

Fluorescence Microscope Study of the Binding of Added C Protein to Skeletal Muscle Myofibrils

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ABSTRACT The binding of extra C protein to rabbit skeletal muscle myofibrils has been investigated by fluorescence microscopy with fluorescein-labeled C protein or unmodified C protein plus fluorescein-labeled anti-C protein. Added C protein binds strongly to the I bands, which is consistent with its binding to F actin in solution (Moos, C., C. M. Mason, J. M. Besterman, I. M. Feng, and J. H. Dubin. 1978. *J. Mol. Biol.* 124:571-586). Of particular interest, the binding to the I band is calcium regulated: it requires a free calcium ion concentration comparable to that which activates the myofibrillar ATPase. This increases the likelihood that C protein-actin interaction might be physiologically significant. When I band binding is suppressed, binding in the A band becomes evident. It appears to occur particularly near the M line, and possibly at the edges of the A band as well, suggesting that those parts of the thick filaments that lack C protein in vivo may nevertheless be capable of binding added C protein.

C protein is a component of the thick filaments in skeletal muscle, constituting ~3-4% of the filament mass (8, 10). It is a single polypeptide chain of 140,000 mol wt, and hydrodynamic measurements indicate a prolate ellipsoid ~35 × 3 nm (10).

Although the C protein in muscle is attached to the shaft of the thick filaments, it has been shown that, in solution, C protein can bind to F actin (7). A physiological role for C protein-actin interactions is not inconsistent with its localization on the thick filaments because the molecule is of sufficient size to span the distance between the thick and thin filaments in the overlap region of the sarcomere. Evidence of specificity for the interaction is provided by the observation that C protein competes with myosin subfragment-1 (S-1) for actin (7). Before considering possible functional implications of C protein-actin interactions in muscle, however, one needs to know whether C protein can bind to native thin filaments which contain the regulatory tropomyosin-troponin complex. To answer this question, the binding of added C protein to myofibrils was investigated by fluorescence microscopy. Using fluorescein-labeled C protein, or unmodified C protein followed by fluorescein-labeled antibody against C protein, it is observed that C protein can indeed bind to the I band. Furthermore, this binding is calcium-dependent: I-band labeling is inhibited in the presence of EGTA, but it is restored at pCa 5.5 or below.

A second aspect of this work relates to the structural locali-

zation of C protein within the A bands of muscle. Antibody-labeling studies using fluorescence microscopy (12) and electron microscopy (2, 12) have shown that C protein is restricted to specific portions of the thick filament: it occurs only at seven discrete sites in approximately the middle one-third of each half of the A band, called the "C zone" by Sjöström and Squire (14). A possible explanation for this localization is that some feature of the myosin assembly in the filament backbone makes it unable to bind C protein anywhere except at these particular sites. It was of interest, therefore, to determine whether or not added C protein can bind to other portions of the A bands of myofibrils. Under conditions which suppress binding to the I bands, our fluorescence microscope observations suggest that added C protein can indeed bind to the central portion of the A band, and perhaps to the periphery as well.

MATERIALS AND METHODS

Myofibrils

Strips of fresh rabbit psoas muscle were tied to lucite rods at rest length or partially stretched. They were soaked for 2 d at 5°C in 50% (vol/vol) glycerol containing 0.1 M KCl and 10 mM potassium phosphate, pH 7, and then stored at -10°C in the same solution. For each day's experiments, strips of glycerinated muscle were minced in cold 0.1 M KCl, 1 mM MgCl₂, 10 mM K-phosphate, pH 7, and blended in a 5 ml MSE homogenizer or Sorvall Omnimixer (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) in ice. When the myofibrils were suitably dispersed, as judged by phase-contrast microscopy, they were pelleted in a bench centrifuge and then washed several times in 10-15 vol of the

KCl-Mg-buffer solution. Treatment with C protein or antibody solutions was carried out by dispersing the myofibrils in 10–15 vol of the same solution containing the added protein at 1 mg/ml, incubating at 0°C, and centrifuging and washing as before.

