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The Proteins in the Z Line of Insect Flight Muscle

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Z discs were isolated from *Lethocerus* flight muscle by removing the contractile proteins from myofibrils with a solution of high ionic strength. The protein composition of the Z discs was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; the major proteins were α-actinin, actin and tropomyosin. Z lines were selectively removed from intact myofibrils by digestion with crude lipase and chymotrypsin, but not by purified lipase.

Insect fibrillar muscle has the special property of undergoing oscillatory contraction (Pringle, 1967). Oscillation is possible because the relaxed fibres are unusually stiff compared with vertebrate muscle fibres, and the contractile system is activated by small degrees of stretch in addition to Ca^{2+} (Machin & Pringle, 1959). The myosin filaments of insect fibrillar muscle extend the whole length of the sarcomere and there is evidence that they are attached to the Z line at both ends (Auber & Couteaux, 1963; Reedy, 1971). Activation by stretch may be due to these connexions.

The Z line of insect fibrillar muscle is structurally different from that of vertebrate muscle (Ashhurst, 1967) and is wider; in *Lethocerus* the Z line is 110–140nm wide compared with about 60nm in rabbit psoas muscle. Structural studies of the Z line in the flight muscle of Diptera (Auber & Couteaux, 1963), Hemiptera (Ashhurst, 1967) and Hymenoptera (Saide & Ullrick, 1973) have shown that actin filaments enter the Z line and interdigitate to form a lattice surrounded by an amorphous material. We have analysed the protein composition of the insect Z line as part of an investigation into the connexion between myosin filaments and Z line.

Previous work on the composition of the Z line in both vertebrates and invertebrates is conflicting. The Z line of vertebrates has been selectively removed from the myofibril by extracting at low ionic strength or with urea. Tropomyosin was found in the extracts (Corsi & Perry, 1958; Rash *et al.*, 1968) and was thought to be a Z-line protein. But Stromer *et al.* (1969) found the Z line was reconstituted into the myofibril best by an extract containing α-actinin but little tropomyosin. The similarity between the lattices of the vertebrate Z line and tropomyosin crystals (Huxley, 1963) suggested there might be tropomyosin in the Z line.

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Antibody labelling experiments have shown that antibodies to tropomyosin do not bind to the Z line, whereas antibodies to α-actinin bind in the Z line region only (Masaki *et al.*, 1967). Schollmeyer *et al.* (1973) have suggested that the amorphous material in the vertebrate Z line is α-actinin. α-Actinin has been isolated from insect flight muscle (Hammond & Goll, 1975) and is likely to be present in insect muscle Z line.

The Z line has been selectively removed from the myofibril by enzymic digestion, and the release of α-actinin associated with tryptic digestion of the Z line in vertebrate myofibrils is further evidence that this protein is in the Z line (Goll *et al.*, 1969). Garamvölgyi (1965, 1968) and Garamvölgyi & Guba (1967) found that lipase removed the Z line from both rabbit and insect muscle and suggested that the Z line contained lipoprotein. However, Goll & Reedy (cited by Stromer *et al.*, 1969) have pointed out that the removal of Z lines by lipase might be due to proteolytic contaminants, and Saide & Ullrick (1974) found that the lipid content of bee Z discs was less than 1% of the Z-disc protein.

The myofibrils of asynchronous insect muscle cannot be simply fractionated into thin and thick filaments as can rabbit myofibrils, probably because both kinds of filament are attached to the Z line. This difficulty can be overcome by first removing the Z line. We have investigated further the enzyme in impure lipase that removes the Z line.

Z discs can be isolated both from insect and from rabbit striated muscle by dissolving the rest of the myofibril (Ernst *et al.*, 1958; Garamvölgyi *et al.*, 1962; Harsanyi & Garamvölgyi, 1969). The free Z discs remain intact and are useful for determining the protein composition of this region of the myofibril. Saide & Ullrick (1974) investigated the protein composition of bee Z discs, prepared by extracting the myofibril with lactic acid, and they found proteins of subunit mol.wt. 87000, 113000, 158000 and 175000, but no

α-actinin and only traces of proteins with lower subunit weights.

