The Site of Calcium Binding in Relation to the Activation of Myofibrillar Contraction

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ABSTRACT Skeletal muscle myofibrils, in the presence of 2 mM $MgCl_2$ at pH 7.0, were found to have two classes of calcium-binding sites with apparent affinity constants of 2.1×10^6 M^{-1} (class 1) and $\sim 3 \times 10^4$ M^{-1} (class 2), respectively. At free calcium concentrations essential for the activation of myofibrillar contraction ($\sim 10^{-6}$ M) there would be significant calcium binding only to the class 1 sites. These sites could bind about 1.3 μmoles of calcium per g protein. Extraction of myosin from the myofibrils did not alter their calcium-binding parameters. Myosin A, under identical experimental conditions, had little affinity for calcium. The class 1 sites are, therefore, presumed to be located in the I filaments. The class 1 sites could only be detected in F actin and myosin B preparations which were contaminated with the tropomyosin-troponin complex. Tropomyosin bound very little calcium. Troponin, which in conjunction with tropomyosin confers calcium sensitivity on actomyosin systems, could bind 22 μmoles of calcium per g protein with an apparent affinity constant of 2.4×10^6 M^{-1}. In view of the identical affinity constants of the myofibrils and troponin and the much greater number of calcium-binding sites on troponin it is suggested that calcium activates myofibrillar contraction by binding to the troponin molecule.

INTRODUCTION

There is now considerable experimental support for the hypothesis that the contraction of striated muscle is coupled to excitation through a translocation of calcium from the sarcoplasmic reticulum to the myofibrils (1–3). Consistent with this hypothesis is the demonstration that the ATPase activity and superprecipitation of in vitro actomyosin systems require the presence of a low concentration ($\sim 10^{-6}$ M) of calcium (4–8). The activating effects of calcium have been related by Weber et al. (5–8) to the reversible binding to the myofibril of about 1.5–2.0 μmoles of exchangeable calcium per g of myofibril protein. Removal of this calcium, either by chelating agents or isolated sarcotubular

vesicles, is associated with inhibition of myofibrillar ATPase activity and superprecipitation. The mechanism by which calcium activates contraction remains obscure. To elucidate this mechanism it is first necessary to identify the site on the myofibril at which calcium binds. At present there is very little evidence to indicate which of the known myofibrillar proteins might contain this site.

Any investigation of this problem must be considered in the light of the recent discovery that the requirement for calcium, as evidenced by the inhibitory effects of calcium-chelating agents, is not a characteristic of highly purified actomyosin preparations. When they elaborated on earlier observations (5, 9) indicating a difference in calcium sensitivity between "natural" and reconstituted actomyosin, Ebashi and Ebashi (10) showed that the calcium sensitivity of actomyosin was dependent on the presence of another protein closely associated with actin. This protein was partially purified and designated "native" tropomyosin in view of its physicochemical similarities to the classical Bailey tropomyosin (11). However, tropomyosin prepared by the Bailey procedure was ineffective in conferring calcium sensitivity on calcium-insensitive actomyosin. The ability of "tropomyosin-like" proteins to sensitize actomyosin to calcium and the inability of Bailey tropomyosin to do so were confirmed by several workers (12–16) and led to suggestions that the calcium-sensitizing factor was either a different molecular form of tropomyosin (10) or some other protein associated with tropomyosin (12, 13, 15). Apparent support for the first possibility came from Mueller's (16, 17) observation that tropomyosin prepared in the presence of a sulfhydryl protecting agent (SH-tropomyosin) had calcium-sensitizing activity whereas unprotected tropomyosin (S-S-tropomyosin) did not. That the state of SH groups might be related to the difference in activity of Bailey tropomyosin and "native" tropomyosin appeared reasonable in the light of earlier observations (18–21) that treatment of myosin B with SH reagents rendered it insensitive to calcium. This view was modified, however, by the discovery of Ebashi and Kodama (22) of a new protein, troponin, found in close association with tropomyosin, and the report by the same workers (23), recently confirmed by Hartshorne and Mueller (24), that the calcium-sensitizing factor is a tropomyosin-troponin complex. The previous observation of Mueller (16, 17) on the role of SH groups can now be explained by the finding of Yasui et al. (25) that the SH groups of troponin, not tropomyosin, are essential for calcium-sensitizing activity.

These discoveries on the nature and function of the calcium-sensitizing factor raise the question of whether the calcium which activates the myofibrils binds to actin, myosin, or to a component of the calcium-sensitizing complex. That the physiologically relevant calcium-binding site is part of the tropomyosin-troponin complex is suggested by earlier studies from this

laboratory (14, 26) showing that metin (27) and Mueller type tropomyosin (16, 17), both relatively impure preparations with calcium-sensitizing activity, had a high affinity for calcium.

In the present investigation we have measured calcium binding to isolated myofibrils and various proteins derived from myofibrils with a view to determining the molecular location of myofibrillar calcium-binding sites and obtaining quantitative information on the affinity of calcium for these sites. Since the activation of myofibrillar contraction requires concentrations of free calcium in the range of only $\sim 10^{-6}$ M we can reasonably conclude that the affinity constant for the binding of calcium to the activating site must be quite high. This criterion has been used to localize calcium-binding sites of potential importance in the regulation of contractility.

METHODS

A. *Solutions and Reagents*

Unless otherwise stated, protein fractions were stored in and calcium-binding measurements were carried out with a solution containing 60 mM KCl, 30 mM imidazole, and 2 mM $MgCl_2$, pH 7.0. This will be referred to as standard buffer solution. All solutions were prepared with distilled, deionized water.

Protein concentrations were estimated by the method of Lowry et al. (28), using bovine serum albumin as a standard.

Chelex-100 was obtained from Bio-Rad Laboratories (Richmond, Calif.). The disodium salt of ATP_1[1] was purchased from P-L Biochemicals (Milwaukee, Wis.) and the imidazole and EGTA[1] were products of Eastman Organic Chemicals (Rochester, N. Y.).