Fluorescence Microscopy

In most cases (Figs. 1–6), myofibrils were examined in the aqueous KCl-Mg-phosphate buffer specified above. However, for the more recent experiments illustrated in Fig. 7, the samples were fixed and dehydrated as follows. The myofibril suspension was applied to a cover slip, and, after a brief rinse with buffer, the adhering myofibrils were fixed for 1 min with 5% glutaraldehyde in the same buffer. (This fixation was found to be sufficient to render myofibrils resistant to disruption by 0.5 M KCl.) The cover slip was then rinsed sequentially in water, 30, 70, 95, and 100% ethanol, 1:1 ethanol-xylene, and pure xylene, and was finally mounted and examined in xylene. In this non-aqueous medium the fluorescence fades more slowly, which facilitates photography of weakly fluorescent samples. Comparison of identical samples examined both in aqueous buffer and after fixation and dehydration showed that the latter treatment caused some lateral shrinkage of the myofibrils but no change in the longitudinal distribution of fluorescence within the sarcomere.

Photographs were taken with a $\times 100$ phase-contrast objective and an epifluorescence attachment with a mercury arc light source. For Figs. 1–6, a Zeiss microscope was used which gave $\times 400$ magnification at the photographic film, whereas a Leitz Diavert microscope with a $\times 630$ film magnification was used for Fig. 7. All figures are printed at $\times 2,000$ total magnification. Each field was photographed with both phase-contrast and fluorescence illumination, and unequivocal identification of the A and I bands in the fluorescence picture was obtained by superimposing the two photographic negatives and also by observing the microscope image visually while switching from fluorescence to phase-contrast illumination. The location of a Z line is indicated in each figure.

Due to the variable fading of the fluorescence, no significance can be attached to apparent differences in absolute fluorescence among different figures; rather, the printing of each picture was adjusted to show the relative fluorescence intensity in different parts of the sarcomeres.

Proteins

C protein was prepared from rabbit muscle, according to Offer et al. (10), and further purified by chromatography on hydroxylapatite.¹ Antibodies against this purified C protein were produced in a goat (11),¹ and a crude immunoglobulin fraction was isolated from the antiserum by precipitation with ammonium sulfate at 40% saturation (4). S-1 was prepared from column-purified myosin (10) by papain digestion in 0.2 M ammonium acetate (5). C protein and immunoglobulin were labeled by treatment with fluorescein isothiocyanate-coated Celite (Calbiochem-Behring Corp., La Jolla, Calif.) (13), and the proteins were separated from free dye and Celite on a Sephadex G-25 column.

The protein concentrations and the amounts of bound fluorescein were calculated from absorbance measurements at 280 and 495 nm. The measured A_{280} values were corrected for bound fluorescein by subtracting 0.35 A_{495} (18), and protein concentrations were determined using extinction coefficients of 1.09 (mg/ml)⁻¹ cm⁻¹ for C protein, 1.4 (mg/ml)⁻¹ cm⁻¹ for immunoglobulin, and 0.77 (mg/ml)⁻¹ cm⁻¹ for S-1. Bound fluorescein was determined from A_{495} using a molar extinction coefficient of 60,000 M⁻¹ cm⁻¹ (16). The labeled proteins contained 1–3 mol bound fluorescein/mol.

Gel Electrophoresis

Samples containing ~0.3 mg/ml total protein, 20 mM Tris base, 20 mM Bicine, 0.7% (wt/vol) sodium dodecyl sulfate (SDS), 0.7% (vol/vol) β -mercaptoethanol, 9% (vol/vol) glycerol, and a small amount of bromphenol blue tracking dye, were heated for 5 min at 100°C. Electrophoresis was carried out in a 6% acrylamide gel according to Weber and Osborn (17), except that the gel was a vertical slab 8 cm wide, 5 cm high, and 2 mm thick, and 0.1 M Tris base plus 0.1 M Bicine was used in place of phosphate buffer.

ATPase Measurements

Myofibrillar ATPase was measured on a Radiometer pH stat system at pH 7.0 and 25°C, in a sample volume of 7.5 ml. The reaction mixture was prepared containing 0.1 M KCl, 1 mM MgCl₂, and either 0.1 mM CaCl₂ or 4 mM EGTA-calcium buffer. The pH was adjusted to 7.0, and 0.25 ml of myofibrils suspended in KCl-Mg-phosphate buffer were then added, giving ~0.5 mg/ml protein in the

final mixture. The ATPase reaction was started by the addition of equimolar MgCl₂ plus ATP at pH 7 to give a final concentration of 2 mM MgATP (i.e., 3 mM total MgCl₂). The ATPase rates were the same if the myofibrils were added last instead of the MgATP. No absolute calibration of ATPase rates was necessary because relative rates only were required, and every reaction mixture contained an equal volume of the same stock myofibril suspension.