The Z discs are an integral part of the myofibril, with actin filaments, and probably myosin filaments, attached to them, and an analysis of the protein composition will include remaining attachments. Also the procedures designed to remove non-Z-disc proteins may extract proteins from the Z disc. However, an estimate of the protein composition of Z discs can be obtained by comparing the composition at different stages of purification with that of the whole myofibril.

Methods

Myofibrils were prepared from the flight muscles of two or three specimens of the tropical water bug *Lethocerus cordofanus* (1–2g of muscle) as described previously (Bullard *et al.*, 1973). Z discs are best prepared from fresh, not glycerinated, muscle and all procedures were completed in 1 day. Z discs were isolated by extracting the myofibrils with solutions of high ionic strength. The method is similar to that used by Garamvölgyi *et al.* (1962) for preparing Z discs from bee myofibrils and by Harsanyi & Garamvölgyi (1969) for preparing Z discs from rabbit psoas muscle. Actomyosin was extracted from washed myofibrils by suspending them in an equal volume of 1.4M-KCl/0.08M-$NaHCO_3$ buffer, pH8.8 for 5min at 20°C. The suspension was centrifuged at 1000*g* for 20min to remove incompletely extracted myofibrils, and the supernatant, containing free Z discs and soluble protein, was centrifuged at 18000*g* for 20min. The pellet from the high-speed centrifugation contained Z discs. These were washed twice to remove soluble proteins by resuspension in 0.7M-KCl/0.04M-$NaHCO_3$ buffer, pH8.8, and centrifuging at 18000*g* for 20min. The washed Z discs were resuspended in 0.1M-KCl/0.01M-potassium phosphate buffer (pH7.0)/0.001M-$MgCl_2$. In some cases the Z discs were extracted further by resuspending in 0.4M-KI/0.01M-phosphate buffer, pH6.9, after washing in the 0.7M-KCl solution. The suspension was spun at 18000*g* for 20min and the Z discs were resuspended in the 0.1M-KCl solution.

In order to remove incompletely extracted myofibrils and trachea, some Z-disc preparations were centrifuged on a sucrose density gradient, by using a method similar to that used by Saide & Ullrick (1974) with bee Z discs. A discontinuous gradient was made by layering a solution of 9ml of 0.4M-sucrose/0.1M-KCl over 9ml of 2.5M-sucrose/0.1M-KCl. The Z discs (1ml) in 0.1M-KCl/0.01M-potassium phosphate buffer (pH7.0)/0.001M-$MgCl_2$ were layered on the gradient and centrifuged in a swing-out rotor at 90000*g* for 16h at 20°C. The layers with refractive index between 1.3900 and 1.4075 contained Z discs. The fractions were pooled, and the Z discs were sedimented at 140000*g* for 3h and analysed by SDS*/polyacrylamide-gel electrophoresis.

