The Z-Band: 85,000-dalton Amorphin and Alpha-Actinin and Their Relation to Structure

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ABSTRACT The conclusions arrived at as a result of this work can be summarized as follows: (*a*) We have found that there is an 85,000-dalton protein, which we have called 85K amorphin, associated with the Z-band of chicken pectoralis muscle myofibrils. We have isolated and purified this protein. It is not a structural component of the Z-filaments since it can be extracted completely without extraction of the Z-filaments. Extraction of 85K amorphin results in loss of specific staining of the Z-band with fluorescence specific anti-85K amorphin. (*b*) We have found that alpha-actinin is the structural component of the Z-filaments, since extraction of alpha-actinin is accompanied by loss of the Z-filament structure.

The earliest suggestions for the protein composition of the Z-band were based on the similarity between the structure of the Z-band and the structure of tropomyosin paracrystals. On this basis it was suggested that tropomyosin might be a component of the Z-band (14). From fluorescence antibody staining of myofibrils, it was found that antibody to tropomyosin did not stain the Z-band (27). Furthermore, studies of the reconstitution of Z-bands in myofibrils from which the Z-bands had previously been removed showed that protein fractions rich in tropomyosin did not reconstitute the Z-band, whereas fractions in which tropomyosin was absent did reconstitute the Z-band (40, 41).

From a number of different studies, alpha-actinin has been implicated as a component of the Z-band, although it is not clear what structural features of the Z-band are related to the alpha-actinin. Using specific antibody to alpha-actinin, Masaki et al. (24) showed that alpha-actinin was present in the Z-band. A number of different treatments of the myofibrils have specific effects on the Z-band and these effects can be related to the presence of alpha-actinin. For instance, a calcium activated factor which specifically removes the Z-bands of myofibrils (5) has been shown to effect the loss of alpha-actinin from the myofibril in parallel to the loss of the Z-band (31). Studies of the binding of alpha-actinin to F-actin have also suggested that alpha-actinin may be involved in the end-to-end linking of actin filaments in the Z-band (1, 3, 9, 13, 33, 39). The binding of alpha-actinin to F-actin was found to be dependent on temperature (9, 39). At 0°C the binding of alpha-actinin to F-actin occurred along the entire length of the F-actin and was strong enough to dislodge tropomyosin from the F-actin filament. At 37°C there was very limited binding of alpha-actinin to F-actin. When tropomyosin was present at 37°C, two molecules of alpha-actinin were bound per every 1 μm of F-actin filament. These findings are consistent with the possibility that the two molecules of alpha-actinin are present at one end of the polar F-actin filament, one binding to each of the two strands of the actin polymer; and, therefore, with the possibility that alpha-actinin is present at the end which binds to the Z-band, implicating alpha-actinin as the possible cross-linker of F-actin filaments at the Z-band. The presence of alpha-actinin in isolated Z-disks has also been shown by fluorescence antibody staining (11, 12, 22). The alpha-actinin is present in the Z-band itself, whereas the localization of desmin and vimentin is confined to the surface of the myofibril, i.e., around the periphery of the Z-band. Therefore, alpha-actinin is not present in the region of the Z-band where desmin and vimentin are localized. Although alpha-actinin has generally been regarded as the principal component of the Z-bands of most vertebrate muscles (24, 32, 36, 37, 42), as well as invertebrate muscles (2, 4, 10, 35), other proteins have also been considered as Z-band constituents. A protein with a chain weight of 55,000 daltons, which has a different amino acid composition than desmin, has been localized to the Z-band by fluorescence antibody staining of myofibrils (26). In studies of the Z-bands of insect fibrillar flight muscles, proteins with chain weights of 87,000, 113,000, 158,000, and 175,000 daltons have been related to the Z-band (10, 35).

The Z-band has been recognized as having a filamentous component as well as an amorphous or structureless component which is present between the Z-filaments. Some studies of both vertebrate and invertebrate muscle Z-bands have suggested that alpha actinin is the amorphous component of the Z-band

THE JOURNAL OF CELL BIOLOGY · VOLUME 94 SEPTEMBER 1982 565-573
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(2, 10, 38). The main purpose of the work presented in this paper is to identify the proteins which make up the Z-filaments and which contribute to the amorphous, structureless material in the Z-band. We have found that in chicken pectoralis muscle the Z-filaments are alpha-actinin and that a protein with a chain weight of 85,000 daltons, which we have named 85K amorphin, is also present in the Z-band but is not a structural component of the Z-filaments.

MATERIALS AND METHODS

Differential Extraction of the Z-band

Glycerinated muscle was shredded into very thin bundles of fibers by teasing with needles. The following procedures were then carried out:

(*a*) The teased muscle bundles were washed with 3 vol of standard salt solution (0.1 M KCl, 0.01 M phosphate, 1 mM $MgCl_2$, pH 7.0) and centrifuged.

(*b*) The washed muscle was extracted with 3 vol of buffered sucrose (0.25 M sucrose, 50 mM Tris, 1 mM NaN_3, 1 mM EDTA, pH 8.0) for 15 min and centrifuged. The muscle was reextracted overnight with 3 vol of buffered sucrose. This supernatant (Fig. 1 *a*) was saved. An aliquot of the extracted muscle was fixed and embedded for electron microscopy (Fig. 2 *b*).

(*c*) The muscle was then extracted with 3 vol of pyrophosphate buffer (0.1 M $Na_4P_2O_7$, 10 mM $MgCl_2$, pH 7.2) for 15 min and centrifuged. This was followed by extraction with 3 vol of 0.6 M KCl for 10 min, then centrifugation. The muscle was reextracted overnight in 3 vol of pyrophosphate buffer, then centrifuged, and the supernatant (Fig. 1 *b*) was saved. An aliquot of the extracted muscle was fixed and embedded for electron microscopy (Fig. 3 *a*).

(*d*) The muscle was briefly suspended in 2 vol of 5 mM Tris, pH 8.0, and centrifuged. The supernatant was discarded. The muscle was then extracted with 3 vol of 5 mM Tris, pH 8.0, for 1.5 h and centrifuged. The supernatant (Fig. 1 *c*) was saved and an aliquot of the muscle was fixed and embedded for electron microscopy (Fig. 3 *b*).