Calcium Buffers

For the experiment in Fig. 7, pCa levels were maintained by means of EGTA-calcium buffers. Stock 0.2 M EGTA and CaEGTA were prepared by weight from well-dissociated H₄EGTA and CaCO₃ and adjusted to pH 7.0 with NaOH. Various proportions of these two solutions were added to the experimental mixtures to give the desired pCa, assuming an effective pK of 6.4 for the dissociation of CaEGTA (D. E. Allen and J. R. Blinks, unpublished observations, quoted in reference 3) and neglecting any other complexes of Ca or EGTA. The total EGTA concentration was 4 mM in every case.

RESULTS

Binding of C Protein to I Bands

The binding of fluorescein-labeled C protein to myofibrils is illustrated in Fig. 1. These myofibrils had been incubated for 2 h in FITC-labeled C protein and washed three times as described in Materials and Methods. Labeling of the I bands is clearly evident, with a gap at the Z line visible in some cases, indicating that C protein can indeed bind to the actin in native thin filaments.

A similar result is also obtained with unmodified C protein, using labeled antibody to reveal the location of the bound C protein. The myofibrils in Fig. 2*a* and *b* were incubated with C protein, washed, and then treated with fluorescein-labeled anti-C protein. These myofibrils, like those in Fig. 1, showed strong fluorescence in the I bands. In other experiments, myofibrils washed as many as six times before treatment with labeled antibody showed little if any decrease in the I band fluorescence.

No control experiment was carried out with nonimmune goat immunoglobulins, but control myofibrils not treated with C protein (Fig. 2*c*) bound fluorescent antibody only in the C zones of the A bands, as described by Pepe and Drucker (12). Therefore, the I band fluorescence in Fig. 2*a* and *b* cannot be explained by nonspecific adsorption of fluorescent immunoglobulin in the absence of bound C protein.

In these experiments, unlike those in which the added C protein itself was fluorescein-labeled, the observed fluorescence indicates the location of all the C protein in the myofibrils including the intrinsic C protein of the A bands. Therefore the intense fluorescence of the I bands relative to the A bands implies that the I bands bind a larger amount of C protein than is present in the A bands. Analysis of control and C protein-treated myofibrils by SDS gel electrophoresis (Fig. 3) showed that a large increase in myofibril-bound C protein did indeed occur.

Comparison of the phase and fluorescence images at different sarcomere lengths in Fig. 1 and in Fig. 2*a* and *b* indicates that binding of C protein to the thin filaments is limited to the I band and does not extend significantly into the overlap region of the A band. Because S-1 inhibits the binding of C protein to F actin in solution (7), it is probable that the binding of myosin heads to the actin in the A band in the absence of ATP prevents the actin in this region from binding C protein. The effect of myosin heads on C-protein binding in myofibrils was tested directly by adding C protein to myofibrils in the presence of S-1. As is shown in Fig. 4, by both labeling methods (fluores-

¹ Purified C protein and antiserum were generously provided by R. Starr and G. Offer of King's College, University of London.