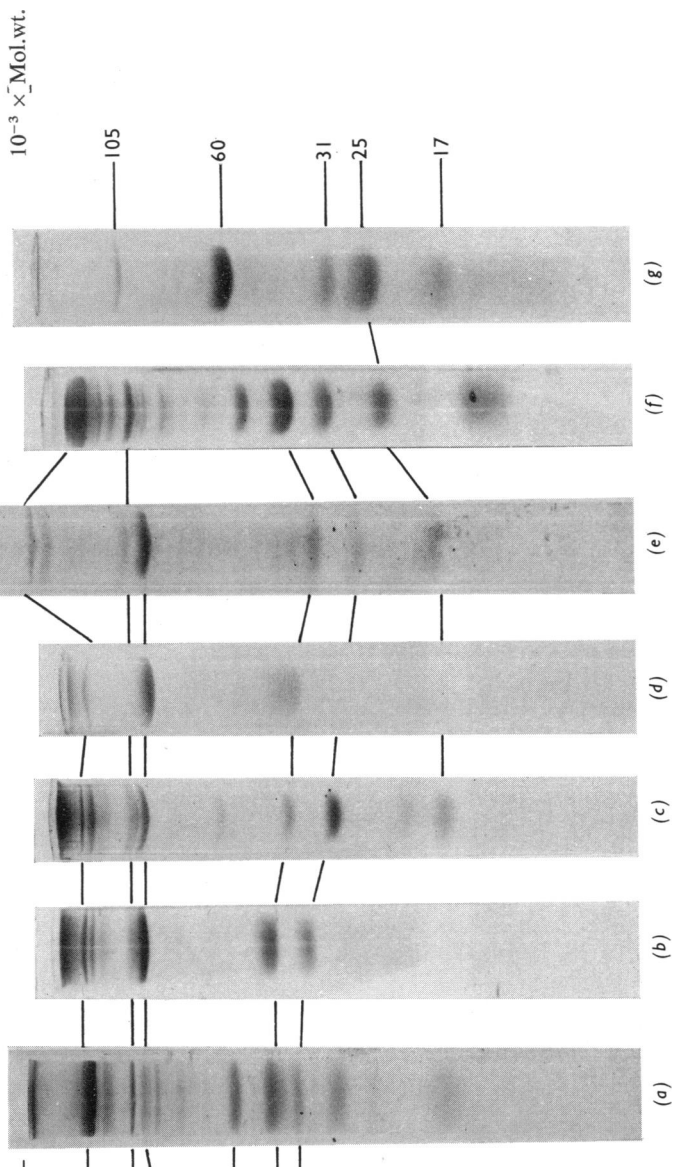
Z lines were removed from myofibrils by enzymic digestion. Lipase [0.1%, w/v, hog pancreatic, type II, Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] in 0.07M-potassium phosphate buffer, pH7.0 was centrifuged at 1000*g* for 5min to remove large particles. This lipase has impurities with amylase and protease activity (Sigma catalogue 1976, p. 270). Trypsin inhibitor (5 mg, Sigma type 1-S) was added to 10ml of the centrifuged lipase to inhibit contaminating trypsin activity. The lipase/trypsin-inhibitor mixture (2ml) was added to 2ml of washed myofibrils in 0.1M-KCl/0.01M-potassium phosphate buffer (pH7.0)/0.001M-$MgCl_2$/0.001M-EGTA (rigor solution). The suspension was incubated at 35°C for 8min, then centrifuged at 1000*g* for 5min and the myofibrils washed by resuspending in rigor solution and centrifuging.

Chymotrypsin and α-amylase were tested for activity in digesting the Z line from myofibrils. Pure lipase was also tested for activity in removing the Z line. Chymotrypsin [1ml of 0.05% (w/v) solution of Sigma type II enzyme in 10mM-potassium phosphate buffer, pH7.0] and pure lipase [1ml of 0.1% (w/v) solution of Sigma type VII enzyme from *Candida cylindracea* in 10mM-potassium phosphate buffer, pH7.0] were added to equal volumes of myofibrils in rigor solution; 5μl of the α-amylase suspension from Sigma (type 1-A) was added to 1ml of myofibrils in rigor solution. The suspensions were incubated at 20°C for 2min for chymotrypsin digestion and 35°C for 8min for lipase and α-amylase digestion. The chymotrypsin digestion was stopped by adding 5μl of 1-chloro-4-phenyl-3-L-tosyl-*p*-sulphonamidobutan-2-one (TPCK, Sigma). The myofibrils were centrifuged at 1000*g* for 5min and washed in rigor solution. The digested myofibrils were examined by phase-contrast microscopy to see if the Z line was removed. A sample of the digested myofibrils was analysed by SDS/polyacrylamide-gel electrophoresis.