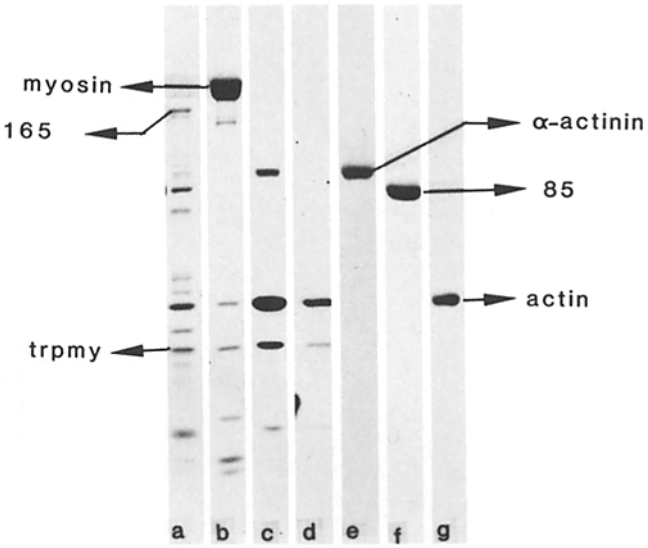

FIGURE 1 SDS PAGE of proteins obtained by stepwise extraction of glycerinated myofibrils. (*a*) Proteins extracted by low ionic strength sucrose solution (0.25 M sucrose, 50 mM Tris, 1 mM EDTA, 1 mM NaN_3, pH 7.9) as described in the text. Note the presence of a protein with a chain weight of 165 kdaltons which might be M-band protein. Also an 85-kdalton protein (85K amorphin) is extracted in addition to some actin, tropomyosin, and other proteins. (*b*) After extraction with low ionic strength sucrose as in *a*, extraction with a pyrophosphate buffer (0.1 M $Na_4P_2O_7$, 10 mM $MgCl_2$, pH 7.2) as described in the text removed mostly myosin. (*c*) After pyrophosphate extraction as in *b*, extraction with 5 mM Tris, pH 8.0, for 1.5 h removed primarily alpha-actinin, actin, and tropomyosin. (*d*) After 5 d of extraction with 5 mM Tris, pH 8.0, the myofibrillar proteins that are left include only actin and tropomyosin with a trace of troponin. (*e*) Purified alpha actinin. (*f*) Purified 85K amorphin. (*g*) Purified actin.

(*e*) The muscle was reextracted with 3 vol of 5 mM Tris, pH 8.0, for 5 d in the cold and centrifuged. The supernatant was saved and the pellet was embedded for electron microscopy (Fig. 3 *c*). An SDS gel of the contents of the pellet is shown in Fig. 1 *d*.

Transverse sections of the muscle bundles were also taken for electron microscopy (Fig. 4).

Isolation and Purification of the 85,000-dalton Z-Band Protein (85K Amorphin)

Fresh chicken pectoralis muscle was ground in a meat grinder and washed three or four times with 0.1 M KCl, 5 mM EDTA, pH 7.0, using 3 vol of wash solution each time. The washed muscle was extracted with 4 or 5 vol of 5 mM Tris, pH 8.0, for 2 h. After centrifugation, the supernatant was collected and the pH of the supernatant was lowered to 6.8. The precipitate which formed was collected by centrifugation and it was dissolved in 5 mM Tris, pH 8.0. The solution was clarified by centrifugation at 27,000 *g* for 10 min in a Sorvall centrifuge using an SS-34 rotor (DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Wilmington, DE). This crude fraction contained a major component (85K amorphin) with a chain weight of 85,000 daltons.

The clarified crude fraction was made 0.1 M in KCl and 1 mM in $MgCl_2$ by adding 2 m KCl and 1 M $MgCl_2$. After standing at room temperature for 0.5 h, the solution was centrifuged at 100,000 *g* for 3 h. The supernatant was dialyzed against 0.1 M phosphate, 2 mM NaN_3, pH 7.9, and applied to a DEAE Sephadex A-50 column, in the same buffer solution. Protein was eluted from the column by a KCl gradient from 0 to 0.6 M KCl in the same buffer solution. The peak containing the 85K amorphin was eluted almost at the end of the gradient (Fig. 5). The peak was separated into three portions as indicated in Fig. 5. The ascending portion of the peak contained only 85K amorphin (Fig. 5 *b*). The other two portions contained impurities (Fig. 5 *c* and *d*) and these were mixed and reapplied to the DEAE Sephadex A-50 column to obtain more 85K amorphin free of contaminants. A column 90 × 2.5 cm was used and it was loaded with ~250 mg of the crude 85K amorphin. About 15 mg of pure 85K amorphin was recovered in the ascending portion of the peak (Fig. 5 *b*) during the first separation. On rechromatographing the mixture of the peak and descending portions (Fig. 5 *c* and *d*) that totaled ~162 mg, a total of ~100 mg more of pure 85K amorphin could be obtained.

Preparation of Antiserum and Isolation of Specific Antibody

Column purified 85K amorphin obtained as described above was then run on preparative SDS PAGE for futher purification before being used as an immunogen. About 5 mg of the 85K amorphin was loaded onto an 8% polyacrylamide gel 1mm thick and the run was made essentially as described by Laemmli (19). The gel slab was then soaked briefly in a cold solution of 0.25 M KCl containing 1 mM DTE which made the 85K amorphin band visible. The 85K amorphin band was cut out of the slab and crushed and the protein was extracted for 2–3 h at room temperature, with 4–5 vol of a solution containing 0.15 M NaCl, 0.1% SDS, 0.1 mM EDTA, 5 mM DTE, 50 mM Tris, pH 7.9. The filtered solution was then dialyzed against 5 mM Tris, pH 8.0, at room temperature. After dialysis, the protein solution was concentrated by wrapping the dialysis bag in dry Sephadex G-200.

Approximately 0.8 mg of 85K amorphin was homogenized with complete Freund's adjuvent and injected subcutaneously in a rabbit. 1 wk later, the rabbit received a second injection and, 1 wk after that, blood was collected. The IgG was obtained by ammonium sulfate precipitation of the serum. This was dialyzed against 0.1 M KCl, 10 mM Imidazole buffer, pH 7.0, containing 0.2% NaN_3. In the same buffer, it was then passed over an alpha-actinin affinity column to remove any contaminating antibodies specific for alpha-actinin.

