BRIEF COMMUNICATION

ON THE RELATION BETWEEN FILAMENT OVERLAP AND THE NUMBER OF CALCIUM-BINDING SITES ON GLYCERINATED MUSCLE FIBERS

FRANKLIN FUCHS, *Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261 U.S.A.*

ABSTRACT The formation of rigor complexes between the thick and thin filaments of glycerinated rabbit psoas muscle fibers causes the fibers to bind more calcium at any given level of free calcium. I studied the maximum amount of calcium bound as a function of filament overlap under rigor conditions. Fibers stretched to zero filament overlap (sarcomere length > 3.8 μm) bound exactly 75% as much calcium as fibers with maximum overlap. Between these extremes a linear relationship was found between maximum bound calcium and the length of the overlap zone. The results support the hypothesis that in the intact filament lattice one of the four calcium-binding sites of troponin depends for its existence on attachment between myosin and actin. In addition, the linear relation between maximum bound calcium and filament overlap is consistent with the assumption that the cooperative effect of rigor complex formation on calcium binding is limited to the binding site in the immediate vicinity of the rigor complex.

The ability of nucleotide-free actin-myosin linkages (rigor complexes) to modulate the Ca^{2+}-binding properties of troponin was first reported by Bremel and Weber (1) and has since been confirmed by others (2,3). The effect of rigor complexes is presumably mediated through tropomyosin (4). As shown by X-ray diffraction studies (5–7), there are characteristic positional changes of the tropomyosin molecule associated with both the rigor state and Ca^{2+}-activated contraction in the presence of ATP. Recent work in this laboratory (8) has shown that in glycerinated rabbit psoas muscle fibers in rigor the number of binding sites which can be occupied by Ca^{2+} is related to the number of cross-bridge attachments between the actin and myosin filaments. The latter can be varied by adjusting the length (and hence filament overlap) of the psoas muscle bundles before glycerination. It has been found that in the presence of 5 mM $MgCl_2$ (no ATP), fibers in which all of the cross-bridges lie within the overlap zone (sarcomere length 2.1–2.3 μm) bind a maximal amount of Ca^{2+} equivalent to about 4 mol/mol troponin. This ratio is in agreement with the experimentally determined

number of Ca^{2+}-binding sites on purified troponin (9). Fibers prestretched to a sarcomere length at which filament overlap is lost (sarcomere length > 3.8 μm in rabbit psoas; see ref. 10) bind an amount of Ca^{2+} equivalent to about 3 mol/mol troponin. Since these measurements were carried out in the presence of 5 mM Mg^{2+}, it is considered unlikely that Ca^{2+} binding to myosin influenced these results ((1,12). These observations suggest that in the intact filament lattice there is a Ca^{2+}-binding site, presumably on troponin, which comes into existence only when the actin and myosin filaments are linked.

Further evidence in support of this hypothesis is as follows: (*a*) If fibers with optimum filament overlap are immersed in 5 mM MgATP, a procedure which causes cross-bridge detachment, there is a reduction in the number of Ca^{2+} binding sites equivalent to that caused by prestretching the fibers (2,8). (*b*) If fibers with only slight overlap (sarcomere length 3.6–3.7 μm) are allowed to undergo ATP-induced shortening to sarcomere length ~2.1 μm (followed by removal of ATP) the maximal bound Ca^{2+} is increased from 3 mol/mol troponin (before shortening) to 4 mol/mol troponin (after shortening) (13). The latter observation is important in that it shows that the reduced Ca^{2+} binding by stretched fibers is not an artifact arising from a selective loss of Ca^{2+}-binding protein during extraction. The physiological significance of the "labile" Ca^{2+} binding site is still not clear. However, insofar as it is an expression of cooperative interactions between the contractile proteins, it might be a useful marker for studying conformational changes in thin filaments located in the intact myofilament lattice. It is of interest that at sarcomere length 2.1–2.3 μm, where all of the cross-bridges are within the overlap zone, some 28–30% of the thin filament Ca^{2+}-binding sites are still outside of the overlap zone. Are these sites influenced by actin-myosin links formed some distance away within the overlap zone? This is not an unreasonable expectation in light of recent evidence (14) indicating end-to-end interactions between adjacent tropomyosin molecules lying in the groove of the actin filament. In that case the binding at longer sarcomere lengths would not be reduced in direct proportion to the reduction in the number of rigor complexes. On the other hand, if the rigor complex influences only the troponin-tropomyosin complex in the immediate vicinity of the cross-bridge, the number of Ca^{2+}-binding sites should decrease linearly with the decrease in the length of the overlap zone.