Isolated Z discs were digested with the lipase/trypsin-inhibitor mixture. The Z discs were suspended in rigor solution and an equal volume of lipase/trypsin-inhibitor solution was added. The suspension was then incubated at 35°C for 3min. The suspension was centrifuged at 18000*g* for 20min and the supernatant examined by SDS/polyacrylamide-gel electrophoresis to find what proteolytic fragments were released from the Z discs.

SDS/polyacrylamide-gel electrophoresis was by the procedure of Weber & Osborn (1969). Samples were dialysed against 1% SDS/0.01M-sodium phosphate buffer, pH7.0, and incubated at 100°C for 5min in 1% SDS/0.01M-sodium phosphate buffer (pH7.0)/20% (w/v) 2-mercaptoethanol, 12% (v/v) glycerol/0.1% Bromophenol Blue; portions 15–30μg were applied to the gels. The gels were 7.5 or 5% (w/v)

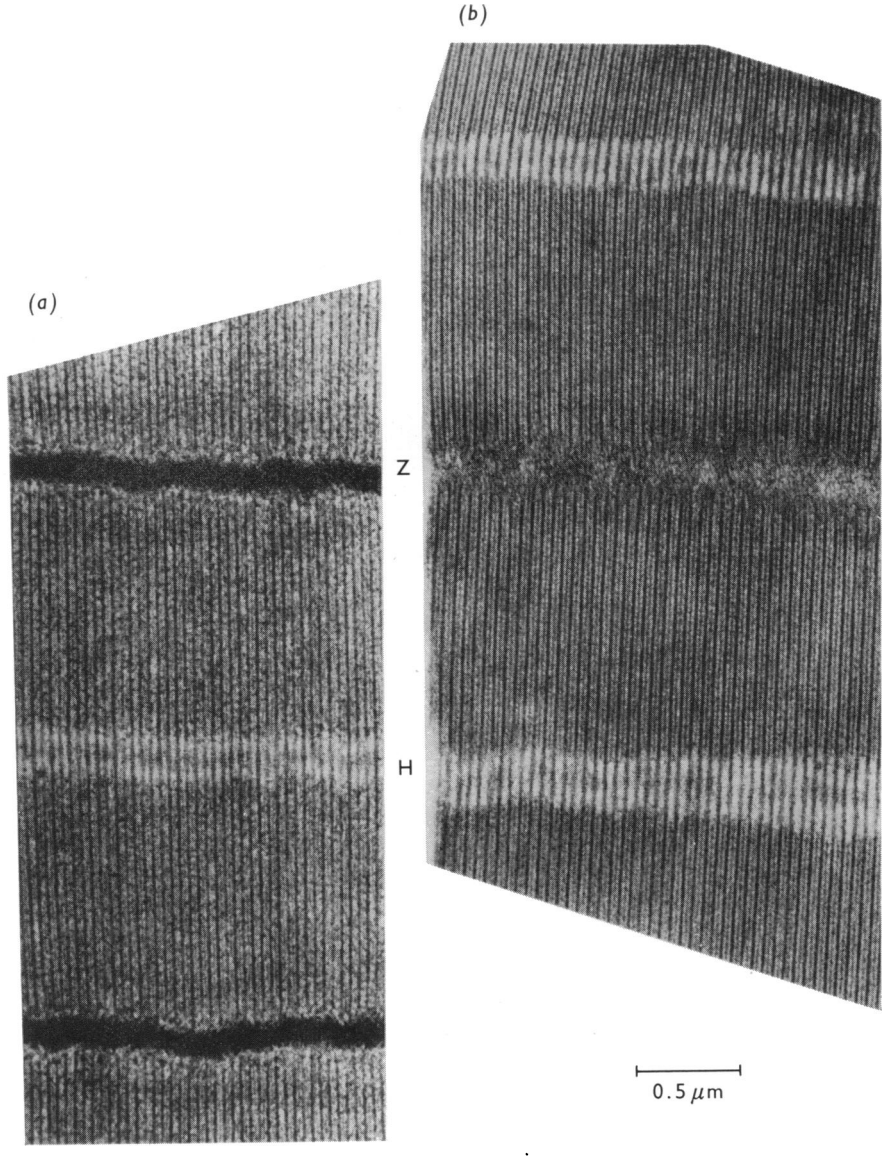
* Abbreviation: SDS, sodium dodecyl sulphate.