The IgG obtained in this way was shown to be specific for 85K amorphin. Whole myofibrils, crude Z-band extract, purified alpha-actinin, and purified amorphin were run on minislab SDS polyacrylamide (8%) gels using the procedure described by Laemmli (19). Samples run in one-half of the slab were duplicated in the other half. At the end of the run, the slab was cut in half and one-half was stained with Coomassie Blue for identification of the bands and the other half was used to electrophoretically transfer the bands to a nitrocellulose sheet as described by Towbin et al. (43). The nitrocellulose sheet was then treated as follows: (*a*) Washed overnight in buffer containing 0.1% bovine serum albumin (BSA), 0.05% NonidetP-40, 0.15 M NaCl, 0.025 M Tris, pH 8.0, and rinsed with fresh buffer. (*b*) Soaked in the same buffer containing 5% BSA for ½ h. (*c*) Stained with a mixture of the specific rabbit IgG (0.05 mg/ml) and normal goat gamma-globulin (0.4 mg/ml) in the same buffer without BSA for 1 h. (*d*) Stained with a mixture of peroxidase-labeled goat anti-rabbit gamma-globulin obtained from Bionetics (50 times dil) (Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, MD) and normal goat gamma-globulin (0.4 mg/ml) in the

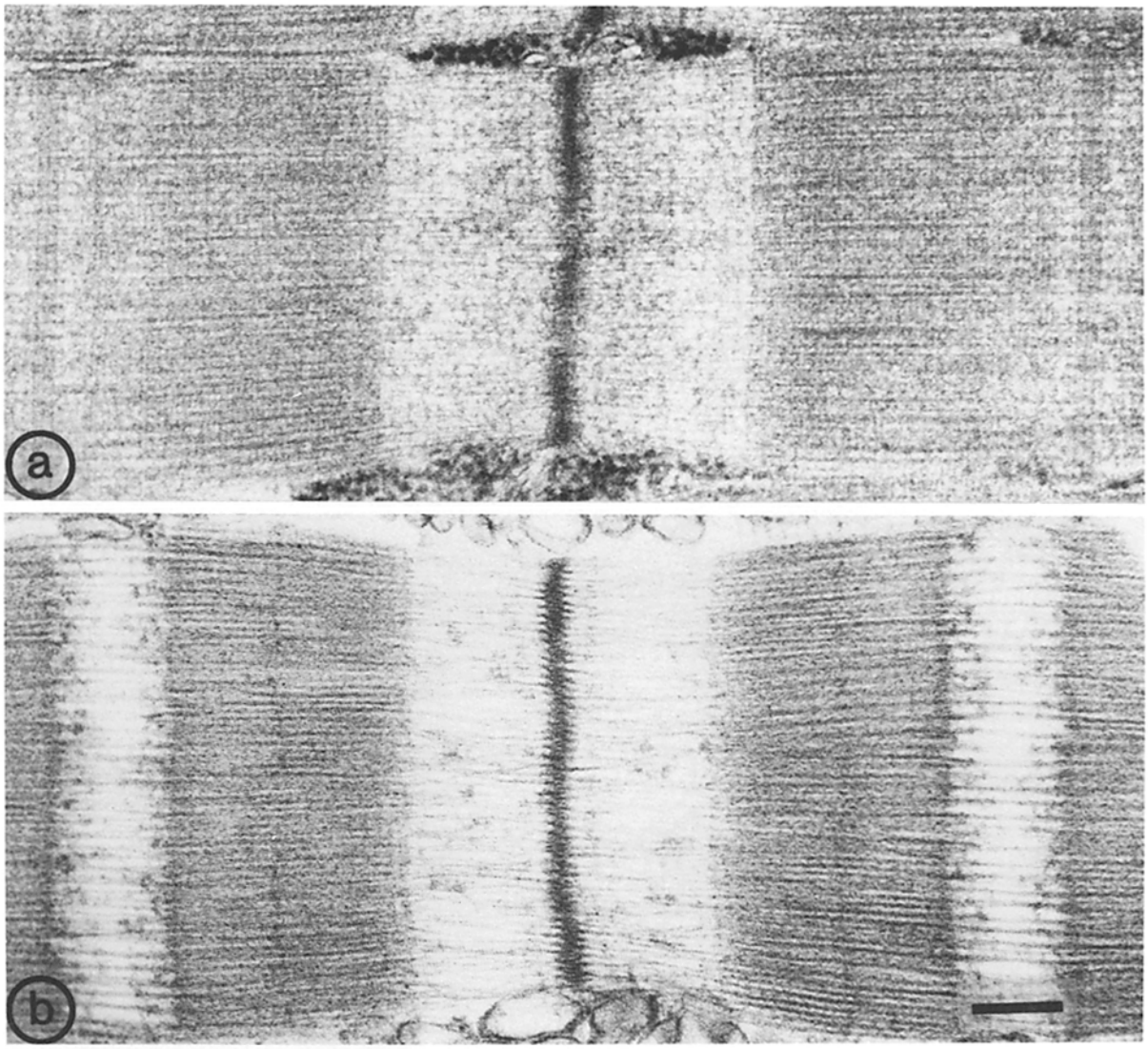

FIGURE 2 Longitudinal sections of myofibrils of chicken pectoralis muscle. (*a*) The intact fresh muscle was fixed and embedded for electron microscopy. The Z-band of the sarcomere is in the middle of the micrograph. (*b*) Glycerinated muscle after extraction with 0.25 M sucrose, 50 mM Tris, 1 mM EDTA, 1 mM NaN_3, pH 8.0, as described in the text. The Z-filaments are clearly present. Bar, 0.2 μm. × 60,000.

same buffer without BSA for ½ h. (*e*) Washed with PBS (0.15 M NaCl, 0.01 M phosphate, pH 7.3) for 10–15 min. (*f*) Washed with 50 mM Tris, pH 7.6, buffer for 10 min. (*g*) Soaked in the following mixture to develop the peroxidase reaction product: 10 mg of diaminobenzidine (DAB) was mixed with 30 ml of 50 mM Tris, pH 7.6, buffer and to this solution was added 20 μl of 30% H_2O_2. (*h*) On color development, washed with 50 mM Tris, pH 7.6, buffer, then with water, and finally dried. The stained bands were identified by comparing them with the corresponding Coomassie-Blue-stained gel.

Fluorescence Antibody Staining of Myofibrils

Myofibrils were used for all the fluorescence antibody staining studies. About 2 g of glycerinated muscle was homogenized to myofibrils in ~25 ml of buffer as previously described (29), except that the buffer used was 0.1 M KCl, 10 mM Imidazole, pH 7.0, with 0.2% NaN_3. After washing by centrifugation and resuspension, the myofibrils were finally suspended in 10 ml of buffer. One drop of this suspension was added to 0.5 ml of specific anti-85K amorphin at 4 mg/ml for staining. One drop of this suspension was added to 0.5 ml of specific anti-alpha-actinin at a concentration of 5 mg/ml for staining. The specificity of this anti-alpha-actinin has been previously determined (21). The antibody and myofibril mixture was allowed to stand overnight at 0°–4°C for antibody binding. The myofibrils were then washed by centrifugation and resuspension in 0.1 M KCl, 10 mM Imidazole buffer, pH 7.0, containing 0.2% NaN_3. The washed myofibrils were then suspended in 0.5 ml of the same buffer and to the suspension was added 50 μl of fluorescein labeled goat anti rabbit gamma globulin (Bionetics Laboratory Products). The mixture was allowed to stand for at least ½ h before the stained myofibrils were observed in fluorescence microscopy. Phase-contrast and fluorescence micrographs of the same myofibril were obtained as previously described (27).

