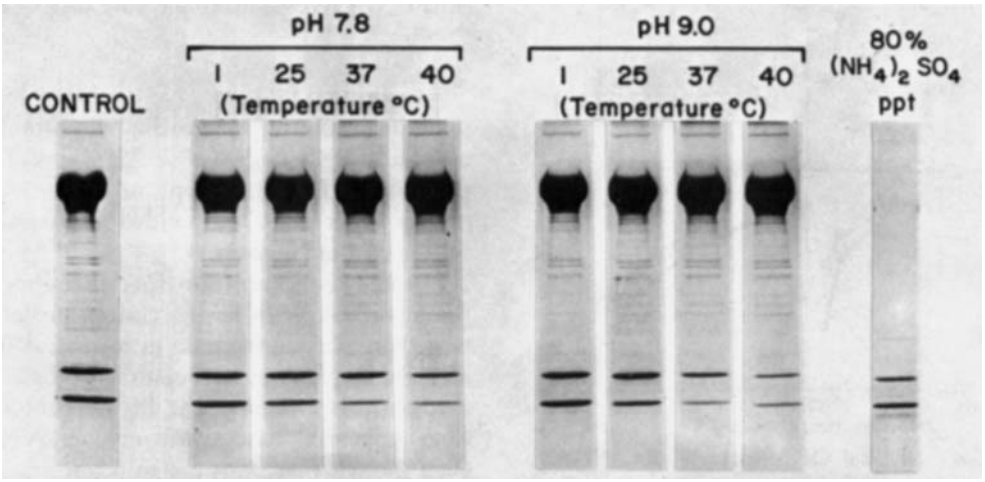

Fig. 2. *Gel patterns for canine cardiac myosin (first slot), light-chain-deficient myosin (slots 2–9) and dissociated light chains (last slot).* Myosin was solubilized in 0.1 M Tris · HCl, pH 7.8, 0.65 M KCl, 5 mM ATP, and 1 mM EDTA and incubated at 37 °C for 10 min at a protein concentration of 0.1 mg/ml. At the end of the reaction time, light-chain-deficient myosin was precipitated by bringing the solution to 45% $(NH_4)_2SO_4$ saturation. The light chains remaining in the supernatant were then precipitated by bringing the supernatant to 80% $(NH_4)_2SO_4$ saturation (last slot). Myosin and myosin light chains were solubilized in 0.05 M Tris · HCl, pH 7.5, 0.5 M KCl, and 1 mM dithiothreitol and dialyzed overnight against the same buffer and then analyzed on a polyacrylamide gradient (5–20%) slab gel containing sodium dodecylsulfate. In these analyses the effects of increments in temperature at various pH values were studied relative to the degree of light chain dissociation

Studies were carried out to determine whether temperature or pH caused a dissociation of myosin light chains and further studies were made to ascertain how these alterations were related to myosin ATPase activity. When myosin was treated at pH 7.8 and 9.0, each with increasing temperatures as shown in Fig. 3, there was a decrease in the ATPase activity with increases in temperature. Myosin denaturation did occur at 37 °C when the pH was alkaline. However, light-chain-deficient myosin retained an elevated ATPase activity when dissociation was carried out at 37 °C for 10 min at pH 7.0. Denaturation did not occur as rapidly at higher temperatures when the pH was neutral. Light chain dissociation occurred at higher temperatures regardless of the pH range from 7.0 to 9.0.