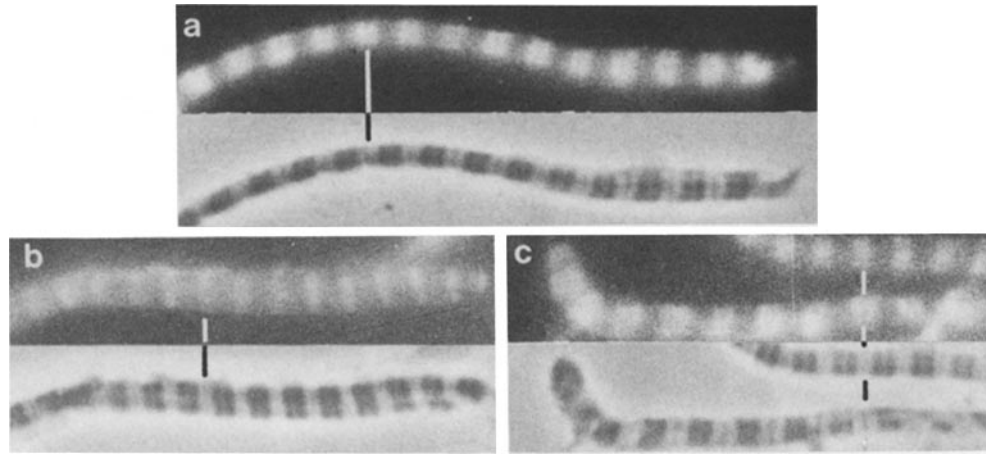


FIGURE 1 Binding of FITC-labeled C protein to myofibrils. Myofibrils were incubated for 2 h with labeled C protein and washed three times, as described in Materials and Methods. a-c, three samples at different sarcomere lengths. The location of a Z-line is indicated. $\times 2,000$.

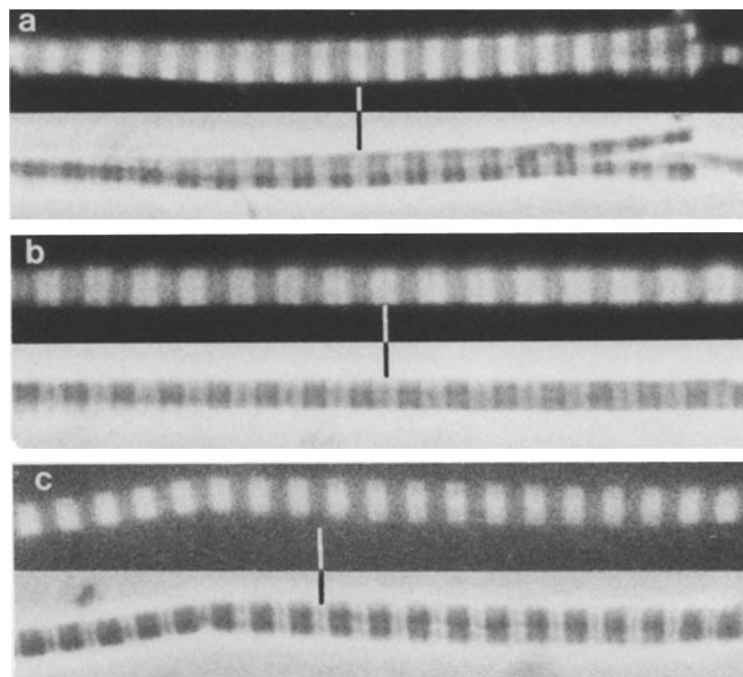


FIGURE 2 Binding of unmodified C protein to myofibrils. Myofibrils incubated with C protein for 2 h (a) or 30 min (b), washed twice, then treated for 5 min with FITC-labeled anti-C protein and washed two more times. a and b show different sarcomere lengths. c, control myofibrils prepared simultaneously with a, but without addition of C protein, showing antibody labeling of the intrinsic C protein of the myofibrils. $\times 2,000$.

cent C-protein or fluorescent antibody), S-1 did indeed inhibit the labeling of the I band. This supports the conclusion that the I band fluorescence observed in the absence of S-1 does represent binding of C protein to actin.

Calcium-dependence of C-Protein Binding

Knowing that the thin filaments in myofibrils contain the tropomyosin-troponin regulatory complex, it was of interest to investigate the effect of calcium ions on C-protein binding. Samples of myofibrils were treated with C protein in the presence of either 0.1 mM CaCl_2 or 1 mM EGTA, with the results shown in Figs. 5 and 6. Labeling in the presence of calcium was the same as described above, but in the presence

of EGTA the I-band fluorescence was nearly obliterated, suggesting that C-protein binding to the thin filaments is calcium regulated.