B. *Preparation of Protein Fractions*

MYOFIBRILS Minced rabbit muscle was homogenized in the cold for 1 min in a Waring Blendor with 3 volumes of standard buffer solution. The homogenate was centrifuged at 4000 *g* for 10 min and the sediment was resuspended in standard buffer solution to which 2 mM EGTA was added to bind free calcium. The suspension was filtered through several layers of cheesecloth and the myofibrils were washed four times with standard buffer solution (the first two centrifugations at 1500 *g* for 10 min, the next two at 1000 *g* for 10 min).

I FILAMENTS In the context of this paper I filaments are defined as myofibrils from which myosin has been extracted. They are assumed to be identical to the actin filaments seen in electron micrographs of the myofibril (29). The starting material was myofibrils prepared as described above (15–20 mg per ml in standard buffer solution). To remove myosin, sodium pyrophosphate, pH 7.0, was added to a concentration of 10 mM followed by solid KCl to a concentration of 0.5 M. After brief stirring in the cold the mixture was subjected to centrifugation at 12,000 *g* for 15

[1] The abbreviations used are ATP, adenosine triphosphate and EGTA, ethylene glycol bis (β-aminoethylether)-*N*,*N'*-tetraacetic acid.

min, the supernatant discarded, and the residue extracted three more times with KCl-pyrophosphate solution. The I filaments were suspended in standard buffer with the aid of a glass Teflon homogenizer and 2 mM EGTA was added to remove extraneous calcium. The EGTA was removed by washing three times with standard buffer solution. Calcium-binding experiments were carried out immediately after preparation as the I filaments showed a marked tendency toward aggregation upon standing. In some cases we followed Huxley's (29) method of first mechanically separating the actin and myosin filaments in a plasticizing medium with the aid of a Virtis homogenizer, followed by dissolution of the myosin filaments with 0.5 M KCl. There was no apparent difference in the experimental results.

The essentially complete removal of myosin was verified by the loss of Mg^{++}-stimulated ATPase activity and ATP-induced superprecipitation. The actin filaments were still present as evidenced by the fact that when such filament suspensions were mixed with purified myosin A there was an enhancement of Mg^{++}-stimulated myosin ATPase activity.

MYOSIN A Minced rabbit muscle was extracted in the cold with Guba-Straub solution according to Mommaerts (30) and purified by three cycles of precipitation. Following the final precipitation the myosin was dissolved in 0.6 M KCl, 10 mM imidazole, 1 mM $MgCl_2$, and 5 mM ATP, pH 7.0, and subjected to centrifugation at 150,000 *g* for 3 hr to remove traces of actin. The top two-thirds of the supernatant was removed, and the myosin was precipitated in the presence of EGTA. The precipitate was washed twice and suspended in standard buffer.

MYOSIN B Two preparations were studied, the conventional calcium-sensitive myosin B (Weber-Edsall myosin B) and the calcium-insensitive myosin B recently described by Perry et al. (31).

Minced rabbit muscle was extracted overnight in the cold with 5 volumes of Weber-Edsall solution (30). The insoluble residue was removed by centrifugation and the myosin A was precipitated by dilution in the presence of ATP (30). The myosin B was collected by centrifugation, dissolved in 1 M KCl, 30 mM imidazole, pH 7.0, and precipitated two more times, the last time in the presence of 2 mM EGTA. The precipitate was washed twice with standard buffer solution and finally suspended in the same solution.

To prepare calcium-insensitive myosin B, once precipitated myosin B obtained as described above was treated according to the procedure of Perry et al. (31).

ACTIN The procedure employed was that of Carsten and Mommaerts (32), with some modifications as required. To obtain *tropomyosin-free* actin the temperature during extraction was maintained at 0°–2°C (33, 34) and the first cycle of polymerization was induced by the addition of 0.6 mM $MgCl_2$ (33, 34). For the preparation of *tropomyosin-rich* actin the procedure was similar except that the acetone powder was extracted at 35°C and the initial polymerization was induced by the addition of 100 mM KCl and 2 mM $MgCl_2$.

TROPOMYOSIN The preparation employed in this study was purified in the presence of 0.5 mM dithiothreitol (SH-tropomyosin) as recommended by Mueller (16, 17), with modifications to reduce troponin contamination. The procedure is described in detail by Yasui et al. (25).

TROPONIN Isolation and purification were carried out in the presence of 0.5 mM dithiothreitol (SH-troponin) according to Yasui et al. (25).

C. *Measurement of Superprecipitation*

Superprecipitation of actomyosin and myosin B was measured by the light-scattering technique (4) as described by Yasui et al. (25).

D. *Measurement of Calcium Binding*

Calcium-binding measurements were performed with myofibrils, I filaments, myosin B, myosin A, F actin, tropomyosin, and troponin. Since some of the proteins (F actin, tropomyosin, and troponin) were soluble and others insoluble under the chosen experimental conditions, two different procedures were employed.

The binding of calcium to the soluble proteins was measured according to the procedure of Briggs and Fleishman (35). This method is based on the partition of ^{45}Ca between the calcium-chelating resin Chelex-100 and the soluble phase containing the calcium-binding proteins. The Chelex-100 was washed and equilibrated with standard buffer as already described (35). 5 ml of standard buffer containing protein, 0.2 μc per ml ^{45}Ca, and varying concentrations of ^{40}Ca, was mixed with resin at room temperature in conical centrifuge tubes and stirred for 15 sec. The resin was sedimented by centrifugation and 0.5 ml of supernatant was removed for measurement of radioactivity with a Beckman LS-100 liquid scintillation counter. The packed volume of resin in all experiments was 0.25 ml. Each experimental run usually consisted of a series of 10 tubes plus a series of protein-free standards and a resin-free control. The total calcium concentration in each tube, which varied from $\sim 5 \times 10^{-6}$ M to $\sim 80 \times 10^{-6}$ M, included the added calcium plus the contaminating exchangeable calcium present in the protein, resin, and buffer solution. For evaluation of the latter, see below.

The measurement of calcium binding to the insoluble proteins was carried out by a variation of the above procedure in which the ^{45}Ca is partitioned between the insoluble protein and a soluble phase containing citrate as a calcium-complexing species. The procedure was as described for the soluble proteins except that the assay mixture contained insoluble protein in place of Chelex-100 and 2 mM citrate was added to the standard buffer.

