Biochemical and Ultrastructural Aspects of M_r 165,000 M-Protein in Cross-striated Chicken Muscle

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ABSTRACT   To better understand the relationship between the M_r 165,000 M-line protein (M-protein) and H-zone structure in skeletal and in cardiac muscle, as well as the possible interaction of M-protein with another skeletal muscle M-line component, the homodimeric creatine kinase isoenzyme composed of two M subunits (MM-CK), we performed biochemical, immunological, and ultrastructural studies on myofibrils extracted by different procedures.

In contrast to MM-CK, M-protein could not be completely removed from myofibrils by low ionic strength extraction. Fab-fragments of antibodies against M-protein could not release M-protein quantitatively from either breast or heart myofibrils but remained bound to the myofibrillar structure, whereas monovalent antibodies against MM-CK cause the specific release of MM-CK and the concomitant disappearance of the M-line from chicken skeletal muscle myofibrils. When MM-CK was removed from skeletal myofibrils by low ionic strength extraction or, more specifically, by incubation with anti-MM-CK Fab, M-protein was still not released quantitatively upon treatment with anti-M-protein Fab as judged from immunofluorescence data. In the ultrastructural investigation of low ionic strength extracted muscle fibers, M-protein could be localized in two stripes on both sides of the former M-line, suggesting a reduced attachment to the residual H-zone structure, whereas the specific removal of MM-CK resulted in the same dense staining pattern for M-protein within the M-line as observed in untreated fibers. However, the binding of M-protein to the residual M-line structure seemed to be reduced, as a considerable amount of this protein could be identified in the supernate of sequentially incubated myofibrils. The results indicate a strong binding of M-protein within the H-zone structure of skeletal as well as heart myofibrils.

The M-line, the electron-dense region running transversely through the middle of the H-zone of vertebrate skeletal muscle, has been the object of rather intensive investigation during recent years. Two approaches, namely biochemical and ultrastructural analyses, have been used to obtain more detailed information on the composition, structure, and function of the M-line.

M-line material has been isolated either from low ionic strength extracts known to remove mainly the electron-dense M-line material (2, 17, 38), or from high ionic strength extracts that, in addition, solubilize the whole A-band (15). Among several proteins extracted by either of these procedures (4, 8, 22-25, 40, 41), only the homodimeric creatine kinase isoenzyme composed of two M subunits (MM-CK, M_r = 88,000, dimer)

(25, 41) and an M_r 165,000 (5S) protein designated now as M-protein (40), besides myosin, probably represent "true" M-line constituents.

Based on recent and refined electron microscope investigations, models for the structure of the M-line in skeletal muscle have been described (16, 20). High resolution electron microscopy using ultrathin cryosections showed that the M-line is actually composed of substriations, with the number of striations varying among different muscle types (32, 33). Three structural elements have been identified: primary and secondary (Y-shaped) M-bridges and M-filaments (14, 16, 20, 27, 33, 39). Only vague notions exist on the function of this complex structure running across the sarcomere at the height of the bare zone of thick myosin filaments (6, 10, 21). To obtain more

insight into this problem it would be of importance to learn more on the common features that make up the several structural components of the M-line region of the different types of muscle fibers. The relationship, however, between the biochemical data and the ultrastructural results causes considerable difficulties in interpretation at present.

To close this gap, a further approach was undertaken to integrate biochemical and immunological data with those obtained from ultrastructural studies. Two unexpected results opened new possibilities for such an approach: (a) M-protein was found to be localized in the middle of the H-zone in chicken cardiac myofibrils (37), organelles that lack an electron-dense M-line (35, 42) and from which MM-CK, another skeletal muscle M-line protein, is absent (3, 42). This opens an interesting aspect of H-zone (M-line) structure in different types of muscles and we therefore decided to investigate skeletal as well as cardiac muscles. (b) Monovalent antibodies against MM-CK (a homodimeric creatine kinase isoenzyme composed of two M subunits) specifically and quantitatively extract the myofibrillar-bound MM-CK and concomitantly remove the electron-dense M-line from skeletal muscle (43).