Electron Microscopy

For electron microscopy fibers were fixed in 5% glutaraldehyde in standard salt (0.1 M KCl, 0.01 M phosphate, 1 mM $MgCl_2$, pH 7.0) for 30 min. This was followed by osmium tetroxide fixation, dehydration in alcohol, embedding in araldite, and sectioning as previously described (28). Electron micrographs were obtained on a Siemen's Elmiskop I.

RESULTS

Differential Extraction of the Z-Band

The muscle which served as starting material for the differential extraction of the Z-band is shown in Fig. 2*a*. The Z-filaments of the Z-band link between the actin filaments of neighboring sarcomeres. Extraction of the thin muscle bundles with low ionic strength buffered sucrose does not affect the integrity of the Z-filaments (Fig. 2*b*). However, from many

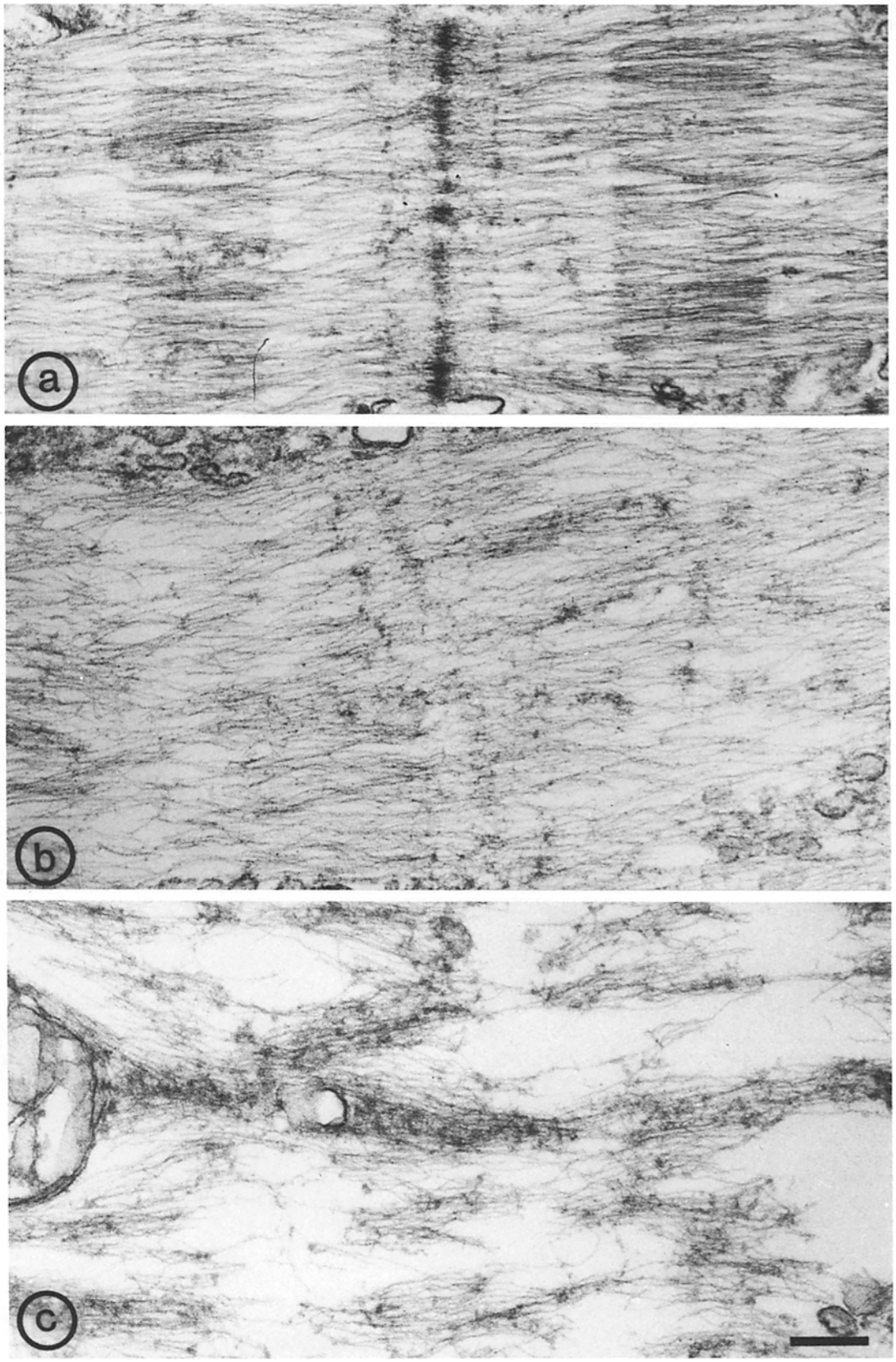

FIGURE 3 Longitudinal sections of myofibrils of chicken pectoralis muscle after sequential extractions. (*a*) After extraction with low ionic strength sucrose solution as in Fig. 2 *b*, the muscle was extracted with 0.1 M $Na_4P_2O_7$, 10 mM $MgCl_2$, pH 7.2, and 0.6 M KCl as described in the text. The Z-filaments are still present in the Z-band, but the A-band is completely extracted. The denser regions in the middle of the sarcomeres on each side of the Z-band are areas of double overlap of the actin filaments. (*b*) After extraction as in *a*, the muscle was extracted for 1.5 h with 5 mM Tris, pH 8.1. Note that the Z-bands have been extracted. Some remnants of Z-filament material still remains in occasional places as shown here, but the structure is disorganized. (*c*) After 5 d of extraction with the 5 mM Tris, pH 8, there is no evidence of Z-filaments present. Bar, 0.2 μm. × 60,000.