To minimize calcium contamination plastic disposable pipets and centrifuge tubes were used in all experiments.

E. *Calculation of Calcium-Binding Parameters*

The concentrations of free calcium and protein-bound calcium, as a function of total calcium, were calculated from the ^{45}Ca partition measurements. The data were then treated according to the method of Scatchard (36) to estimate the number of each class of binding sites and the affinity constant for each class of sites.

In the Chelex-protein partition system, the free calcium and the protein-bound calcium were calculated by means of the equations (35)

$$[Ca^{++}] = [Ca_T] - ([Ca_R] + [Ca_P]) \quad (1)$$

$$[Ca_P] = [Ca_T] - \frac{[Ca_R]}{f_R} \quad (2)$$

where

$$[Ca_R] = [Ca_T] \times \left(1 - \frac{x}{x_T}\right) \quad (3)$$

$$f_R = \frac{[Ca_R]}{[Ca_R] + [Ca^{++}]} \quad (4)$$

The following symbols are employed

$[Ca^{++}]$ = free calcium concentration
$[Ca_P]$ = protein-bound calcium concentration
$[Ca_R]$ = resin-bound calcium concentration
$[Ca_T]$ = total calcium concentration
x = cpm in supernatant
x_T = cpm in resin-free control

As long as $[Ca_T]$ is small relative to the concentration of resin-binding sites, f_R is a constant which can be evaluated independently. For the method of determining f_R and the derivation of equation (2) consult the paper of Briggs and Fleishman (35). All concentrations were expressed in moles per liter. If $[Ca_P]$ is divided by the protein concentration (grams per liter), we obtain Ca *Pr*, the moles of calcium bound per gram of protein. The relevant binding parameters were then determined from a plot of $\frac{\text{Ca } Pr}{[Ca^{++}]}$ vs. Ca *Pr*.

The citrate-protein system is formally identical to the Chelex-protein system. It differs experimentally in that the insoluble protein replaces the resin and the citrate replaces the soluble calcium-binding protein. Hence, the following expressions are applicable.

$$[Ca^{++}] = [Ca_T] - ([Ca_c] + [Ca_P]) \quad (5)$$

$$[Ca_P] = [Ca_T] \times \left(1 - \frac{x}{x_T}\right) \quad (6)$$

or

$$[Ca_P] = [Ca_T] - \frac{[Ca_c]}{f_c} \quad (7)$$

where

$[Ca_c]$ = calcium citrate concentration

$$f_c = \frac{[Ca_c]}{[Ca_c] + [Ca^{++}]} \quad (8)$$

Rearranging equation (7) we have

$$[Ca_c] = f_c([Ca_T] - [Ca_P]) \quad (9)$$

To evaluate f_c it is necessary to have values of Ca_c as a function of total *soluble* calcium. These values were derived by carrying out a ^{45}Ca partition between Chelex-100, with a known value of f_R, and standard buffer containing 2 mM citrate. By analogy with equation (2) we then have

$$[Ca_c] = [Ca_T] - \frac{[Ca_R]}{f_R} \quad (10)$$

Since

$$[Ca^{++}] + [Ca_c] = [Ca_T] - [Ca_R] \quad (11)$$

we can substitute

$$f_c = \frac{[Ca_c]}{[Ca_T] - [Ca_R]} \quad (12)$$

Over the range of total calcium concentrations employed in this study f_c was constant, hence $[Ca^{++}]$ could be readily calculated from equations (5), (6), and (9).

F. *Determination of Exchangeable Calcium*

Preliminary experiments showed that, despite careful precautions, the calcium contamination in the assay system was of sufficient magnitude (2 to 10 $\times$ 10^{-6} M) to introduce appreciable error into the calculation of affinity constants if neglected. This calcium was contributed by the protein and, to a lesser extent, the Chelex-100 and buffer solution. Not all the protein-bound calcium is exchangeable (37, 38). Therefore, it was necessary in each experiment to determine the calcium initially present in the protein, resin, and buffer, and the fraction of the protein-bound calcium which was nonexchangeable.

The determination of total calcium in protein solutions and buffer was carried out by a modification (39) of the method of Kepner and Hercules (40). In the case of the insoluble proteins, aliquots of protein suspension were placed in Vycor tubes, dried *in vacuo*, and ashed for 24 hr at 500°C. To determine the calcium in the resin a fixed amount of Chelex-100 was extracted with an amount of EGTA equivalent to 10 times the number of equivalents of divalent cation-binding sites on the resin. The calcium in the extract was then analyzed following combustion of the EGTA. Control experiments with ^{45}Ca showed that recovery of calcium from the resin was complete.

Nonexchangeable protein-bound calcium was determined by equilibrium dialysis. The protein, dissolved or suspended in standard buffer, was dialyzed for 48 hr in the cold against 1½ volumes of standard buffer, to which 0.1 μc per ml ^{45}Ca was added. The dialysis tubing was pretreated with EGTA solution and exhaustively washed

with the deionized water. Since the ^{40}Ca present as contamination was sufficiently high to be measured accurately by our analytical method, none was added. At the end of the dialysis period the contents of the dialysis bag and the outside solution were analyzed for ^{40}Ca and ^{45}Ca. Negligible Donnan effects were assumed, and the bound radioactivity and bound calcium were determined from the difference between inside and outside values. The exchangeable bound calcium *at equilibrium* was calculated from the formula,

$$\text{Exchangeable bound Ca} = \text{bound Ca} \times \frac{\text{specific activity bound Ca}}{\text{specific activity free Ca}}$$