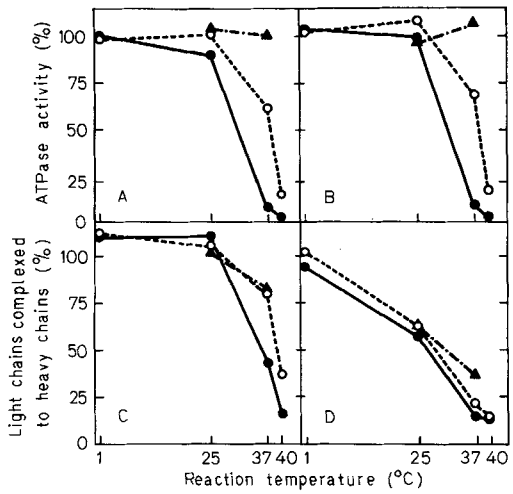

Fig. 3. *Potassium-activated (A) and calcium-activated (B) light-chain-deficient myosin ATPase activities of canine myosin, prepared as described for gel electrophoresis in Fig. 2, at pH 7.0 (▲), 7.8 (○) and 9.0 (●); percentage of light chain C_1 complexed to myosin heavy chains after this treatment (C) and the percentage of light chain C_2 remaining (D)*

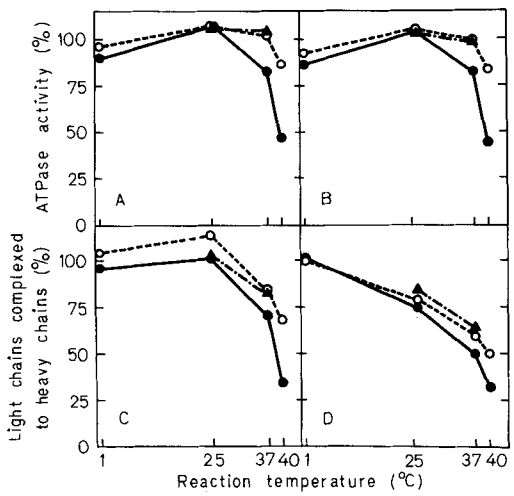

Fig. 4. *K^+-stimulated (A) and Ca^{2+}-stimulated (B) light-chain-deficient myosin ATPase activity and percentage of light chains C_1 (C) and C_2 (D) complexed to heavy chains at pH 7.0 (▲), 7.8 (○) and 9.0 (●).* Rat cardiac myosin was treated as described in Fig. 2. Both ATPase activities were assayed as described in Methods. The percentage of light chains C_1 and C_2 complexed to myosin heavy chains after treatment were also measured. Light-chain-deficient myosin ATPase activity, like analyses of light chain dissociation, was measured by precipitating myosin after incubating at 37 °C in the absence of divalent cations, dialyzing, and then assaying myosin at 25 °C

Rat cardiac myosin showed greater stability in the absence of divalent cations in that there was less light chain dissociation and the light-chain-deficient myosin retained a higher ATPase activity at more alkaline pH values. There was no decrease in enzymatic activity at pH 7.0 or 7.8 at either 25 °C and 37 °C; there was only a small decrease in the enzymatic activity at pH 9.0 with higher temperatures (Fig. 4). There was

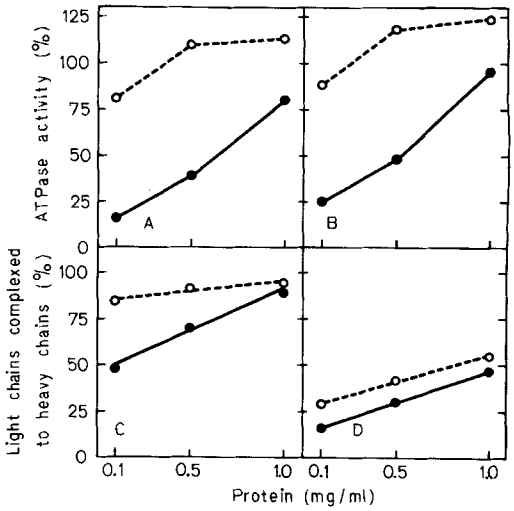

Fig. 5. *K^+-stimulated (A) and Ca^{2+}-stimulated (B) light-chain-deficient myosin ATPase activity and percentage of light chains C_1 (C) and C_2 (D) complexed to heavy chains at pH 7.8 (○) and 9.0 (●).* Canine myosin was treated as described in Fig. 2, at increasing protein concentrations as indicated, at both pH 7.8 (○——○) and pH 9.0 (●——●) for 5 min in the presence of 1 mM EDTA and 5 mM ATP. Potassium-activated (A) and calcium-activated (B) light-chain-deficient myosin were assayed as described in Methods. The percentage light chain C_1 (C) and light chain C_2 (D) attached to myosin heavy chain after treatment are shown