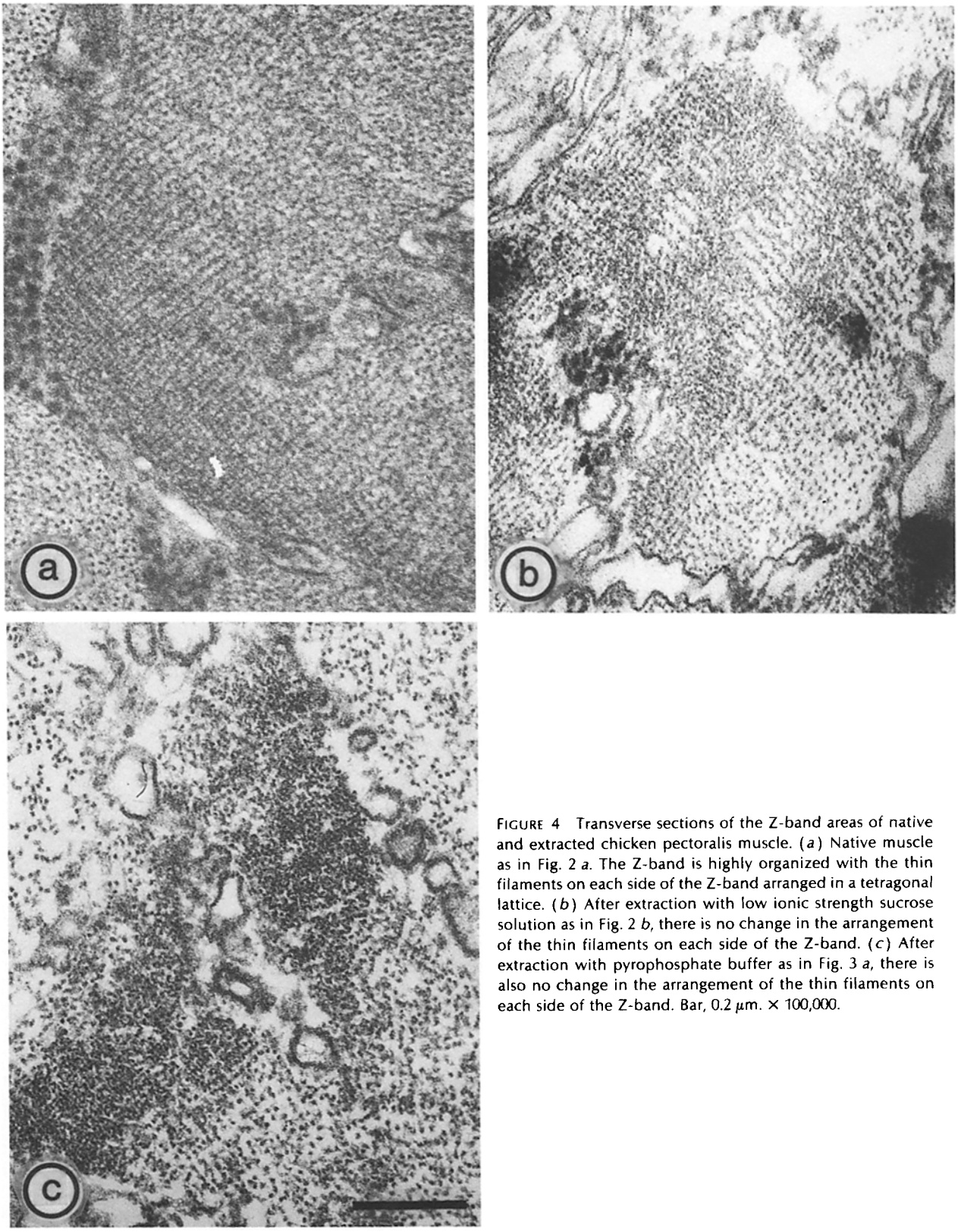

FIGURE 4 Transverse sections of the Z-band areas of native and extracted chicken pectoralis muscle. (*a*) Native muscle as in Fig. 2 *a*. The Z-band is highly organized with the thin filaments on each side of the Z-band arranged in a tetragonal lattice. (*b*) After extraction with low ionic strength sucrose solution as in Fig. 2 *b*, there is no change in the arrangement of the thin filaments on each side of the Z-band. (*c*) After extraction with pyrophosphate buffer as in Fig. 3 *a*, there is also no change in the arrangement of the thin filaments on each side of the Z-band. Bar, 0.2 μm. × 100,000.

electron micrographs it appears that some unstructured density between the Z-filaments of the Z-band has been removed. In addition, it is clear that the M-band has been entirely extracted. The protein components extracted by the buffered sucrose are shown in Fig. 1 *a*. These include a protein with a chain weight of 85,000 daltons (amorphin). The structure of the Z-band as observed in transverse section after extraction with sucrose showed the square lattice pattern typical of normal Z-bands (Fig. 4 *a* and *b*).

On further extraction with pyrophosphate buffer (0.1 M $Na_4P_2O_7$, 10 mM $MgCl_2$, pH 7.2), the major component removed was myosin (Fig. 1 *b*). From electron micrographs of

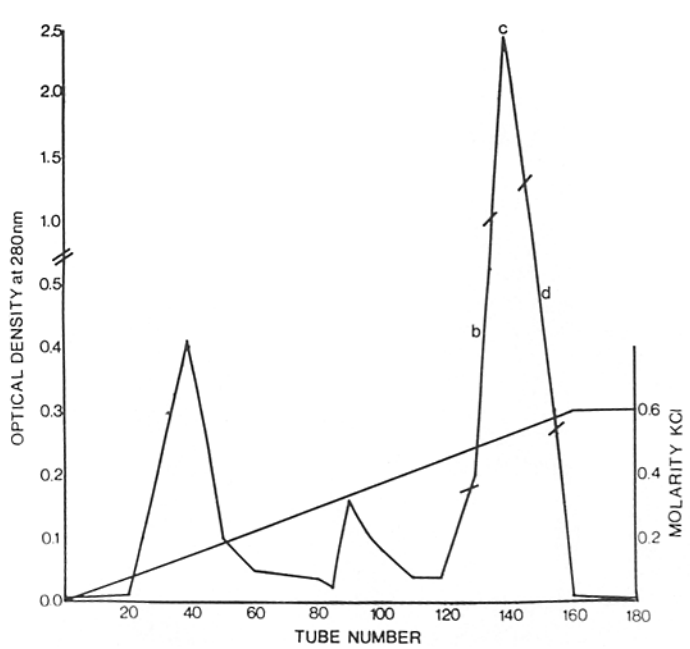

FIGURE 5 Purification of 85K amorphin by column chromatography on DEAE Sephadex A-50. The protein was applied to a 2.5 × 90 cm column in 0.1 M potassium phosphate buffer at pH 7.9 containing 2 mM NaN_3. The protein was eluted with a salt gradient from 0–0.6 M KCl in the same buffer. The 85K amorphin was eluted almost at the end of the gradient. Different portions of the peak were kept separate as indicated on the elution curve by *b*, *c*, and *d*. (*a*) SDS gel electrophoresis of the crude 85K amorphin preparation which was applied to the column. (*b*) The protein collected from the ascending portion of the peak marked *b* on the elution curve. This portion of the curve gave the most highly purified protein. (*c*) The protein collected in the peak portion marked *c* on the elution curve. (*d*) The protein collected in the descending portion of the peak marked *d* on the elution curve.