The nonexchangeable calcium could then be readily calculated and this was sub-

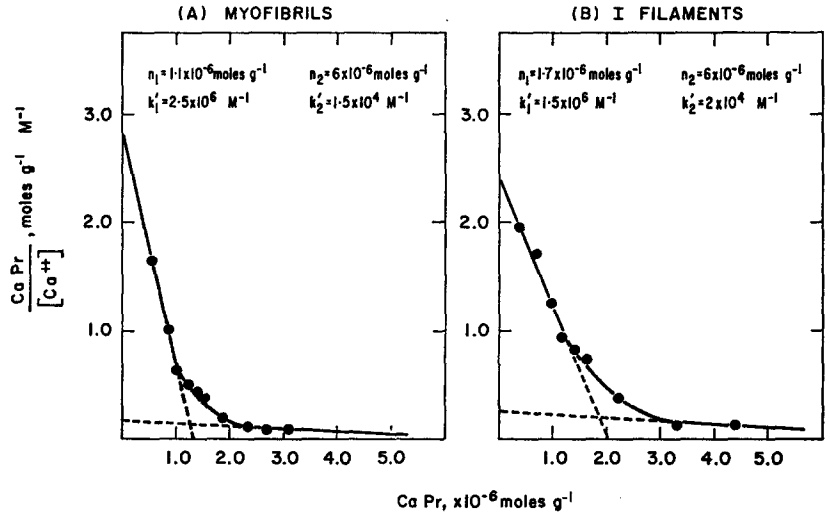

FIGURE 1. Scatchard plots of calcium binding to myofibrils and I filaments. See Methods section for assay conditions and meaning of symbols. Protein concentrations, 5 mg per ml.

tracted from the *initial* calcium to give the exchangeable calcium present as contamination in the protein preparation.

RESULTS

The Binding of Calcium to Myofibrils A Scatchard plot of the data derived from a typical myofibril-citrate partition is shown in Fig. 1 A. The curve is closely approximated by two straight lines, indicating the presence of two classes of binding sites. These are designated as *class 1* sites and *class 2* sites, respectively. From the intercepts and slopes of the straight lines approximate values for the affinity constants and the number of binding sites were obtained and these were then adjusted to fit the experimental data, using the equation

TABLE I

CALCIUM-BINDING PARAMETERS OF MYOFIBRILLAR PROTEINS

Preparation	n_1	k_1'	n_2	k_2'
	$\times 10^{-6}$ μmoles/g	$\times 10^{6}$ M^{-1}	$\times 10^{-6}$ moles/g	$\times 10^{4}$ M^{-1}
Myofibrils				
1	1.2	2.0	3	5
2	1.3	1.5	7	1
3	1.1	2.8	5	5
4	1.1	2.5	6	2
5	1.7	1.8	5	1
Mean	1.3	2.1	5	3
I filaments				
1	1.7	1.5	6	2
2	2.0	1.0	12	2
3	1.3	0.8	9	1
4	1.0	1.1	6	1
5	4.5	1.1	12	2
6	0.5	1.1	5	1
Mean	1.8	1.1	8	2
Myosin B				
1	0.7	3.5	3	3
2	2.0	2.8	5	5
3	1.5	5.5	3	2
Mean	1.5	3.9	4	3
Warm actin				
1	2.8	2.5	60	1
2	2.4	2.0	∽100	1
3	3.3	2.7	9	5
Mean	2.8	2.4	∽60	2
Tropomyosin				
1	4.5	2.4	—	—
2	2.5	2.8	—	—
3*	3.6	1.4	—	—
Mean	3.5	2.2	—	—
Troponin				
1	20.8	2.7	—	—
2	21.0	2.2	—	—
3*	23.6	2.2	—	—
Mean	21.8	2.4	—	—

Myosin A, no measurable calcium binding. Cold actin, no measurable calcium binding. Perry myosin B, no measurable calcium binding.

* Prepared by reduction of the S-S-form of the protein (25).

for binding to two sets of independent, noninteracting sites (41):

$$CaP_r = \frac{n_1 k_1'[Ca^{++}]}{1 + k_1'[Ca^{++}]} + \frac{n_2 k_2'[Ca^{++}]}{1 + k_2'[Ca^{++}]}$$

Here n_1 and n_2 are the moles of calcium per gram of protein which can bind to each class of site at saturation and k_1' and k_2' are the respective apparent affinity constants. These are considered to be apparent affinity constants since calcium binding was studied in the presence of 2 mM Mg^{++} at pH 7.0. It is likely that both hydrogen ions and magnesium (8) compete with calcium for the binding sites on the protein. These conditions were chosen because they are known to be appropriate for the demonstration of calcium control of contractility (1, 3).

The calcium-binding parameters of the five different myofibril preparations are listed in Table I. The average value of n_1 was 1.3 μmoles Ca per g protein

TABLE II

BOUND NONEXCHANGEABLE CALCIUM

Preparation	No. of measurements	Mean	Range of values
		μmoles/g	*μmoles/g*
Myofibrils	8	0.19	0.00–0.40
I filaments	5	1.95	0.00–6.90
Myosin B	7	0.28	0.00–0.70
Cold actin	1	4.75	
Warm actin	3	0.11	0.00–0.30
Tropomyosin	2	1.91	1.53-2.30
Troponin	3	1.35	0.00–2.80

and the average k_1' was $2.1 \times 10^6 M^{-1}$. The corresponding values for the class 2 sites were about 5 μmoles per g and about $3 \times 10^4 M^{-1}$, respectively. The estimates of n_2 and k_2' are somewhat uncertain since at higher total calcium concentrations only a few per cent of the initial radioactivity was removed from solution by the myofibrils. The error in the calculated values of $CaPr$ and $[Ca^{++}]$ will increase as the total calcium concentration increases. Nevertheless, we can estimate from the difference in slopes of the two linear portions of the curve that k_2' must be at least two orders of magnitude less than k_1'. Therefore, in the range of free calcium concentrations at which myofibrils are activated (10^{-7} to 10^{-5} M) there would be significant binding of calcium only to the class 1 sites. The value of k_1' is of the order of magnitude predicted from the calcium-dependence studies of Weber and Herz (8). Hence we assume that the class 2 sites represent nonspecific calcium-protein interactions and that only the class 1 sites are relevant to the problem of calcium activation. This assumption is supported by the close agreement between our values

of n_1 and the amount of calcium bound to myofibrils during maximal superprecipitation (8).