a dissociation of rat cardiac myosin light chains, especially light chain C_2 at 25 °C and 37 °C with all pH values (Fig. 4). Sheep and beef cardiac myosin, on the other hand, responded very similarly to that of dog cardiac myosin.

Protein concentration was an important facet in these studies. With an increase in protein concentration there was an increase in myosin ATPase activity and an increase in association of light chains with myosin heavy chains. The higher protein concentrations appeared to activate myosin ATPase activity even though there was a significant amount of dissociated light chains, particularly light chain C_2 (Fig. 5).

There was a continuous decrease in myosin ATPase activity as well as dissociation of light chain C_1 with an increase in exposure time at elevated temperatures (Fig. 6), whereas dissociation of light chain C_2 was rapid and complete in 10 min. The addition of calcium or magnesium to the incubation mixture prevented light chain dissociation and stabilized myosin ATPase activity (Fig. 6). If ATP was omitted from the incubation medium, myosin ATPase activity was strongly decreased (Table 1). To prove that temperature plus the absence of divalent cations but not $(NH_4)_2SO_4$ caused light chain dissociation, myosin was treated as described in Fig. 2 and passed through a Sephadex G-100 column equilibrated at 37 °C with 0.05 M Tris, pH 7.8, 5 mM ATP, 1 mM EDTA and 0.65 M KCl. Light-chain-deficient myosin was recovered in the

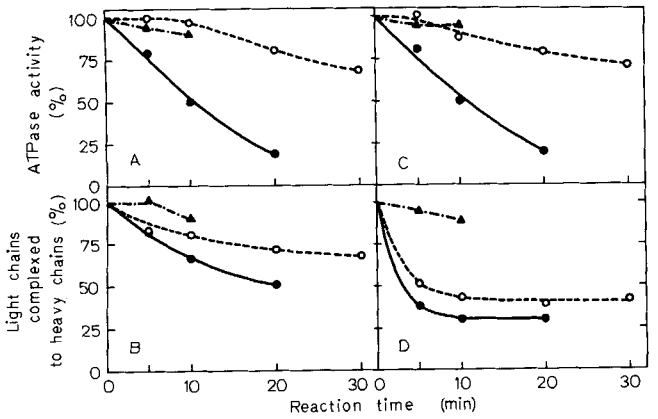

Fig. 6. *K^+-stimulated (A) and Ca^{2+}-stimulated (B) light-chain-deficient myosin ATPase activity in the presence of EDTA and/or Ca^{2+} and the percentage of light chains C_1 (C) and C_2 (D) complexed to heavy chains.* Ventricular myosin from the dog was treated at 37 °C, 0.1 mg/ml, pH 7.8, for varying lengths of time, except EDTA was omitted (▲) and 10 mM calcium was added (▲); (●) EDTA present, Ca^{2+} absent. Potassium-activated (A) and calcium-activated (B) light-chain-deficient myosin ATPase activities are shown. The percentage of light chain C_1 (C) and light chain C_2 (D) complexed to heavy chains are shown for light-chain-deficient myosin

Table 1. *Effect of treatment of myosin at 37 °C and pH 7.8 or 9.0, in the presence of EDTA and/or ATP, on light chain dissociation and ATPase activity*

Myosin (0.1 mg/ml) was treated for 5 min at 37 °C in the presence of 0.65 M KCl and 5 mM ATP and/or 1 mM EDTA at the indicated pH. Results are given as a percentage of the control values (i.e. untreated myosin); the control value for K^+-ATPase was 0.884 μmol P_i mg^{-1} min^{-1}, for Ca^{2+}-ATPase it was 0.549 μmol P_i mg^{-1} min^{-1}