TABLE I

Amino Acid Composition of 85K Amorphin and Alpha-actinin

	Z-band protein		Sarcoplasmic reticulum proteins*	
Amino acid	85K amorphin	Alpha-actinin	Ca^{++}-pump protein	Ca^{++}-binding protein
Asp-	11.7	11.4	8.72	17.85
Thr-	4.3	5.3	5.90	3.21
Ser-	4.6	5.3	5.00	4.00
Glu-	11.5	18.5	10.60	17.70
Pro-	4.6	3.7	5.50	4.73
Gly-	7.2	4.6	7.03	4.33
Ala-	8.6	8.4	8.48	6.30
Val-	6.2	3.5	7.66	6.54
Met-	2.0	3.1	3.39	1.89
Ile-	4.4	6.2	6.18	5.21
Leu-	10.0	9.2	9.75	9.00
Tyr-	4.2	2.9	2.33	3.03
Phe-	4.3	3.0	5.30	4.94
His-	3.3	2.7	1.27	1.97
Lys-	5.3	6.0	5.42	5.66
Arg-	7.5	6.3	5.08	2.24

Amino acid composition of 85K amorphin and alpha-actinin obtained from chicken pectoralis muscle in mole percent: comparison with sarcoplasmic reticulum proteins.

* Meissner et al. (25).

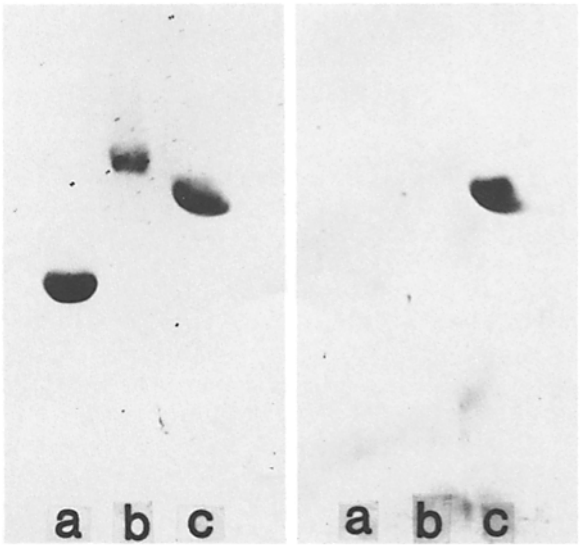

FIGURE 6 Specificity of anti-85K amorphin. On the left is a Coomassie-Blue-stained SDS polyacrylamide gel containing BSA in lane *a*, alpha-actinin in lane *b*, and 85K amorphin in lane *c*. On the right are the comparable proteins after having been transferred from the gel to nitrocellulose sheets and stained with specific anti-85K amorphin followed by horse radish peroxidase-labeled anti-rabbit gamma-globulin. No reaction occurred in lanes *a* or *b*. Strong reaction occurred in lane *c*.

the muscle after extraction with pyrophosphate, it is clear that the A-bands of the sarcomeres are completely removed (Fig. 3*a*). It is also clear that the Z-band structure remaining after sucrose extraction (Fig. 2*b*) has not been affected by the pyrophosphate extraction. Therefore, pyrophosphate extraction does not affect the integrity of the Z-filaments. The structure observed in transverse sections of the Z-band after pyrophosphate extraction (Fig. 4*c*) was also typical of that observed in unextracted fibrils (Fig. 4*a*).

On further extraction with 5 mM Tris, pH 8, for 1.5 h, the major protein components extracted were alpha-actinin, actin, tropomyosin, and some troponin (Fig. 1*c*). The myofibrils in the extracted fibers clearly show loss of Z-band structure. Only rarely can evidence for remnants of Z-band structure be seen in some fibrils (Fig. 3*b*). The 5 mM Tris, pH 8, has clearly disrupted the structural organization of the Z-filaments. After 5 d extraction with 5 mM Tris, pH 8, all evidence of Z-band structure is gone. The Z-filament structure has been completely removed (Fig. 3*c*). The myofibrillar proteins remaining after this period of extraction are primarily actin, tropomyosin, and troponin (Fig. 1*d*). The loss of alpha-actinin parallels the loss of Z-filaments.

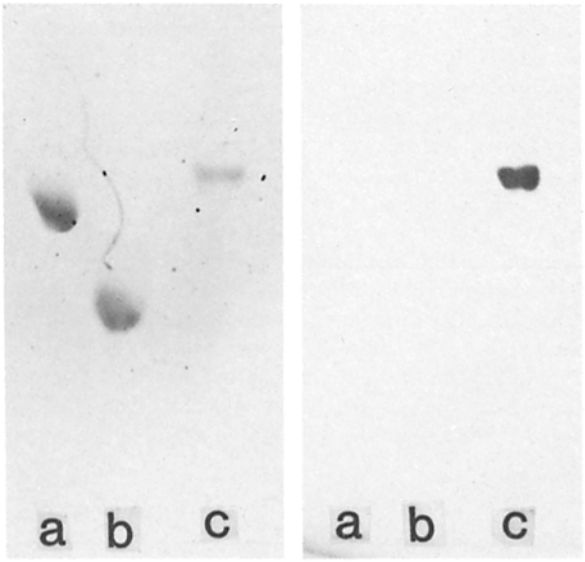

FIGURE 7 Specificity of anti-alpha-actinin. On the left side is a Coomassie-Blue-stained SDS polyacrylamide gel containing 85K amorphin in lane *a*, BSA in lane *b*, and alpha-actinin in lane *c*. On the right are the comparable proteins after having been transferred from the gel to nitrocellulose sheets and stained with specific anti-alpha-actinin followed by horse radish peroxidase-labeled anti-rabbit gamma-globulin. No reaction occurred in lanes *a* or *b*. Strong reaction occurred in lane *c*.

Amino Acid Composition of 85K Amorphin

Amino acid analysis of purified 85K amorphin and purified alpha-actinin were performed by Dr. Ruth Hogue-Angeletti (University of Pennsylvania) and the results are given in Table I. It is clear that these proteins have distinctly different amino acid compositions. There is from 1.4 to 1.8 times more phenylalanine, tyrosine, glycine, and valine in 85K amorphin than there is in alpha-actinin, and there is from 1.4 to 1.6 times more isoleucine, glutamate, and methionine in alpha-actinin than there is in 85K amorphin. The amino acid composition of purified 85K amorphin is also distinctly different from that reported by Meissner et al. (25) for the Ca^{++} pump protein and Ca^{++}-binding sarcoplasmic proteins. From Table I, the Ca^{++}-pump protein has from 1.4 to 1.7 times more threonine, isoleucine, and methionine, whereas the 85K amorphin has from 1.5 to 2.6 times more arginine, tyrosine, and histidine than the Ca^{++}-pump protein. Comparison of 85K amorphin with the Ca^{++}-binding protein shows that there is 1.5 times more aspartate and glutamate in the Ca^{++}-binding protein, whereas there is from 1.4 to 3.3 times more alanine, glycine, histidine, and arginine in the 85K amorphin. The Ca^{++}-pump protein has a chain weight of 110,000 daltons and the Ca^{++}-binding protein has a chain weight of 60,000 daltons.