In the present work four main approaches were undertaken: (a) The selective extraction (low ionic strength) of the M-line proteins (MM-CK and M-protein) with respect to their relative binding affinities to the M-line region was studied. (b) The question was examined of whether M-protein is selectively extracted by incubation of the myofibrils with monovalent antibodies against M-protein. (c) The question was examined of whether the specific removal of MM-CK from skeletal muscle myofibrils by Fab-fragments of antibodies directed against MM-CK affects the binding of M-protein to the M-line structure. (d) The extractability of MM-CK after incubation of skeletal muscle myofibrils with antibodies (or Fab-fragments thereof) against M-protein was also studied.

MATERIALS AND METHODS

Chicken breast and heart muscle was obtained from Kneuss, Mägenwil, Switzerland.

Buffers

Washing buffer: 0.1 M KCl, 5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), pH 7. Low ionic strength buffer: 5 mM Tris-HCl, 1 mM DTT, pH 7.7. High ionic strength buffer: 0.6 M KCl, 1 mM EDTA, 1 mM MgCl$_2$, 10 mM Na$_4$P$_2$O$_7$, 0.3 mM DTT, 0.1 M potassium phosphate buffer, pH 6.4. Phosphate-buffered saline (PBS): 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.2. Boric saline buffer: 0.15 M NaCl, 0.02 M boric acid, pH to 8 with NaOH.

Purification of the Proteins

All solutions also contained 10^{-6} M Pepstatin (Sigma Chemical Co., St. Louis, Mo.) and 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) as protease inhibitors. M-protein, debranching enzyme, and phosphorylase b were purified from chicken breast muscle as described previously (37, 40).

Homogeneous debranching enzyme as well as phosphorylase b were either obtained in the course of the M-protein preparation after the DEAE-cellulose step (40) or isolated separately according to earlier published procedures (8, 9). The homogeneity of the proteins is illustrated in Fig. 1.

Protein Determination

The protein concentrations were determined using the biuret method (19) or calculated for the purified proteins from their A$_{280nm}^{1\%}$: M-protein, 12.2 (40); debranching enzyme, 17.8 (9); and phosphorylase b, 13.2 (8).

Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (NaDodSO$_4$) was carried out according to Laemmli (18).
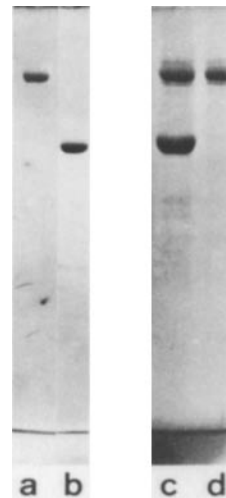

FIGURE 1 8% Polyacrylamide slab gels in the presence of 0.1% NaDodSO$_4$ of: (a) glycogen-debranching enzyme (2 µg), (b) phosphorylase b (3 µg), (c) before 5'-AMP-Sepharose 4B column purification (M-protein + phosphorylase b) (8 µg), and (d) M-protein (breakthrough of the 5'-AMP-Sepharose 4B column) (4 µg).

Preparation and Purification of the Antibodies

Rabbit antiserum against chicken MM-CK, prepared as described (28) was kindly provided by Dr. M. Caravatti. Antisera against purified chicken breast muscle M-protein (37) and debranching enzyme (isolated as described by Trinick and Lowey [40]) were elicited in rabbits. 0.3 mg of debranching enzyme, dissolved in 0.5 ml of 50 mM Tris-HCl, 1 mM EDTA, 0.3 mM DTT, 0.1 mM PMSF, 10^{-6} M Pepstatin, pH 7.9, were diluted with 1.5 ml of 0.9% NaCl and emulsified with 2 ml of complete or incomplete Freund's adjuvant, and the animals were immunized as described for the M-protein (37). The antisera against M-protein and debranching enzyme were tested by Ouchterlony double immunodiffusion (26). Preimmune serum (control) was taken from the rabbits before immunization. The IgG fraction of preimmune serum was prepared by ammonium sulfate fractionation and DEAE-cellulose chromatography (34). The purification of the antisera against MM-CK and M-protein was done by antigen-affinity chromatography as described (28). The affinity matrices were prepared by covalently linking the purified antigens to commercially available CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.).