The amount of nonexchangeable calcium bound to the myofibrils, about 0.2 μmole Ca per g protein (Table II), is in agreement with values given by Weber (3) but is considerably less than expected from previous studies on the nonexchangeable calcium content of F actin (37, 38). If we assume that 10–15% of the myofibrillar protein is F actin (42) and that there is 1 mole of bound nonexchangeable calcium per mole of G actin (37, 38), there would be about 2 μmoles of nonexchangeable calcium per g of myofibrillar protein. It thus appears that F actin in its native state contains little or no nonexchange-

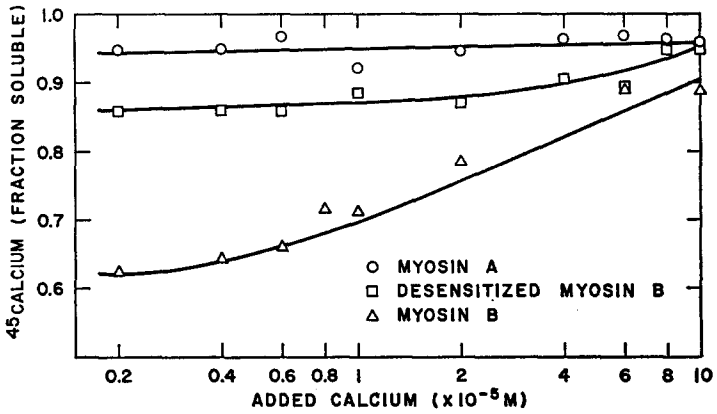

FIGURE 2. Partition of ^{45}Ca between the insoluble proteins, myosin A, myosin B, and Perry myosin B and a solution containing 2 mM citrate. The final concentration of the proteins, suspended in the standard buffer solution, was 5 mg per ml. The ordinate shows the fraction of the calcium remaining in the solution after the proteins were removed by centrifugation.

able calcium. This possibility has also been suggested by Weber (3) on the basis of the considerable difference between the nonexchangeable calcium content and the actin-bound ADP content of the myofibril. It is conceivable that the presence of nonexchangeable calcium in purified F actin may be a preparative artifact.

The Ultrastructural Site of Calcium Binding

In terms of the "sliding filament" model (43) of myofibril ultrastructure one can envision the binding of calcium to either the thick (A) filaments or thin (I) filaments, or, conceivably, to both. The A filaments contain myosin (43) and the I filaments contain actin (43), tropomyosin (44, 45), troponin (45, 46), and possibly other proteins (47, 48). The differential solubility of the A filament and I filament proteins (43) provides a method for the morphological localization of the myofibrillar calcium-binding sites.

As shown by the Scatchard plot in Fig. 1 B and the data in Table I, the I filaments had essentially the same calcium-binding parameters as the myofibrils. Purified myosin A, assayed under the same conditions, bound no measurable amounts of calcium (Fig. 2). As pointed out, this method of measuring calcium binding is not well suited to the detection of low affinity binding sites. If the myosin does bind calcium, there can be no class of sites with an apparent affinity constant greater than $\sim 10^3$ M^{-1}. Hence we conclude that the class 1 sites of the myofibril are located on the I filaments.

The average value of n_1 of the I filaments (1.8 μmoles Ca per g protein) was somewhat higher than that of the myofibrils but, as shown in Table I, the range of individual values was much greater (0.5 to 4.5 μmoles Ca per g protein). Myosin constitutes about 50% of the myofibrillar protein (43). If the extraction of myosin was quantitative and selective, the value of n_1 of the I filaments should have been twice that of the myofibrils. However, extraction was not selective as evidenced by the fact that dilution precipitates from the KCl-pyrophosphate extracts showed an EGTA-sensitive superprecipitation in the presence of ATP. It would seem reasonable to assume, then, that variable amounts of I filament proteins were removed during the myosin extraction, thus accounting for the variation in values of n_1. The similarly large variation in the nonexchangeable calcium content of the I filament (Table II) is consistent with this assumption.

The Binding of Calcium to Actin

F actin is considered to be the major I filament protein (43), although the true actin content of the myofibril is still subject to some uncertainty (42). It is now known that both the purity and biological properties of actin depend upon the method of preparation. Actin prepared from low temperature extracts and polymerized with dilute $MgCl_2$ is relatively free of tropomyosin whereas actin extracted at room temperature or above and polymerized with 0.1 M KCl may contain up to 20% tropomyosin (34, 49). Katz (15) has shown that actomyosin reconstituted from tropomyosin-free actin ("cold" actin) was not sensitive to calcium while actomyosin prepared with tropomyosin-rich actin ("warm" actin) required calcium for ATPase activity and superprecipitation.

We have confirmed the observation of Katz (Fig. 3 A) and have found, in addition, that the preparative differences stated above also affect the calcium-binding properties of actin. Cold actin, unlike warm actin, bound no measurable amounts of calcium (Fig. 4). Like the myofibrils and the I filaments, the warm F actin had two classes of binding sites (Fig. 5 B and Table I). The high affinity sites could bind about 2.8 μmoles Ca per g protein with a k_1' of 2.4 $\times$ 10^6 M^{-1}. This value of k_1' is almost identical to the k_1' of the myofibrils and leads us to conclude that the high affinity sites of the warm actin are identical

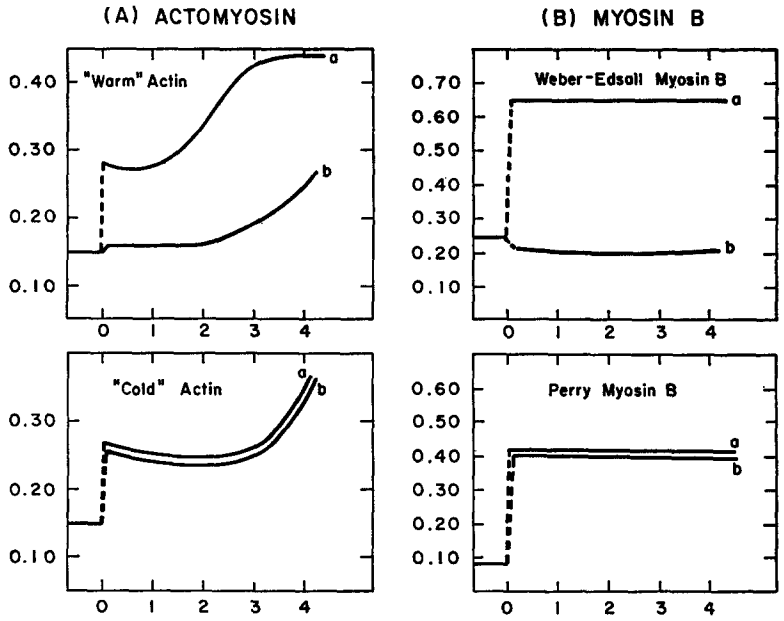

FIGURE 3. Superprecipitation studies with reconstituted actomyosin and myosin B. Protein concentrations were 0.3 mg per ml. Superprecipitation was measured in standard buffer solution. At time 0 ATP was added to a final concentration of 0.25 mM. Abscissa, minutes; ordinate, optical density. Curve a, control; curve b, with 0.1 mM EGTA.