EXPLANATION OF PLATE 1

Electrophoresis to show the composition of isolated Z discs from Lethocerus flight muscle and the effects of digesting Z lines enzymically

SDS/7.5% polyacrylamide-gel electrophoresis was by the method of Weber & Osborn (1969). All samples were prepared from *Lethocerus cordofanus* flight muscle (for details see the text). (*a*) Whole myofibrils. (*b*) Washed Z discs. (*c*) Z discs after extraction with 0.4M-KI. (*d*) Proteins extracted from washed Z discs by 0.4M-KI. (*e*) Washed Z discs separated on sucrose density gradient. (*f*) Myofibrils after digestion with lipase to remove Z lines. (*g*) Peptides released by digestion of isolated Z discs with lipase.



EXPLANATION OF PLATE 2

Removal of the Z line from Lethocerus myofibrils

Electron micrographs of longitudinal sections of *Lethocerus cordofanus* flight muscle: (*a*) undigested myofibril; (*b*) myofibril digested with impure lipase. The bar represents 0.5 μm.

acrylamide. Protein concentrations were measured by the micro biuret method (Goa, 1953) standardized by Kjeldahl N determinations.

For electron microscopy a suspension of digested or undigested myofibrils in rigor solution was centrifuged at 1000*g* for 5 min. A small sample of the pellet was fixed in 2.5% glutaraldehyde/0.15 M-sucrose/0.05 M-sodium cacodylate buffer (pH 7.0), dehydrated, embedded in Araldite and sectioned. Sections were stained with 2% (w/v) uranyl acetate followed by lead citrate (Reynolds, 1963) and viewed under an AEI 801A electron microscope. Myofibrils that were sectioned parallel to the fibre axis were selected from the micrographs.

Results

Composition of isolated Z discs

Z discs appeared as free plates by phase-contrast microscopy and could be kept for several days in a solution containing 0.1 M-KCl, 0.01 M-potassium phosphate buffer, pH 7.0, 0.001 M-$MgCl_2$, 0.001 M-NaN_3, with no change in microscopic appearance.

In Plate 1 the protein compositions of Z-disc preparations and extracts are compared with that of the whole myofibril. The proteins in the gel of the myofibril (Plate 1*a*) were identified by comparing the mobilities of the bands with those of purified proteins. The band in Plate 1 (a) with slightly lower mobility than actin is a protein of subunit mol.wt. 55000 which is bound to actin in insect muscle (Bullard *et al.*, 1973). This protein does not occur in the Z discs. The proteins of the Z disc with the same subunit weight as proteins in the myofibril were assumed to be the same protein.

The protein composition of six washed Z-disc preparations was similar to that shown in Plate 1(b). Z discs contained proteins of subunit weight 35000, 42000, 95000 and 105000 and a pair of proteins of about 200000 subunit weight. These correspond respectively to the proteins tropomyosin, actin, α-actinin and paramyosin; the two high-molecular-weight proteins have about the same mobility as myosin. The protein present in the greatest amount is α-actinin. The amount of paramyosin in the isolated Z-disc preparation is small compared with that in the whole myofibril. The proportions of actin and tropomyosin are more nearly equal than in the whole myofibril. Densitometry of gel scans gave a ratio of actin/tropomyosin of about 2:1 (w/v) in Z bands and about 8:1 in myofibrils. The larger of the two high-molecular-weight proteins has the same mobility as myosin. This was shown by lightly loading SDS/5% polyacrylamide gels with Z discs or myofibrils or with both on the same gel. The gels were run until the two high-molecular-weight proteins were well separated. The predominating high-molecular-weight protein in the whole myofibril is myosin, and this coincided with the lower-mobility band in the Z discs. The protein with slightly higher mobility than myosin had a subunit mol.wt. of about 180000. Z-disc preparations that were centrifuged in a sucrose gradient to remove incompletely extracted myofibrils and other non-Z-disc material had a protein composition similar to that of the washed Z discs (Plate 1*e*). However, there was a protein of subunit weight 25000 which was not found before application of the Z discs to the gradient, and this was probably produced by degradation of one of the Z-disc proteins.