Preparation of the Fab-Fragments

Aliquots of the affinity-purified antibodies as well as the control IgG were digested with papain (Worthington Biochemical Co., Clifton, N. J.) in the presence of 10 mM cysteine (29). The reaction was stopped by the addition of iodoacetamide (final concentration, 23 mM). The Fab preparation was dialyzed against boric saline buffer for 12 h at 0°C, any precipitates removed by centrifugation, and the supernate dialyzed against washing buffer (12 h, 0°C). The Fab-fragments were concentrated by vacuum dialysis and stored frozen at −20°C.

Extraction of Isolated Myofibrils

Myofibrils from adult chicken breast and heart muscles were prepared according to Kundrat and Pepe (17) with the precautions given by Heizmann et al. (7).

0.5 ml of a suspension (~20 vol/wt) of well-washed breast or heart muscle myofibrils were centrifuged for 1 min in a microcapillary centrifuge and the pellet resuspended and extracted in 0.5 ml of either low or high ionic strength buffer at 4°C for the time indicated in the text. After extraction, the suspension was centrifuged for 1 min (low-ionic-strength-extracted myofibrils) or for 5 min (high-ionic-strength-extracted myofibrils) in the microcapillary centrifuge. The supernate was either directly analysed by gel electrophoresis or Ouchterlony double immunodiffusion, or stored at −20°C. The pellet was washed extensively and then stored at 4°C in washing solution until use.

Incubation of Myofibrils with Antibodies and Indirect Immunofluorescence Localization

Isolated myofibrils were incubated at 4°C for different times (see text) in 0.5

ml of either specific or control IgG (or Fab) at the concentrations indicated in the figures. After incubation the myofibrillar suspensions were centrifuged for 1 min in the microcapillary centrifuge. Proteins in the supernates were precipitated with 2 ml of cold (−10°C) acetone and centrifuged at −10°C for 15 min at 20,000 g; the pellets were dissolved in 40 μl of NaDodSO₄ (1%) sample buffer (18) and separated by polyacrylamide slab gel electrophoresis.

The myofibrils were washed thoroughly with washing solution and processed for the indirect immunofluorescence localization as described elsewhere (44). Fluorescein-conjugated goat anti-rabbit IgG (Grand Island Biological Company, Grand Island, N. Y.) was diluted 1:100 in washing solution.

Immunoreplica Gels

Antigens separated on polyacrylamide (8%) slab gels in the presence of 0.1% NaDodSO₄ were identified by the immunoreplica technique (31). Antisera were diluted 1:2 to 1:3 in the 0.6% agarose overlay gel. After incubation at 37°C for 4–12 h in a humidity chamber, the overlay gel was removed from the slab gel and washed for ~3 d in PBS. Immunoprecipitates were stained for 3–5 min with 0.05% Coomassie Brilliant Blue R in 50% methanol/10% acetic acid.

Preparation of Fiber Bundles

A small fiber bundle (2 mm × 20 mm) was untied from the breast muscle, bound under stretching to a toothpick and then excised from the muscle. To make the cell membranes permeable and to extract the cytoplasm, the fiber bundles were glycerinized by alternating washing buffer which contained 70% glycerol and washing buffer without glycerol every 3 h for 24 h. Fiber bundles could be stored at −20°C in the glycerol solution. Before use the fiber bundles were extensively washed to remove the glycerol. To prevent contraction the muscles were chemically fixed in their stretched state by treatment with 2.5% glutaraldehyde for 5 min in washing buffer, then removed from the toothpicks, and teased in very small fiber bundles by means of an injection needle.

Extraction of Muscle Fiber Bundles

The extractions were performed on glycerinized but not pre-fixed muscles that still were tied onto the toothpicks. For low salt extractions, the muscle fibers were incubated in low ionic strength buffer for 24 h. Extractions with the Fab-fragments of anti-MM-CK IgG were performed for 48 h using the antibody (0.1 mg/ml) in washing buffer; after this extraction the fibers were extensively washed to remove remaining Fab-fragments. The extracted fiber bundles were pre-fixed and teased in pieces as already described.