Fig. 7 shows, first, that this effect of EGTA is due to calcium removal, and second, that the free-calcium ion concentration necessary for C-protein binding to the I band corresponds to that at which the actin-myosin interaction is activated. In this experiment, myofibril samples were incubated overnight with FITC-labeled C protein at various free-calcium concentrations. The first sample (Fig. 7a) was washed with 4 mM EGTA and then resuspended in 0.1 mM CaCl_2 before incubation with C protein to verify that the effect of EGTA could indeed be reversed by calcium. Three other samples (Fig. 7b-d) were treated with C protein at pCa 5.5, 6.4, and 7.0, respectively,

with EGTA as a calcium-buffer. The ATPase activity of the same stock myofibril suspension was also measured (without added C protein) at approximately the same four concentrations of free-calcium ions, and the ATPase rates at pCa 5.5, 6.4, and 7.0 are presented in Fig. 7 as percentages of that in 0.1 mM CaCl₂. In 0.1 mM CaCl₂, and at pCa 5.5, all myofibrils showed equal or greater fluorescence in the I band than in the A bands. This I band labeling in Fig. 7*a* despite prior treatment with 4 mM EGTA, and in Fig. 7*b* in the presence of 4 mM EGTA-calcium buffer, makes it unlikely that inhibition of C-protein binding by EGTA could be due to any other effect than removal of free calcium ions. Furthermore, when the pCa was increased at a constant total EGTA concentration (Fig. 7*b-d*), the labeling of the I bands with fluorescent C protein disappeared at approximately the same pCa, between 5.5 and 6.4, at which the myofibrillar ATPase was switched off.

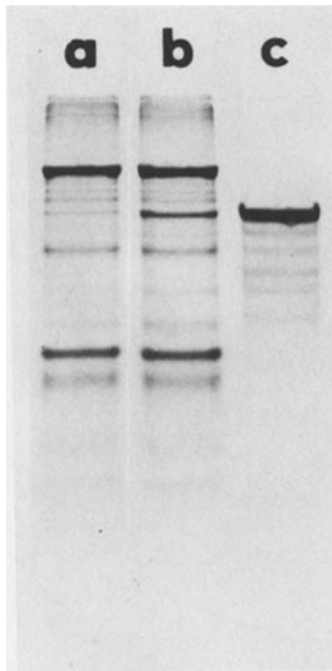


FIGURE 3 Electrophoretic analysis of C-protein binding to myofibrils. *a*, control myofibrils; *b*, myofibrils treated with C protein as in Fig. 2*a* (sample taken before addition of antibody); *c*, C-protein marker.

Binding of Extra C Protein in the A Bands

An important question related to the structural localization of C protein in native thick filaments is whether C protein can bind to those parts of the thick filament that do not normally contain it, particularly the central portion and the ends. When binding of C protein to the I bands of myofibrils was inhibited by either S-1 or EGTA (Figs. 4–7), binding of extra C protein in the A bands could be detected. In myofibrils treated with fluorescent C protein under these conditions and examined in aqueous buffer (Figs. 4*a* and 5*b*), labeling of the A band was consistently observed, suggesting that extra C protein can indeed bind to the thick filaments. However, the precise localization of the added C protein could not be discerned by this method. On the other hand, when C protein plus fluorescent antibody was used (Figs. 4*b* and 6) or, in more recent experiments, when myofibrils labeled with fluorescent C protein were examined in a non-aqueous medium (Fig. 7), more details were visible. Labeling was always evident at the center of the A band near the M line, often of sufficient intensity to be seen even in the presence of I band labeling (Figs. 2*a* and *b*, 6*a*, and 7*b*). The fluorescent antibody binding in the M region was indeed due to C protein binding because it was seen only when C protein was added; control myofibrils treated with FITC-labeled antibody in the absence of added C protein showed a central dark band between the two fluorescent C zones (Fig. 6*d* and *e*). We can conclude with some confidence, therefore, that added C protein can bind to the central portion of the thick filaments between the C zones.

Labeling of the remainder of the A band is less clearly interpretable. Within the C zones, the fluorescent antibody would label intrinsic as well as added C protein, so one cannot tell by this procedure whether extra C protein is bound within the C zones, for example between the seven C protein stripes (2). Likewise, fluorescent C protein might bind to the intrinsic C protein due to the tendency of C protein to aggregate at low ionic strength (10). At the ends of the thick filaments, distal to the C zones, C protein did appear to bind, but labeling in this region was variable. Some myofibrils were strongly labeled only near the M line (Fig. 6*c*), although, more commonly, labeling of both the center and the edges of the A band was more intense than in the C zones, so that three bright lines were seen in each A band (e.g., Figs. 6*b* and 7*c* and *d*). It appears, therefore, that C protein probably can bind near the