Actin and tropomyosin may be present in the fringe on either side of the discs. In an attempt to remove as much as possible of the non-Z-disc proteins, Z discs were extracted twice with KI as described in the Methods section. This resulted in the selective extraction of actin, α-actinin and myosin (Plate 1*d*). At this stage the Z discs appeared paler in the phase microscope, probably owing to the extraction of α-actinin. The composition of the remaining Z discs is shown in Plate 1(*c*). As expected, the proportion of actin, α-actinin and myosin was decreased relative to the other Z-disc proteins. The protein of about 180000 daltons, paramyosin and tropomyosin were still present showing that these proteins are constituents of the Z disc. Whether they are inside the Z disc or tightly bound to the outside cannot be determined from this experiment. In addition to the proteins present before KI extraction there was the protein of subunit mol.wt. 25000, which was probably produced by degradation during long extraction. As this protein does not appear in the KI extract, it must be tightly bound to the Z disc.

Removal of Z lines from the myofibril

Electron micrographs of myofibrils digested by impure lipase with trypsin inhibitor showed selective removal of the Z line (Plate 2). There were disorganized filaments holding the sarcomeres together in the Z-line region. The myosin filaments appeared to be intact, but the H-zone was wider than in untreated myofibrils; this was due to actin filaments being pulled out of the myosin-filament array so that the length of the overlap region was less than in the intact myofibril.

The amount of proteolytic degradation in myofibrils was variable, depending on the time of digestion. Plate 1(*f*) shows a gel from one of the less-degraded cases. The major proteins of the extracted myofibril were the same as those in the intact myofibril, therefore the enzyme acted specifically on a minor protein. Comparison of Plates 1(*a*) and 1(*f*) shows that there is less α-actinin relative to paramyosin in the digested myofibril and the diffuseness of the α-actinin band in the gel of the digested myofibril suggests proteolysis.

Since the digestion of the myofibrils could be so specific for Z lines, it was decided to find what pro-

teolytic fragments were released from the isolated Z discs. After Z discs had been digested by the method used to remove Z lines from myofibrils, no intact discs were seen by phase-contrast microscopy. There was some variation in the extent of degradation of the proteolytic fragments released from the Z discs, but one of the less-digested cases is shown in Plate 1(*g*). The digest contained a large amount of peptide of 60000 daltons, a peptide of 25000 and small amounts of peptides of 105000, 31000, and 17000 daltons. The major peptide of mol.wt. 60000 released by digestion must be derived from a protein of higher molecular weight. Since the major high-molecular-weight protein in Z discs is α-actinin, and this protein is not present in the digest, it is likely that the 60000-mol.wt. peptide is produced by hydrolysis of α-actinin. The 25000-mol.wt. peptide was found in Z discs subjected to centrifugation on a sucrose gradient and in Z discs after extraction with KI; therefore it may be from a Z-band protein that is hydrolysed relatively easily.

Some preliminary attempts were made to find out what enzyme in impure lipase was digesting Z lines. Neither pure lipase nor α-amylase extracted Z lines, and trypsin activity in impure lipase would have been inhibited by trypsin inhibitor; therefore these are not the active enzyme. Chymotrypsin did remove Z lines from myofibrils, and by phase-contrast microscopy the myofibrils without Z lines were indistinguishable from those obtained with impure lipase. SDS/polyacrylamide gels of extracted myofibrils showed as little degradation as was found in the best experiments with impure lipase. These results show that, of the enzymes tried here, the best to use for selective removal of the Z line is chymotrypsin.