System			Light chain associated with myosin		ATPase activity	
pH	ATP	EDTA	C_1	C_2	K^+	Ca^{2+}
			% control			
7.8	+	+	79	30	81	88
	−	+	89	22	30	45
	+	−	81	44	104	113
9.0	+	+	47	16	16	25
	−	+	63	15	18	29
	+	−	53	19	37	44

void volume and light chains in the inclusion volume. All of the light chain C_2 was dissociated under these conditions.

When ATP was present and EDTA absent from the pH-7.8 treatment at the temperatures indicated, there was 100% retention of myosin ATPase activity (Table 2). In another set of experiments, 1 g of cardiac myosin was treated at pH 9.0 in the presence of EDTA and absence of ATP at the temperatures indicated; under these conditions there was a large decrease in ATPase activity with the dissociation of light chains (Table 2). Fig. 7 shows the calcium binding properties

Table 2. *Effect of treatment of myosin at pH 7.8 or 9.0 and 0 °C or 37 °C, in the presence of ATP and/or EDTA, on light chain dissociation and ATPase activity*

Conditions				ATPase activity		Light chain associated with myosin	
pH	temp.	ATP (5 mM)	EDTA (1 mM)	K^+	Ca^{2+}	C_1	C_2
	°C			% control			
7.8	0	+	−	100	100	100	100
	37	+	−	108	106	76	40
9.0	0	−	+	113	109	96	99
	37	−	+	28	26	55	21

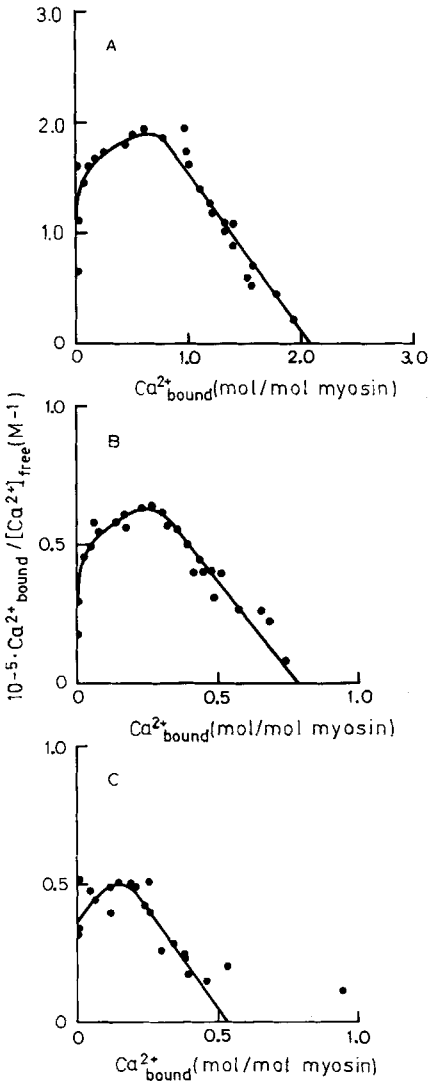

Fig. 7. *Representative Scatchard plots for the binding of calcium by canine cardiac myosin, untreated (A) and treated at 37 °C, pH 7.8 (B) or 37 °C, pH 9.0, plus EDTA (C).* For (A), $n = 2.06$, $K = 1.43 \times 10^5$ M^{-1}. In (B), cardiac myosin was treated at 37 °C for 10 min in 0.05 M Tris, pH 7.8, 5 mM ATP, and 0.65 M KCl at 0.1 mg/ml and then precipitated with 45% $(NH_4)_2SO_4$ dialyzed and assayed relative to calcium binding properties; $n = 0.85$; $K = 1.03 \times 10^5$ M^{-1}. In (C), cardiac myosin was treated as described in B, except at pH 9.0, and containing 1 mM EDTA; $n = 0.55$; $K = 1.3 \times 10^5$ M^{-1}