to the class 1 sites of myofibril. Thus the class 1 sites are part of the actin-containing filament but are located on some component other than actin. Moreover, these results, taken together with the observation of Katz (15), suggest that the sensitivity of contractile systems to calcium, or more spe-

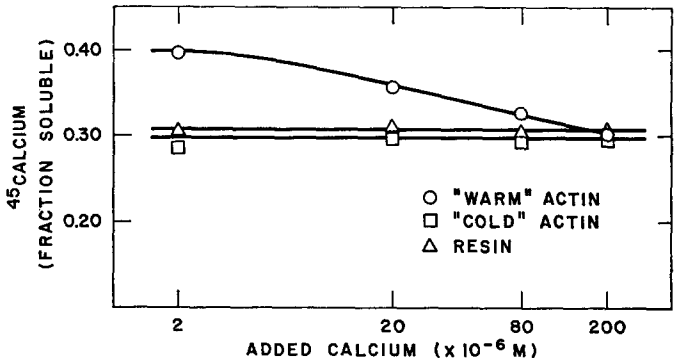

FIGURE 4. Partition of ^{45}Ca between Chelex-100 and solutions of actin. "Resin" shows ^{45}Ca partition in the absence of actin. "Warm" actin and "cold" actin were extracted from the muscle pulp at 35° and 0°C, respectively. "Warm" actin, 1.7 mg per ml, "cold" actin, 2.0 mg per ml in the partition.

cifically, the presence of the "calcium-sensitizing factor" is closely associated with the presence of the class 1 calcium-binding sites.

It is of interest that the warm actin contained only a negligible amount of nonexchangeable bound calcium (Table II). Only one measurement was made with cold actin and this gave a value 70% less than that reported in the literature (38). In view of differences in preparative procedure and methods of measuring bound calcium, direct comparison with earlier studies is probably not warranted. More data are needed before any firm conclusions can be drawn, but the results reported here are consistent with the suggestion (see above) that the calcium content of actin may be related to the preparative conditions.

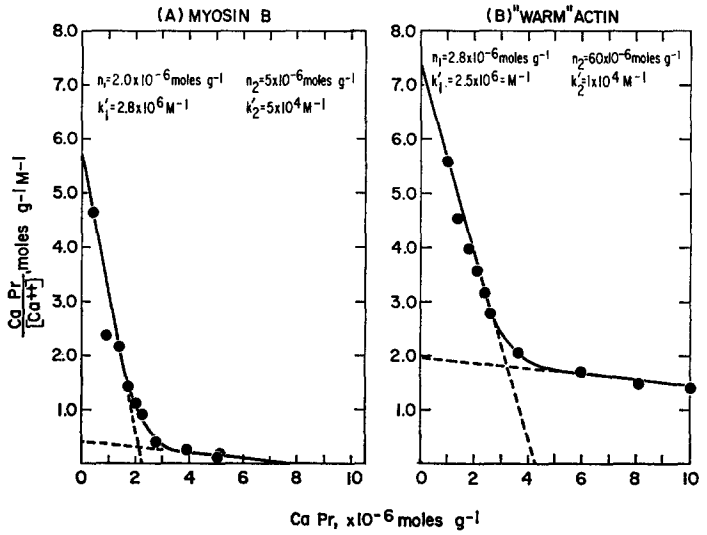

FIGURE 5. Scatchard plots of calcium binding to myosin B and actin extracted at 35°C. See Methods section for assay conditions. Myosin B, 3 mg per ml; actin, 2.1 mg per ml.

The Binding of Calcium to Myosin B

Perry et al. (31) have shown that if myosin B is extracted for 2 days with water at slightly alkaline pH its ability to hydrolyze ATP and superprecipitate is not affected, but the requirement for calcium is lost. According to Hartshorne and Mueller (24) this treatment leads to the removal of the tropomyosin-troponin complex. If our suggestion that the class 1 binding sites are closely associated with the calcium-sensitizing factor is correct, we should expect to find a difference between the conventional myosin B and Perry type myosin B as regards capacity to bind calcium.

As shown in Fig. 3 B, treatment of myosin B by the Perry procedure clearly

eliminated its responsiveness to EGTA. Similarly, the Perry myosin B bound only insignificant amounts of calcium compared to the Weber-Edsall myosin B (Fig. 2). The calcium-binding parameters of Weber-Edsall myosin B were quite similar to those of the myofibrils, as expected (Fig. 5 A and Table I). These data support the suggestion that the calcium-sensitizing factor and the protein carrying the class 1 calcium-binding sites are closely associated, if not identical.