Antibody Incubation

The small fiber bundles were incubated for 24 h with either specific or control antibodies (IgG or Fab), using a concentration of ~1 mg/ml. After the incubation, the muscle fibers were washed extensively (1 h, three changes of buffer) to remove the unbound antibodies.

Electron Microscopy

The incubated fiber bundles were fixed again for 30 min with 2.5% glutaraldehyde in washing buffer, and washed and postfixed for 5 min with 1% OsO₄ in washing buffer. The fixed material was dehydrated in ethanol and aceton and embedded in Epon 812. Longitudinal sections (thickness, ~50 nm) were cut with an Ultrotome (LKB Instruments, Inc., Rockville, Md.) poststained with 2% uranylacetate in H₂O for 45 min, followed by lead citrate for 15 min. A Siemens Elmiskop 102 electron microscope was used at 100 kV.

RESULTS

Characterization of the Antisera

The antiserum against MM-CK has been characterized earlier (28, 44). The antisera against M-protein and against debranching enzyme showed both a single precipitin line when tested against their homologous antigens on Ouchterlony double immunodiffusion tests (Fig. 2a and b). No cross-reaction could be observed of the anti-M-protein serum with purified glycogen-debranching enzyme and phosphorylase b, the two most likely contaminants of the M-protein preparation (Fig. 2a).

The identity of the myofibrillar 7S protein (40) and glycogen

debranching enzyme (9), both isolated from chicken muscle, has been proven earlier (9, 40). This result is further confirmed by the cross-reaction of the anti-7S-protein serum with debranching enzyme isolated by a different procedure (9) (Fig. 2c). The precipitin lines fuse completely without the formation of any spurs.

M-protein and debranching enzyme are hardly distinguishable in the Laemmli gel system (40). Thus the immunoreplica technique is the method of choice for their clear identification. The ability of this method to distinguish between the two protein species is demonstrated in Fig. 3. An overlay gel containing anti-M-protein serum gives a precipitin line only at the location of M-protein but not at that of debranching enzyme (Fig. 3, overlay A), whereas an overlay gel containing
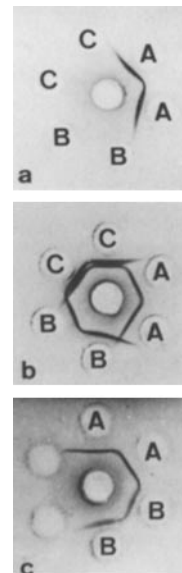

FIGURE 2 Ouchterlony double immunodiffusion tests. Center wells: a, anti-M-protein serum (30 μl); b, anti-M-protein serum (15 μl) + anti-debranching enzyme serum (15 μl); c, anti-debranching enzyme serum (30 μl). Peripheral wells: a: A, purified M-protein (30 μl); B, debranching enzyme (30 μl); C, phosphorylase b (30 μl). b: A, M-protein (30 μl); B, debranching enzyme (30 μl); C, mixture of M-protein and debranching enzyme (15 μl each). c: A, Mᵣ 165,000 (7S) protein (30 μl); B, glycogen-debranching enzyme purified according to reference 9 (30 μl). The concentration of the antigens was 0.3 mg/ml.
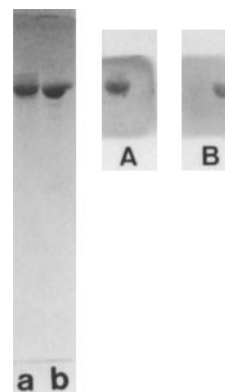

FIGURE 3 NaDodSO₄-polyacrylamide (8%) slab gel electrophoresis of M-protein (9 μg) (a) and glycogen-debranching enzyme (5 μg) (b) and an immunoreplica of a part of the same gel overlaid with an agarose gel containing anti-M-protein serum (A) or anti-debranching enzyme serum (B).

the antiserum against debranching enzyme reacts only at the site of the enzyme (Fig. 3, overlay B).