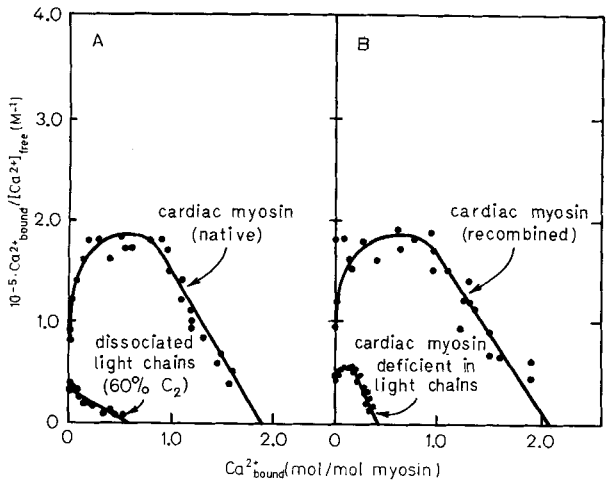

Fig. 8. *Scatchard plots for the binding of calcium by (A) native canine cardiac myosin* (n = *1.9*, K = *1.7×10^5 M^{-1}*) *and its dissociated light chains (60% light chain C_2*, n = *0.6*, K = *5×10^4 M^{-1}*) *and (B) light-chain-deficient myosin* (n = *0.45*, K = *1.7×10^5 M^{-1}*) *and recombination of dissociated light chains with light-chain-deficient myosin* (n = *2.1*, K = *1.5×10^5 M^{-1}*). Light chain dissociation was carried out as in Table 2 at pH 9.0. For reassociation, the recovered dissociated light chains were recombined with light-chain-deficient myosin at 4 °C, dialyzed in 0.2 M Tris · maleate, pH 6.5, for 24 h and precipitated with 45% $(NH_4)_2SO_4$. Conditions for Ca^{2+} binding are as described earlier [19], except the dissociated myosin light chains were also analyzed for Ca^{2+} binding properties at Ca^{2+} concentrations of 1 μM to 1 mM to better assess their affinity binding constant, which was lower than that of native myosin or reconstituted myosin

Table 3. *ATPase activity of light-chain-deficient myosin and reassociated myosin*
Conditions for the dissociation of light chains are as described in Table 2 at pH 9.0, except that the myosin concentration was 0.5 mg/ml. The dissociated light chains were again added to light-chain-deficient myosin, dialyzed against 0.2 M Tris · maleate, pH 6.5, for 24 h, precipitated with 45% $(NH_4)_2SO_4$ and then precipitated with 9 vol. H_2O, pH 6.5

Myosin	ATPase activity	Light chain complexed with myosin	
		C_1	C_2
	% control		
Native	100	100	100
Light-chain-deficient	24	88	24
Reassociated	35	98	103

and the number of calcium binding sites of this myosin described in Table 2 after partial dissociation of the light chains. After dissociation of more than 50% light chain C_2, Scatchard plots still express cooperativity (Fig. 7B). This may indicate random light chain C_2 dissociation, thus negating selective light chain C_2 removal from one of the two myosin heads. However, after an approximate 50% light chain C_1 dissociation cooperativity may have been lost (Fig. 7C). The Scatchard plot in Fig. 7C could also have been fitted to a straight line.