The Binding of Calcium to Tropomyosin and Troponin

That the calcium-sensitizing protein and the calcium-binding protein might be identical was first suggested by earlier studies in this laboratory demonstrat-

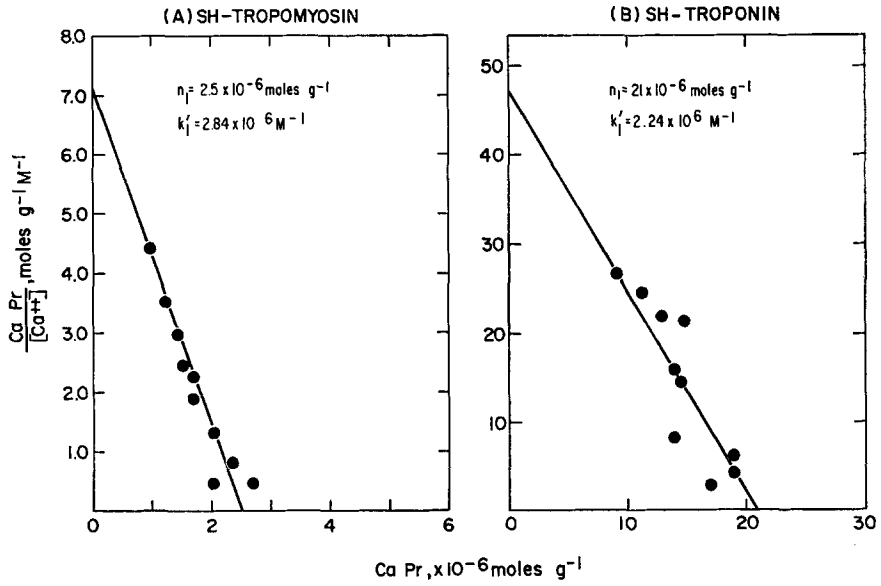

FIGURE 6. Scatchard plots of calcium binding to SH-tropomyosin and SH-troponin. Tropomyosin, 0.3 mg per ml; troponin, 0.1 mg per ml.

ing a strong binding of calcium to metin (14) and Mueller type SH-tropomyosin (26). Since both of these preparations contained unknown impurities no conclusions could be drawn as to the precise localization of the calcium-binding site. A means of resolving this problem was provided by the discovery of troponin (22) and the demonstration that the calcium-sensitizing factor is a tropomyosin-troponin complex (23).

Measurements of calcium binding to SH-tropomyosin and SH-troponin disclosed that each had a single class of binding sites with average k_1' values of 2.2×10^6 M^{-1} and 2.4×10^6 M^{-1}, respectively (Fig. 6 and Table I). However, the average site concentration, n_1, of troponin (22 μmoles Ca per g) was more than six times that of tropomyosin (3.5 μmoles Ca per g). We assume that the

binding sites found in the tropomyosin preparation are in fact located on troponin since the tropomyosin preparations employed in this study did have some residual troponin contamination as disclosed by disc electrophoresis studies (25). On the basis of the high n_1 value of the troponin and the identical k_1' values of the troponin and myofibrils we conclude that the class 1 sites of the myofibril are located on the troponin molecule.

DISCUSSION

Control of myofibrillar contractility is effected through alterations of the free calcium concentrations over a range of 10^{-7} to 10^{-6} M (1, 3). Thus the affinity constant for the binding of calcium to the activating site should be $\sim 10^6$ M^{-1}. We have shown in this communication that the myofibril has two classes of calcium-binding sites, with apparent affinity constants of 2.1×10^6 M^{-1} (class 1) and $\sim 3 \times 10^4$ M^{-1} (class 2), respectively. At physiological free calcium concentrations there would be significant binding of calcium only to the class 1 sites, the value of the apparent affinity constant, k_1', being of the predicted order of magnitude. Furthermore, the binding capacity (n_1) of the class 1 sites (1.3 μmoles Ca per g protein) corresponds closely with the amount of calcium actually bound to contracted myofibrils (1.5–2.0 μmoles Ca per g protein), as measured by Weber and Herz (8). Therefore, we feel confident in assuming that the class 1 sites are the physiological calcium "receptors" of the myofibril.

Evidence has been presented that the class 1 sites are located on the I filaments and, more specifically, on the troponin molecule. This conclusion is based on the following observations: (*a*) I filaments, essentially devoid of myosin, had the same calcium-binding parameters as the intact myofibril. (*b*) Purified myosin A had no measurable affinity for calcium. (*c*) The binding of calcium to F actin was only observed in those preparations containing the tropomyosin-troponin complex, (*d*) The binding of calcium to myosin B was eliminated following treatment known to remove the tropomyosin-troponin complex. (*e*) Purified troponin had a single class of binding sites with a k_1' value identical to that of the myofibrils but with an n_1 value (22 μmoles Ca per g) 17 times greater than the myofibrillar n_1 value.

The values of k_1' for the myofibrils, I filaments, calcium-sensitive myosin B, and warm actin, as well as troponin, were virtually the same, indicating that we were measuring calcium binding to the same sites in all these preparations. The small number of high affinity binding sites present in tropomyosin could be accounted for on the basis of troponin contamination (25). Our preparations of cold actin presumably contained α-actinin (47) and β-actinin (48). The fact that these preparations had no affinity for calcium indicates that the actinins as well as actin lack high affinity calcium-binding sites. The data suggest that troponin is the only myofibrillar protein with an affinity for cal-

cium great enough to account for the binding of calcium to myofibrils in the range of physiological free calcium concentrations. It is now established that troponin, acting in conjunction with tropomyosin, is responsible for the calcium sensitivity of actomyosin systems (23–25). We propose that calcium initiates myofibrillar contraction by forming a complex with the troponin molecule.

Previous measurements of calcium binding to myofibrillar proteins (1), although limited in scope, are consistent with our results. The weak affinity of calcium for myosin was indicated in an earlier study by Nanninga (50). The binding of calcium to G actin was studied by Martonosi et al. (51), who have indicated the presence of one binding site per molecule with an affinity constant of $\sim 10^5$ M^{-1} and a second class of 8 to 11 sites with an affinity constant of 2–4 $\times$ 10^3 M^{-1}. Since the polymerization of actin is known to have profound effects on the exchangeability of actin-bound calcium (37, 38), these results cannot be readily related to our own. According to Parker and Gergely (37) and Bárány et al. (38), F actin contains about one mole of calcium per mole of actin, of which less than 10% is exchangeable. This observation is consistent with our finding that troponin-free F actin bound no exchangeable calcium. On the other hand, Oosawa et al. (52) reported that as much as a third of the actin-bound calcium was exchangeable. In retrospect, it is obvious that the presence of troponin in F actin preparations, a factor not appreciated at the time of the aforementioned studies, may be responsible for misleading results.