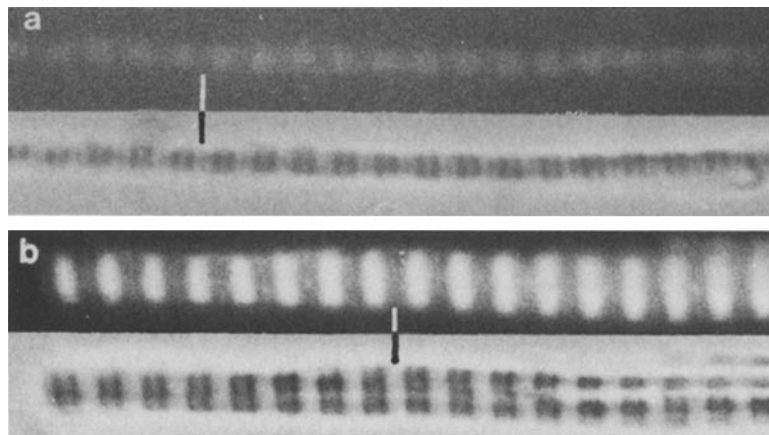


FIGURE 4 Inhibition of C-protein binding by S-1. *a*, myofibril treated with FITC-labeled C protein simultaneously with sample in Fig. 1*a*, but in the presence of S-1 at 1 mg/ml, then washed as in Fig. 1. *b*, myofibril treated with unmodified C protein simultaneously with the sample in Fig. 2*b* but in the presence of S-1 at 1 mg/ml, then washed and labeled with antibody as in Fig. 2*b*. $\times 2,000$.

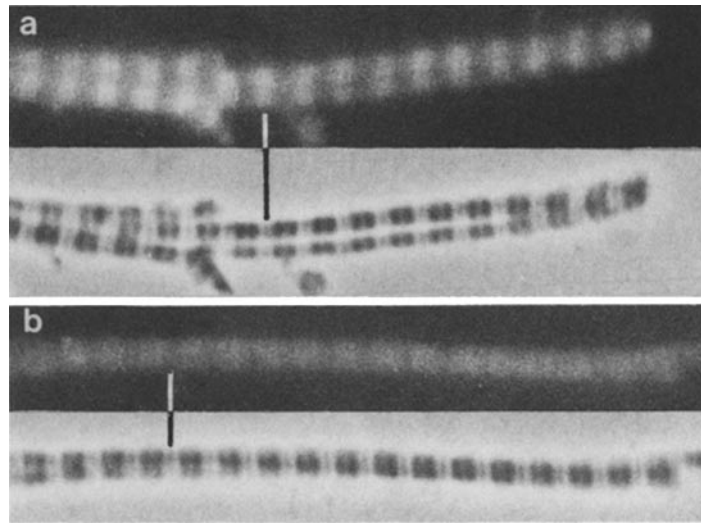


FIGURE 5 EGTA-inhibition of binding of labeled C protein. *a*, myofibril incubated for 2.5 h with FITC-labeled C protein and washed three times, with 0.1 mM CaCl_2 added to incubation and wash solutions. *b*, sample prepared in parallel with *a*, but with 1 mM EGTA added in place of CaCl_2 . $\times 2,000$.