Antibody Specificity

The specificity of the anti-alpha-actinin used in this work has previously been established (21). The specific anti-85K

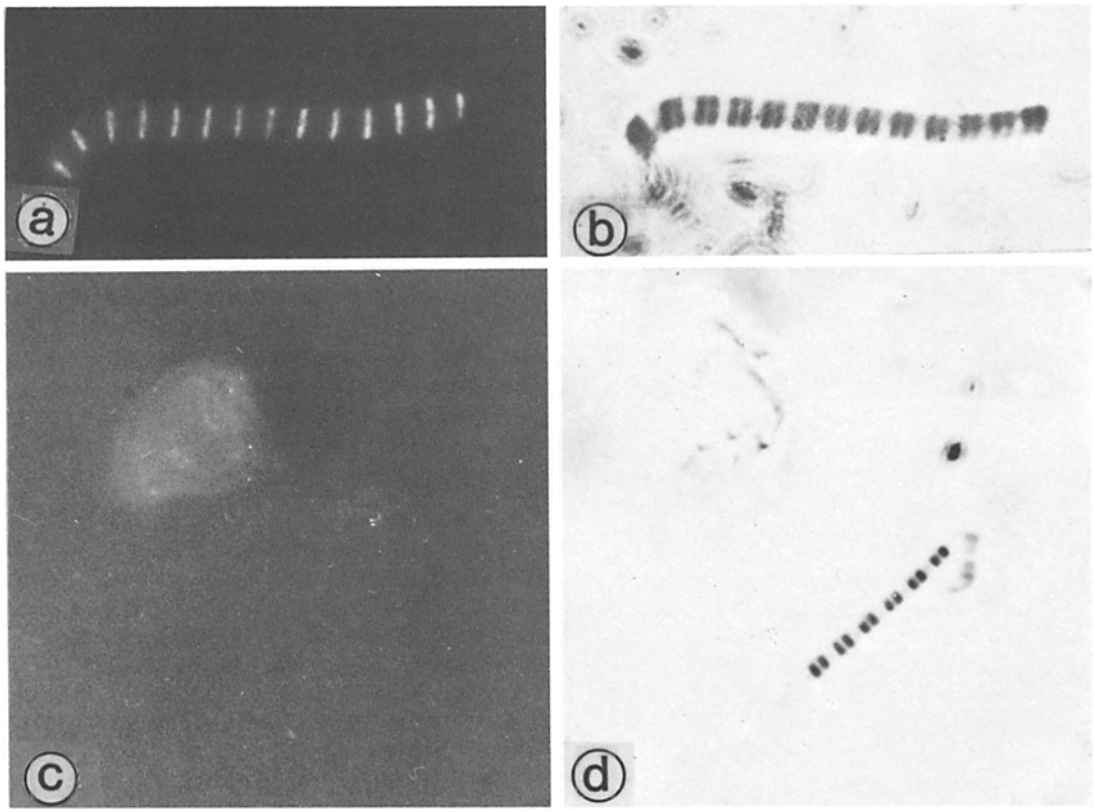

FIGURE 8 Specific anti-85K amorphin staining of myofibrils. (*a*) Fluorescence antibody staining pattern obtained on glycerinated myofibrils. The same fibril in phase-contrast microscopy is shown in *b*. The specific staining with anti-85K amorphin is restricted to the Z-band. (*c*) After sucrose extraction of the myofibril, corresponding to the electron micrographs in Figs. 2 *b* and 4 *b* where Z-filaments are clearly visible, no staining occurs with the anti-85K amorphin. The unstained fibril which is present in this area is clearly visible in phase-contrast microscopy as can be seen in *d*. × 2,000.

amorphin prepared as described in the Materials and Methods stained only the 85,000-dalton band present in gels of either entire myofibrils or of the crude Z-band extract. In addition, when specific anti-85K amorphin was used to stain both purified 85K amorphin and purified alpha-actinin (21), it stained only the 85K amorphin (Fig. 6); and when specific anti-alpha-actinin was used to stain the two purified proteins, only the alpha-actinin was stained (Fig. 7). This clearly establishes that the two antibodies are entirely specific with no indication of cross reactivity between 85K amorphin and alpha-actinin.

Fluorescence Antibody Staining

The specific antiamorphin (Fig. 6) stained only the Z-bands of glycerinated myofibrils as seen in Fig. 8 *a*. Myofibrils which had been extracted with buffered sucrose before staining were not stained by the antiamorphin (Fig. 8 *c*). The corresponding phase-contrast images are seen in Fig. 8 *b* and *d*, respectively.

Glycerinated myofibrils treated with specific anti-alpha-actinin showed bright staining localized only in the Z-band (Fig. 9 *a*). Sucrose extraction of the myofibrils which resulted in no staining with specific antiamorphin had no effect on the anti-alpha-actinin staining of the Z-bands (Fig. 9 *c*). The corresponding phase-contrast images are seen in Fig. 9 *b* and *d*. Similarly pyrophosphate extraction which essentially removed all components except for the Z-band and actin filaments had no effect on the specific staining of the Z-bands with anti-alpha-actinin. However, it was difficult to get structurally well preserved myofibrils on homogenization of the fibers after pyrophosphate extraction.

Purified 85K amorphin can be added back to myofibrils from which it has been completely extracted with buffered sucrose solution (Fig. 8 *c*), by suspending the extracted fibrils in a solution containing 5 mg/ml of 85K amorphin in 2 mM Tris, 1 mM DTE, 0.1 M KCl at pH 7.6 for 3–4 d in the cold. Fluorescence antibody staining of the washed fibrils shows that the 85K amorphin has specifically localized very heavily in the Z-band (Fig. 10). Some light fluorescence can be seen in the M-band region. This, most likely, represents slight nonspecific binding of 85K amorphin to this region of the sarcomere. The striking observation is the heavy specific binding which is restricted to the Z-band.