We have already alluded to the possibility (see Results) that F actin, as it exists in the myofibril, may not contain nonexchangeable calcium. Since nonexchangeable calcium cannot participate in reversible calcium binding, this question has no bearing on the main conclusions of this paper.

There are no data in the literature on the binding of calcium to tropomyosin. Cohen and Longley (53) have described some interesting effects of calcium on the aggregation of tropomyosin but since the calcium concentrations in their study were several orders of magnitude greater than those employed here the relevance of their observations to the present work is doubtful.

Since the troponin content of the myofibril is unknown, it remains to be determined whether the calcium-binding sites of troponin can account for all the high affinity binding sites of the myofibril. Yasui et al. (25) made an indirect estimate of troponin content based on the amount of troponin needed to restore full calcium sensitivity to desensitized myosin B. According to their calculations troponin should be about 8% of the myofibrillar protein. Assuming 22 μmoles of calcium sites per g of troponin this would correspond to about 1.8 μmoles of calcium per g of myofibrillar protein. This value compares favorably with the value of n_1 of the myofibrils (1.3 μmoles Ca per g) and the amount of calcium actually bound to contracted myofibrils (1.5–2.0 μmoles

Ca per g), as determined by Weber and Herz (8). The above estimate of troponin content also agrees with that expected on the basis of the difference in n_1 values between the myofibrils and troponin. On a weight basis, the troponin contains 17 times as many high affinity calcium-binding sites as the myofibrils. The troponin preparations used in these experiments were quite pure, as judged by disc electrophoresis studies (25). If we assume that there are no other myofibrillar proteins with comparable affinity for calcium, then the troponin should constitute $\frac{1}{17}$, or 6%, of the myofibrillar protein. It seems reasonable to suppose, therefore, that the troponin content of the myofibril is in the range of 5 to 10%, in which case there would be no serious discrepancy between the amount of calcium actually bound to contracted myofibrils and that which can be accounted for by binding to troponin.

Ohtsuki et al. (46) have recently shown, using antibody-staining methods, that troponin is distributed along the entire length of the I filament at 400 A intervals. If the calcium released during excitation of the muscle binds to troponin, then it should bind to the entire length of the I filament with the same periodicity. It is of interest to reexamine the early studies of Draper and Hodge (54) on the electron microscopic localization of mineral in microincinerated myofibrils. These authors found a regular deposition of mineral at 400 A intervals in the region we now recognize as occupied by the I filaments. The Z line and H zone regions were relatively free of mineral residue. It is not unreasonable to suppose that these deposits may have been troponin-bound calcium.

An opposite conclusion emerges from the radioautographic studies of Winegrad (55) in which it was shown that in the living muscle fiber stimulated to develop tension there was a preferential deposition of ^{45}Ca oxalate in the region of overlap of the A and I filaments. This finding implies, but does not prove, that the binding of calcium is specifically related to cross-bridge formation between the two sets of filaments. However, caution must be exercised in equating the sites of calcium oxalate deposition, as observed by Winegrad, with the sites of protein-bound calcium. One wonders, for instance, whether there might not be a greater retention of calcium oxalate in the overlap zone during preparation of the sections for radioautography. Suggestive in this regard are the subsequent data of Winegrad (56) showing that the binding of calcium to glycerinated fibers (in the absence of oxalate) was independent of sarcomere length. This latter observation would be predicted on the basis of a uniform binding of calcium along the entire length of the I filament. Nevertheless, it would seem reasonable to assume that only the calcium bound to troponin lying within the overlap zone could activate cross-bridge formation and tension development. Hence, our conclusions do not conflict with the observation of Guld and Sten-Knudsen (57) that the latency of contraction in frog skeletal muscle increases as the distance between the terminal sacs of the

sarcoplasmic reticulum (presumably the calcium storage site) and the overlap zone is increased.

Very little can be said on the mechanism of calcium activation in the absence of further information on the molecular properties of troponin and the organization of the various proteins which comprise the I filament. The fact that the high affinity binding sites are located on a molecule which does not itself participate in superprecipitation supports an earlier suggestion (6) that calcium is not a direct reactant in the superprecipitation phenomenon. It is in apparent contradiction to the proposal of Davies (58) that the activating calcium ions form a bridge between the terminal phosphate groups of adenine nucleotides projecting out from the A and I filaments.

Since the molecular weight of troponin has not yet been reported the stoichiometry of binding remains unknown. If we make the simplifying assumption that there is only one binding site per molecule the troponin would have a minimum molecular weight of 45,000. In this connection it is of interest that the electrophoretic mobility of troponin is considerably greater than that of tropomyosin (25), the latter having a molecular weight of 70,000 (59, 60). Also, Watanabe and Staprans (61) and Drabikowski (personal communication) have found that troponin is retarded to a greater extent than tropomyosin on Sephadex G-200 columns. Thus the above estimate of minimum molecular weight is not unreasonable.

We have reported elsewhere (25) that treatment of troponin with SH reagents caused a loss of calcium-sensitizing activity without any change in the affinity constant for calcium binding. Oddly enough, there was a twofold increase in the total number of binding sites. This dissociation of calcium binding from calcium-sensitizing activity strongly suggests that these two functions are mediated by different sites on the molecule. It is well known that SH groups are essential for the calcium sensitivity of contractile actomyosin systems (18–21). Recently, Levy and Ryan (21, 62) have presented data indicating that calcium activates contraction by interfering with the binding of ATP to an SH-dependent inhibitory site on the myofibril. The possible identity of this site with the calcium-activating site of troponin is currently being investigated in this laboratory.

Addendum While this manuscript was in preparation Ebashi et al. (63) published a preliminary report stating that troponin can bind about 50 μmoles Ca per g with an affinity constant of 6×10^5 M^{-1}. A comparison of their results with ours must await publication of their experimental methods, but considering that the magnesium concentration was higher (4 mM) and the pH slightly lower (6.8) in their study, the agreement is reasonably good. They also report, in agreement with our findings, that tropomyosin bound much less calcium than troponin. A tight binding of calcium to troponin, but not to tropomyosin, has also been observed by Drabikowski (personal communication).

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