In my previous report (8), the data suggested a nonlinear relationship; that is, the amount bound did not significantly decrease until the sarcomere length was extended beyond 3 μm. To ensure that sarcomere length was the only variable, all of the measurements in that particular study were made with bundles obtained from a single rabbit. However, sampling errors could still have influenced the results (e.g. small differences in nonfibrillar protein contamination in different bundles). Since that report additional data have been obtained on Ca^{2+} binding to rigor fibers as a function of sarcomere length. All measurements were made on psoas fibers extracted sequentially with 1% Triton X-100 and 50% glycerol and incubated in 2×10^{-5} M free Ca^{2+}. At that concentration of Ca^{2+} all binding sites are saturated (8). Sarcomere length was determined by phase contrast microscopy and Ca^{2+} binding was measured by a double

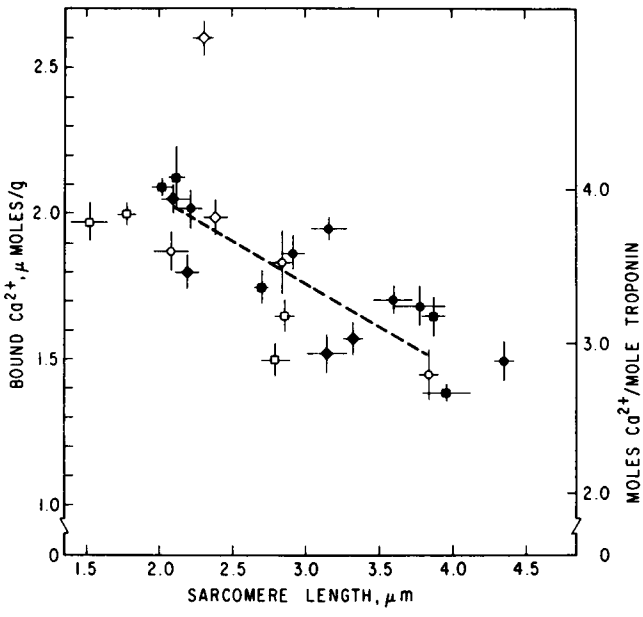

FIGURE 1 Maximum Ca^{2+} bound to rabbit psoas fibers as a function of sarcomere length. Results are expressed both as micromoles of bound Ca^{2+} per gram protein and as moles of bound Ca^{2+} per mole troponin. Horizontal bar indicates standard error of sarcomere length measurement and vertical bar indicates standard error of Ca^{2+}-binding measurement. Each point is the mean of 8–14 sarcomere length determinations and 4–41 Ca^{2+}-binding determinations. Dashed line is least squares regression line for data in sarcomere lengths range 2.08–3.84 μm. Each symbol represents fiber bundles obtained from same rabbit. Slope = -0.55 μmol $\cdot g^{-1}$ μm^{-1}; intercept at zero overlap 1.54 μmol $\cdot g^{-1}$; $r = 0.60$; $P < 0.01$.

isotope technique employing $^{45}Ca^{2+}$ and [^{3}H]glucose, the latter serving as a marker for solvent space. The procedures have been described in detail elsewhere (8).

The data in Fig. 1 are the results of 411 measurements made with 24 psoas bundles obtained from 6 rabbits. Mean sarcomere lengths varied from 1.53 to 4.36 μm. It is evident that for each rabbit bound Ca^{2+} is inversely related to sarcomere length. All of the data at sarcomere lengths more than 3.9 μm and less than 2.5 μm were averaged to

TABLE I

MAXIMUM BOUND Ca^{2+} AT LONG AND SHORT SARCOMERE LENGTHS

Sarcomere length	Maximum bound Ca^{2+}	Moles Ca^{2+}/mole troponin
μm	$\mu mol/g$	
< 2.5	2.01 ± 0.02 (165)	3.9
> 3.8	1.49 ± 0.04 (63)	2.9

Values are given as mean ± SEM. Number of measurements is indicated in parentheses. The ratio of Ca^{2+} to troponin was calculated by assuming that fibers contained 87% myofibril protein with a troponin content of 0.6-μmol/g myofibril protein (see text).

obtain the best estimates of the amounts of Ca^{2+} bound maximally to "exposed" thin filaments and to thin filaments linked to myosin by rigor complexes. The results are shown in Table I. The mean value for the stretched fibers (1.49 μmol Ca^{2+}/g) is equivalent to 2.9 mol Ca^{2+}/mol troponin and the mean value for the short fibers, (2.01 μmol Ca^{2+}/g) is equivalent to 3.9 mol Ca^{2+}/mole troponin. This calculation is based on the assumption that the fibers contain 87% myofibrillar protein (see ref. 8) with a troponin content of 0.6 μmol/g myofibril protein (4). It is of some interest that the ratio of the means, a parameter assumed to be independent of a systematic measurement error, is exactly 4:3. This ratio is consistent with the hypothesis (8) that the Ca^{2+}-binding site, dependent on cross-bridge attachment for its existence, is one of the four sites on troponin. The most likely alternative hypothesis is that the troponin content of the fibers has been overestimated, in which case the troponin binds four Ca^{2+} ions at all sarcomere lengths and the additional Ca^{2+} is bound to myosin. The myosin molecule has two high-affinity divalent cation binding sites (12, 15) and the molar ratio of myosin to troponin in the rabbit myofibril is unity (16). If the two sites are empty when the cross-bridges are detached but occupied by Ca^{2+} when rigor complexes are formed, then the bound Ca^{2+} ratio for these two states would be 3:2. The standard errors given in Table I seem small enough to justify the exclusion of the latter ratio.