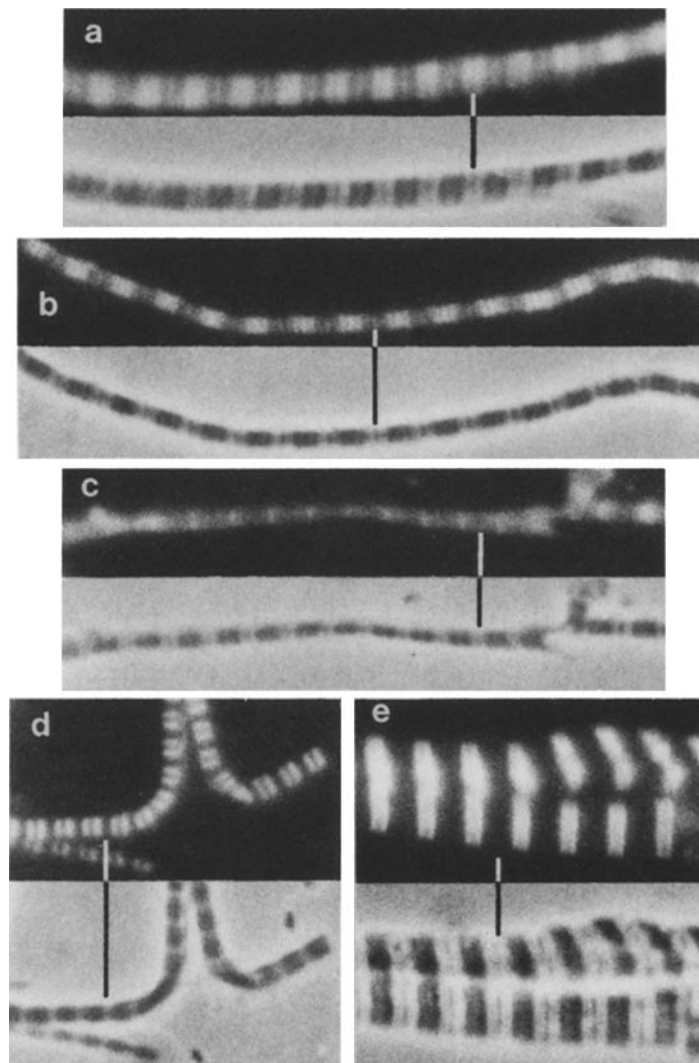


FIGURE 6 EGTA-inhibition of binding of unmodified C protein. *a*, myofibril incubated for 2 h with C protein, washed three times, incubated for 30 min with labeled anti-C protein, and washed three more times, with 0.1 mM CaCl_2 added to all solutions. *b* and *c*, different myofibrils from a sample prepared in parallel with *a* but with 1 mM EGTA added in place of CaCl_2 . *d* and *e*, control myofibrils prepared in parallel with *b* and *c*, but with C protein omitted from the first incubation. $\times 2,000$.

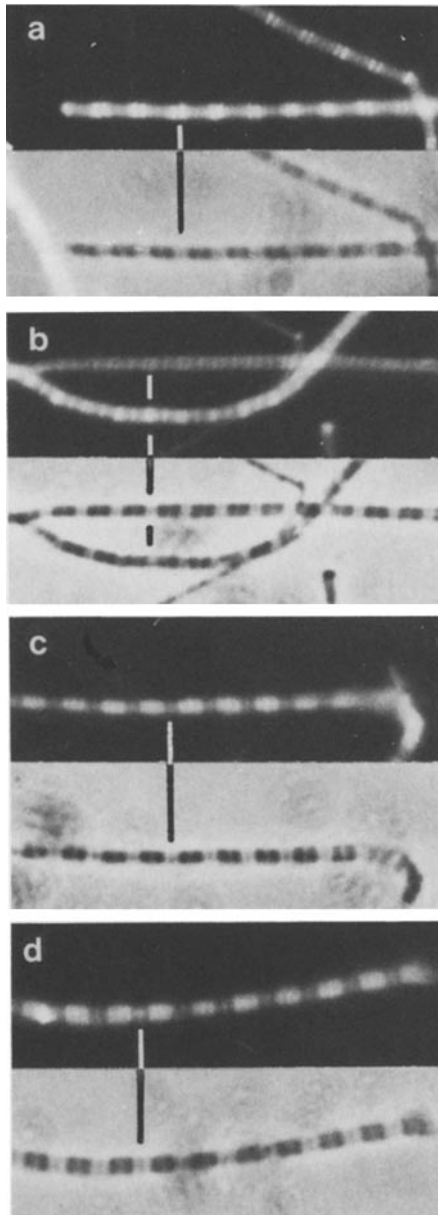


FIGURE 7 Calcium regulation of C-protein binding myofibrillar ATPase. Myofibrils were incubated with C protein at the following concentrations of calcium and EGTA, and relative ATPase rates were measured under the same conditions except for omission of phosphate buffer and addition of MgATP. a, 0.1 mM CaCl_2 , ATPase defined as 100%; b, 0.5 mM EGTA plus 3.5 mM CaEGTA ($\text{pCa} = 5.5$), ATPase = 91%; c, 2 mM EGTA plus 2 mM CaEGTA ($\text{pCa} = 6.4$), ATPase = 26%; d, 3.2 mM EGTA plus 0.8 mM CaEGTA ($\text{pCa} = 7.0$), ATPase = 15%. For microscopy, myofibrils were washed (a) twice in KCl-Mg-phosphate buffer containing 4 mM EGTA, then twice in buffer with 0.1 mM CaCl_2 , or (b-d) four times in KCl-Mg-phosphate buffer containing the above EGTA and CaEGTA concentrations. All samples were then suspended in the respective buffers plus 1 mg/ml FITC-labeled C protein, incubated overnight, washed once and fixed with glutaraldehyde in this buffer, and finally rinsed with water and mounted in xylene as in Materials and Methods. $\times 2,000$.