DISCUSSION

Although the structure of the Z-band of vertebrate skeletal muscle has been studied in considerable detail, there are still unresolved questions about the precise organization of its structural components (6–8, 15–18, 20, 23, 30, 34, 44). The presence of alpha-actinin in the Z-band of vertebrate skeletal muscles (11, 12, 21, 22, 24, 31, 32, 36, 37, 42), as well as in invertebrate muscles (2, 4, 11, 37), is now generally accepted

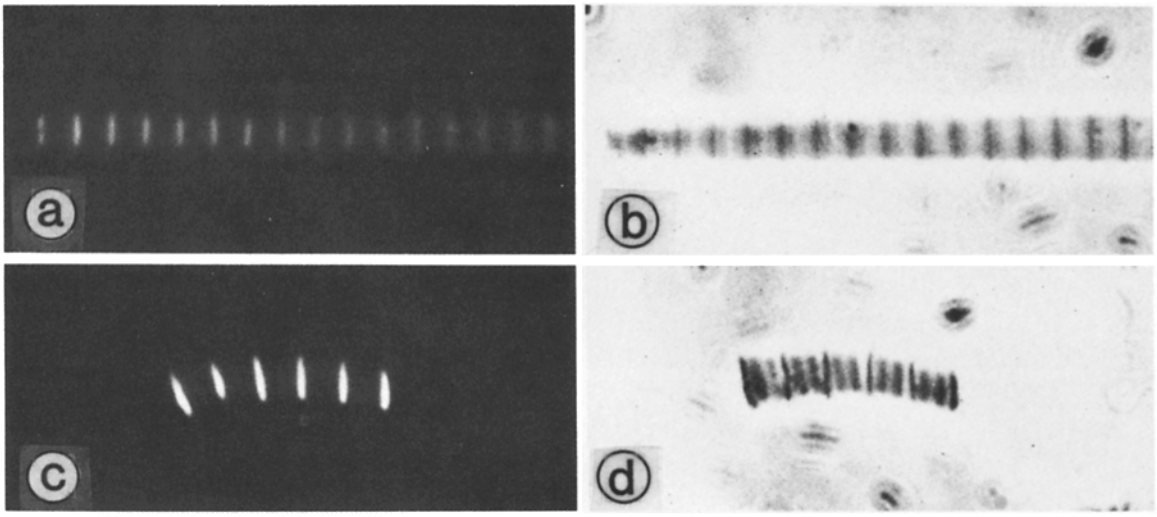

FIGURE 9 Specific anti-alpha-actinin staining of myofibrils. (*a*) Fluorescence antibody staining pattern obtained on glycerinated myofibrils. The same fibril in phase-contrast microscopy is shown in *b*. The specific staining with anti-alpha-actinin is restricted to the Z-band as previously reported (21). (*c*) After sucrose extraction of the myofibril, corresponding to the fibil in Fig. 8 *b* and to the electron micrographs in Figs. 2 *b* and 4 *b* where Z-filaments are clearly visible, the specific staining of the Z-band is still obtained with anti-alpha-actinin. The same fibril in phase-contrast microscopy is shown in *d*. × 2,500.

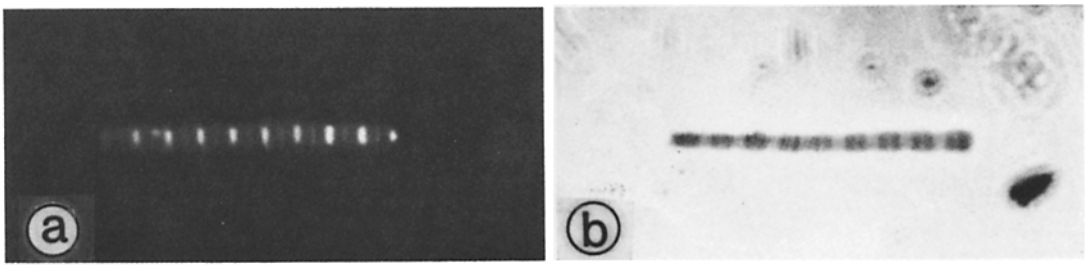

FIGURE 10 Specific rebinding of 85K amorphin to the Z-band of myofibrils from which it has previously been extracted. (*a*) A fibril from which 85K amorphin was extracted using buffered sucrose, comparable to that in Fig. 8 *c* and *d* and to which purified 85K amorphin was added followed by staining with fluorescence anti-85K amorphin. The same fibril is seen in phase-contrast microscopy in *b*. The 85K amorphin binds strongly only to the Z-band of the myofibril. The weak binding to the M-band region is most likely nonspecific. × 2,000.

although the structural feature of the Z-band with which the alpha-actinin is associated has not been determined. Other proteins have also been implicated in the Z-band structure of both vertebrate and invertebrate muscles (10, 26, 35), but these have not been studied as extensively as alpha-actinin.

In this study, we have concentrated on two proteins, alpha-actinin and a protein with a chain weight of 85,000 daltons, which are found in the Z-band of chicken pectoralis muscles. With these studies, we cannot exclude the possibility that proteins, other than these, are also present in the Z-band. Our major goals were: to verify the specific localization of alpha actinin to the Z-band and to show that the 85,000-dalton protein is also localized specifically in the Z-band; and to identify each of these proteins with a structural characteristic of the Z-band. In its simplest terms, the Z-band is made up of Z-filaments which link between the actin filaments of neighboring sarcomeres; and an unstructured amorphous material is present in the Z-band. The unstructured material is more clearly evident in the Z-bands of muscle which has not been glycerinated (10, 38). We have obtained evidence that alpha-actinin makes up the structure of the Z-filaments and that the 85,000-dalton protein is a component of the unstructured amorphous material in the Z-band. For this reason, we have chosen to call the 85,000-dalton protein of the Z-band, amorphin. It is anticipated that other amorphins (unstructured Z-band proteins) in addition to the 85K amorphin will eventually be identified.

Identification of 85K amorphin with the unstructured amorphous component of the Z-band was made by the fact that specific anti-85K amorphin staining of the Z-band was completely eliminated on extraction with buffered sucrose (Fig. 8 *a* and *c*) and that under these same conditions the Z-filaments observed in electron microscopy remained intact (Figs. 2 *b* and 4 *b*). Therefore, the 85K amorphin cannot be a structural component of the Z-filaments. Even after further extensive extraction of the fibers which removed the myosin filaments, the Z-filaments remained intact (Figs. 3 *a* and 4 *c*). Only on subsequent extraction of alpha-actinin was the structure of the Z-filaments lost (Fig. 3 *b* and *c*), thus identifying alpha-actinin as the structural component of the Z-filaments.

It is possible that the 85K amorphin interacts specifically with the alpha-actinin of the Z-filaments in the Z-band. Another possibility is that the 85K amorphin is interacting with the portion of the actin filaments in the Z-band region where tropomyosin may not be present (27). Studies of the interaction of 85K amorphin with purified alpha-actinin and actin are in progress to get more detailed evidence for how they may be related in the Z-band. We have observed very strong rebinding of the 85K amorphin which is highly specific for the Z-band (Fig. 10), but from these experiments we cannot tell whether this represents specific binding to the alpha-actinin or to the portion of the actin filaments which projects into the Z-band region.

We are grateful to Dr. Ruth Hogue-Angeletti of the Department of Pathology for doing the amino acid analysis for us.

Received for publication 23 April 1981, and in revised form 3 April 1982.

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