Also shown in Fig. 1 is the least-squares regression line for data which lie within the sarcomere length range of 2.08 μm (complete overlap of cross-bridges) to 3.84 μm (no overlap). The data are satisfactorily fitted by assuming a linear relation between bound Ca^{2+} and overlap ($r = 0.60$, $P < 0.01$). The intercept at zero overlap is 1.54 μmol Ca^{2+}/g protein, a value in close agreement with the mean given in Table I. From the slope of the regression line it may be calculated that when the ends of the actin filaments just meet in the center of the A band (sarcomere length 2.2 μm), the bound Ca^{2+} would be 1.98 μmol/g (3.8 mol Ca^{2+}/mol troponin).

These data are compatible with the assumption that the effect of cross-bridge attachment on the number of Ca^{2+}-binding sites is confined to the immediate vicinity of the cross-bridge. How this effect is brought about is not clear. It does not seem to be related simply to the positional change of tropomyosin which occurs when rigor bonds are formed between the actin and myosin filaments (6). The same movement of tropomyosin occurs in highly stretched fibers when Ca^{2+} binds to troponin (5, 6); yet, as shown in this laboratory (8) and elsewhere (2, 3), unattached thin filaments bind less Ca^{2+} at any given free Ca^{2+} level than thin filaments, which are linked to myosin by rigor complexes.

I am indebted to Ms. Beverly Black for outstanding technical assistance and to Dr. S. Ishiwata for a valuable discussion.

This work was supported by grants from the National Institutes of Health (AM-10551) and the Muscular Dystrophy Association.

Received for publication 27 July 1977 and in revised form 16 November 1977.

REFERENCES

1. BREMEL, R. D., and A. WEBER. 1972. Cooperation within actin filaments in vertebrate striated muscle. *Nat. New Biol.* **238**:97.
2. MARSTON, S., and R. T. TREGEAR. 1974. Calcium binding and the activation of fibrillar insect flight muscle. *Biochim. Biophys. Acta.* **347**:311.
3. SOLARO, R. J., F. D. BRUNI, and E. N. GLEASON. 1976. Effects of ionic strength on calcium binding to rabbit skeletal myofibrils, thin filaments, and myosin. *Biochim. Biophys. Acta.* **449**:304.
4. WEBER, A., and J. M. MURRAY. 1973. Molecular control mechanisms in muscle contraction. *Physiol. Rev.* **53**:612.
5. HUXLEY, H. E. 1972. Structural changes in the actin- and myosin-containing filaments during contraction. *Cold Spring Harbor Symp. Quant. Biol.* **37**:361.
6. HASELGROVE, J. C. 1972. X-ray evidence for a conformational change in the actin-containing filaments of vertebrate striated muscle. *Cold Spring Harbor Symp. Quant. Biol.* **37**:341.
7. VIBERT, P. J., J. C. HASELGROVE, J. LOWY, and F. R. POULSEN. 1972. Structural changes in actin-containing filaments of muscle. *J. Mol. Biol.* **71**:757.
8. FUCHS, F. 1977. The binding of calcium to glycerinated muscle fibers in rigor: the effect of filament overlap. *Biochim. Biophys. Acta.* **491**:523.
9. POTTER, J. D., and J. GERGELY. 1975. The calcium and magnesium binding sites on troponin and their role in the regulation of myofibrillar adenosine triphosphatase. *J. Biol. Chem.* **250**:4628.
10. PAGE, S. G., and H. E. HUXLEY. 1963. Filament lengths in striated muscle. *J. Cell Biol.* **19**:369.
11. POTTER, J. D. 1975. Effect of Mg^{2+} on Ca^{2+} binding to myosin. *Fed. Proc.* **34**:671.
12. BREMEL. R. D., and A. WEBER. 1975. Calcium binding to rabbit skeletal muscle myosin under physiological conditions. *Biochim. Biophys. Acta.* **376**:366.
13. FUCHS, F. 1977. Cooperative interactions between calcium binding sites on glycerinated muscle fibers: the influence of cross-bridge attachment. *Biochim. Biophys. Acta.* **462**:314.
14. TAWADA, Y., H. OHARA, T. OOI, and K. TAWADA. 1975. Non-polymerizable tropomyosin and control of superprecipitation of actomyosin. *J. Biochem. (Tokyo).* **78**:65.
15. BAGSHAW, C. R. 1977. On the location of the divalent metal binding sites and the light chain subunits of vertebrate myosin. *Biochemistry.* **16**:59.
16. POTTER, J. D. 1974. The content of troponin, tropomyosin, actin, and myosin in rabbit skeletal muscle myofibrils. *Arch. Biochem. Biophys.* **162**:436.