ends of the thick filaments. However, in view of the variability of these results, and also the possibility, which cannot be discounted, that the extra C protein in the periphery of the A band was bound to actin rather than to myosin, one cannot be entirely confident of this conclusion. Further investigation of C-protein binding to native thick filaments is in progress.

DISCUSSION

The observations reported here have two distinct aspects: the first concerns C-protein binding to the thin filaments in the I band, in which it was shown that such binding can indeed occur and that, like the binding to F actin in solution (7), it is inhibited by the binding of S-1 to the actin. Furthermore, the binding to native thin filaments is subject to calcium control. The second aspect is the finding that C protein can bind to parts of the A band where it is not present *in vivo*, particularly to the central region, and perhaps the edges as well.

The localization of C protein at specific sites on the thick filaments (2, 12) and its attachment to the myosin rod assembly in the filament backbone (6) suggested that its function in muscle relates only to thick filament structure (9, 14, 15). However, the discovery that C protein can bind to F actin *in vitro* raised the possibility that it might more directly influence the contractile machinery (7). As has been pointed out before, an interaction of C protein with actin in muscle would not be inconsistent with its localization on the thick filaments because the C-protein molecule is large enough to reach from the thick filament to the overlapping thin filament. The present observation that C protein can bind to intact thin filaments as well as to isolated F actin gives added encouragement to such speculations. Furthermore, it is of particular interest that the binding of C protein to the thin filaments in myofibrils is calcium regulated and is switched on and off in the same range of pCa as is the myofibrillar ATPase.

The calcium-dependence of C-protein binding to thin filaments argues against one of the possible roles we suggested for the actin-C protein interaction (7), namely stabilization of the filament lattice in relaxed muscle by formation of weak cross links between thick and thin filaments, because the thin filaments apparently cannot bind C protein in the relaxed state. However, this finding also leads to a new possibility. One can speculate that, because "switching on" the thin filament with calcium enhances C-protein binding, the binding of C protein might reciprocally help "switch on" the thin filament and thus contribute to the cooperativity, and hence the rapidity, of muscle activation *in vivo*. Quantitative studies of the effect of C protein on the calcium sensitivity of regulated actomyosin systems are currently under way.

The observations on C-protein binding in the A band are less definitive, but they suggest that the restricted localization of C protein within the C zones of the thick filament cannot be explained by an inability of the myosin assembly in other parts of the filament to bind C protein. Added C protein seems to bind to the central portion of the A band and possibly also to the peripheral portions of the A band outside the C zones. In an electron microscope investigation of C protein binding to myofibrils, Craig (1) observed that addition of C protein caused a specific increase in density at positions 3×43 nm inward from the innermost of the seven C protein stripes in each half A band, or ~ 117 nm from the center of the M line. Binding at these locations could probably account for the fluorescence labeling seen here at the center of the A band because two fluorescent bands only 230 nm apart would probably not be resolved optically.

It has been suggested (1, 2, 12) that the C zones are defined by a tendency of C protein to bind only where all the myosin molecules are oriented the same way and the packing of the filament core is uniform, rather than where the transition to antiparallel assembly of myosin tails occurs (near the center of the filament) or where the filament tapers. Binding of added C

protein in the latter regions might seem to argue against this view; however, it may be that although binding outside the C zones is possible, it has a significantly weaker affinity than at the normal C-protein binding sites in each C zone, this difference in affinity being sufficient to account for the localization of C protein in vivo. Preliminary efforts to confirm this idea by selectively washing out the added C protein while leaving the intrinsic C protein in place have not yet been successful; further work will be necessary to test the hypothesis definitively. Of course, a fully satisfactory explanation for the localization of C protein in vivo will also depend on a clarification of the internal structure of the thick filament itself.

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