Discussion

The protein composition of *Lethocerus* Z discs described here differs from that found by Saide & Ullrick (1974) for bee Z discs. However, as pointed out by these authors, the acid extraction used to prepare bee Z discs might have eluted some of the Z-disc proteins. Of the high-subunit-weight proteins found in bee Z discs, the one of 175000 daltons may correspond to the protein of 180000 daltons in *Lethocerus* Z discs. The other high-subunit-weight proteins were not found in *Lethocerus* Z discs. The high proportion of actin, tropomyosin and α-actinin in *Lethocerus* Z discs is consistent with the structural studies. The relatively high ratio of tropomyosin/actin in the Z line suggests that tropomyosin may be important in forming the Z lattice. The lack of anti-tropomyosin binding to the Z line (Masaki *et al.*, 1967) may be due to masking of tropomyosin by α-actinin.

The minor components of the Z-disc preparations, paramyosin and myosin, are probably derived from remains of thick filaments bound to the Z line, and are likely to be associated with the surface of the Z discs. The protein of subunit weight 180000 is unidentified. This protein was resistant to extraction from the Z disc and may be an integral part of the lattice. The amino acid analysis of whole Z discs from bee muscle (Saide & Ullrick, 1974) shows that the Z discs of these insects have a proline content of 82 residues/1000 residues. This is considerably higher than the proline content of any of the Z-disc proteins which we have identified: actin, with 50 residues/1000, has the largest amount of proline. This strongly suggests that there is an unidentified Z-disc protein with an exceptionally high proline content.

An actin-binding protein, which binds to membranes, has been isolated from alveolar macrophages (Stossel & Hartwig, 1975) and *Amoeba castellanii* (Pollard, 1975). This protein has a subunit weight somewhat greater than myosin and is characterized by an unusually high proline content (71 residues/1000 residues; Stossel & Hartwig, 1975). The insect Z line develops from the cell membrane (Auber, 1969) and it is not unreasonable to suppose that a high-molecular-weight protein like the actin-binding protein, usually associated with membranes, may be in the Z line. The 180000-mol.wt. protein may be similar to the actin-binding protein, although its subunit weight is somewhat lower, and it may bind to actin filaments in the Z line. To account for the high proline content of insect Z lines the 180000-mol.wt. protein would have to have more proline than the actin-binding protein. An alternative explanation for the high proline content of Z lines is that there may be a Z-line protein not identified because it has the same mobility on SDS/polyacrylamide gels as one of the main myofibrillar proteins. This could be resolved by amino acid analysis of the individual Z-disc proteins.

The enzymic removal of the *Lethocerus* Z line by impure lipase with added trypsin inhibitor differs from the result of Goll & Reedy (cited by Stromer *et al.*, 1969) who found that impure lipase did not remove the Z line from rabbit striated muscle in the presence of trypsin inhibitor. This may be due to a difference in the susceptibility to enzymes of insect and vertebrate Z lines.

Enzymic digestion of the Z line leaves a myofibril that is held together by a disordered network of filaments in the Z-line region, without the dense material. These filaments are likely to be actin. The isolated Z disc disintegrates when incubated with the same enzyme, therefore the protein digested by the enzyme binds together the components of the Z disc. The isolated Z disc contains actin, and α-actinin has been shown to cross-link actin filaments (Goll *et al.*, 1972) also the major peptide released by digestion of Z discs is probably derived from α-actinin, therefore it is

likely that α-actinin is a cross-linking protein hydrolysed by the enzyme.

One of the proteins of the isolated Z discs probably binds the myosin filaments to the Z line. Tropomyosin, actin and α-actinin are unlikely to have this function, as they do not bind irreversibly to myosin or paramyosin under physiological conditions. But the 180000-mol.wt. protein may possibly be the linking protein.

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