For assessing the reassociation of myosin based on the reconstitution of the calcium binding sites, 2 g of cardiac myosin were prepared. The original myosin was shown to have two high-affinity calcium binding sites (Fig. 8A). When myosin was treated at pH 9.0 as described in Table 2, light-chain-deficient myosin expressed a diminished number of Ca^{2+} binding sites but the same Ca^{2+} binding constant, while the dissociated light chains expressed one Ca^{2+} binding site per light chain C_2 and a decrease in the Ca^{2+} binding constant (Fig. 8A and B). When the dissociated light chains were recombined with light-chain-deficient myosin, the number of high-affinity Ca^{2+} binding sites was regained (Fig. 8B). Reassociation of myosin did not restore a decreased myosin ATPase activity (Table 3), although analyses of calcium binding and quantification of reconstitution by gel electrophoresis showed reassociation of myosin had occurred (Table 3, Fig. 8).

DISCUSSION

Myosin presumably goes through a change in the absence of divalent cations when the incubation temperature is increased to 25 °C and 37 °C, similar to the temperature-dependent dissociation of the spectrin oligomer [20]. A transition in the pH activity curve of heavy meromyosin with increases in temperature has been reported [21]. As described in these studies, light chain dissociation is dependent on temperature, protein concentration, and the absence of divalent cations, but independent of substrate and continues to occur over a pH range from 7.0 to 9.0. Unlike the 5,5′-dithio-bis(2-nitrobenzoate) light chain of skeletal muscle myosin where 5,5′-dithio-bis(2-nitrobenzoate) [22] and/or EDTA [2,23] promote(s) dissociation of the light chain, the cardiac myosin light chains, particularly light chain C_2, are only dissociated with these agents when there are increases in temperature and divalent cations are absent. With the dissociation of cardiac myosin light chain C_2, the Ca^{2+} binding light chain [7], as in the case of the 5,5′-dithio-bis(2-nitrobenzoate) light chain of rabbit skeletal muscle myosin [22,24], there was a corresponding decrease in the number of high-affinity calcium binding sites. Prior to treatment, myosin had two high-affinity calcium binding sites, indicative of one calcium binding light chain per myosin head. The number of binding sites diminished with treatment of myosin, depending on the degree of light chain dissociation. The remaining binding sites on light-chain-deficient myosin retained a high affinity constant. The dissociated light chains continued to bind calcium but with a lower binding constant similar to that reported for cardiac myosin light chain C_2 by Kuayama and Yagi

[7]. When the recovered dissociated light chains were recombined with light-chain-deficient myosin, the same number of high-affinity calcium binding sites as present for native myosin were obtained.

According to earlier reports, including studies on heat denaturation of myosin [25], there was a simultaneous loss of myosin ATPase activity with the dissociation of light chains [11, 12, 25, 26]. Using selective conditions to protect the active site of myosin, e.g. a neutral pH, ATP, and a brief incubation at 25 °C and 37 °C, there was no decrease in ATPase activity even though there was a 20% dissociation of light chain C_1 and 60% dissociation of light chain C_2. Degree of light chain C_1 dissociation greater than 20% was associated with a corresponding decrease in ATPase activity. It cannot be explained at this time why a small (20%) loss of light chain C_1 did not cause a corresponding decrease in ATPase activity since the separation of the comparable rabbit skeletal muscle myosin alkali light chains, A_1 and A_2, were associated with a loss in ATPase activity [10–12]. Reassociation of light chains with light-chain-deficient myosin takes place whether light-chain-deficient myosin has normal or depressed ATPase activity. Where there is depressed ATPase activity reassociation of light chains does not restore this activity; however, conditions are being analyzed to determine if restoration of ATPase activity could possibly occur under certain light-chain-dissociation and reassociation conditions.

If Ca^{2+} was added to the reaction mixture prior to incubation at 37 °C there was no dissociation of light chains, no shift in the pH optimum, and the ATPase activity of myosin was retained. Light chains may be permanently attached at lower temperatures [4, 5] but dissociate at 37 °C. Reassociation again takes place at 4 °C. Low protein concentration is not conducive to reassociation, thus light-chain-deficient myosin is obtained at low myosin concentrations. It may be that light chains, particularly light chain C_2, are important relative to the structure of myosin [27], where calcium or other divalent cations bind one or more light chains more tightly to the heavy chains [3–5].

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