Localization of the M-Protein in Skeletal and Heart Myofibrils

Purified anti-M-protein IgG bind specifically to the H-zone of breast-muscle myofibrils as shown by the indirect immuno-fluorescence technique (Fig. 4a). A deposit of the antibody can sometimes even be seen in the light microscope; the H-zone of relaxed myofibrils usually represented by a lighter zone within the middle of the dark A-band in phase contrast micrographs appears as a black stripe upon the incubation with anti-M-protein IgG and the fluorescence-labeled second antibody against rabbit IgG (Fig. 4a, phase contrast). The same result is found when chicken cardiac myofibrils, known to lack the electron-dense material within the M-line region (35, 42) are incubated with anti-M-protein IgG (Fig. 4c). Fig. 4b and d shows controls with preimmune IgG. As already reported by Trinick and Lowey (40), ultrastructural investigations of breast muscle show that antibodies against M-protein bind to that region of the H-zone where the M-line is localized (data not shown).

Measurements on our electron micrographs indicate that the dimension of the decorated M-region in anti-M-protein-treated breast muscle is broader than in the control muscle, having an average width of 63 nm, whereas the dimension of the controls is ~41 nm. This difference is not only the result of the binding of IgG molecules to the outermost margin of the real M-line, because incubation with anti-MM-CK IgG does not essentially broaden the M-line region. This phenomenon, together with the fact that chicken heart muscle does not show an electron-dense M-line but nevertheless contains M-protein could be explained by assuming that the M-protein is not responsible for the bulk of the electron-dense material making up the M-line structure.

Extraction of M-Protein from Myofibrils

Low ionic strength buffers have been reported to extract M-line material rather specifically (2, 17, 38). After low-salt

extraction (5 mM Tris-HCl, pH 7.7) for ~1 h the myofibril-bound MM-CK activity was released virtually quantitatively (44). This result was confirmed by the absence of immunofluo-rescent staining because of the total lack of binding sites for anti-MM-CK antibodies within the H-zone of extracted my-ofibrils (44).

Most of the M-protein, however, was still bound after incu-bation of skeletal as well as cardiac myofibrils in low ionic strength buffer. This is shown in Fig. 5. Even after 3 d of extraction the myofibrils still showed a regular fluorescence pattern predominantly within the H-zone (Fig. 5 a and b). In addition, the low salt extraction procedure did not result in a significant deterioration of myofibrillar structures (Fig. 5 a and b; phase contrast).

In contrast, high ionic strength extraction (0.6 M KCl, 0.1 M phosphate buffer, pH 6.4), also used for the isolation of M-line proteins (40), resulted in considerable structural damage of the myofibrils after 1 h of extraction (not shown). High salt extrac-tion solubilizes the whole A-band (15) and, as expected, causes the release of M-protein and most other structural components from myofibrils. The supernates of the low and high ionic strength extracts as well as the myofibrillar pellets obtained after centrifugation were directly analyzed for M-protein by polyacrylamide gel electrophoresis in the presence of Na-DodSO₄ in combination with the immunoreplica technique. Washing solution did not remove significant amounts of M-protein from myofibrils (Fig. 6a). Low salt buffer does extract some M-protein (Fig. 6b and c); but the bulk remains bound to the myofibrils, as a considerable amount of M-protein can be shown to remain in the pellets (Fig. 6h and i). High salt buffer causes the quantitative release of M-protein (Fig. 6d and e) even after rather short incubation times; only traces of the M-protein remained within the pellets (Fig. 6k and l).

Ultrastructural Appearance of Low-ionic-strength-extracted Breast Muscle Fibers upon Incubation with Antibodies Against M-Protein

After a 24-h treatment of breast muscle fibers with low ionic strength buffer, the visible electron-dense M-line is completely removed (Fig. 7a). When low-ionic-strength-extracted muscle fibers were fixed for 5 min with 2.5% glutaraldehyde in washing buffer (to prevent a further release of proteins) and then incubated for 24 h with anti-M-protein IgG, the antibody deposit showed a characteristic pattern: the marginal zones of the marked M-bands were heavily stained, whereas the middle part seemed to be brighter (Fig. 7b). Incubation with the much smaller Fab-fragments of the anti-M-protein IgG showed this characteristic staining pattern very clearly (Fig. 7c).
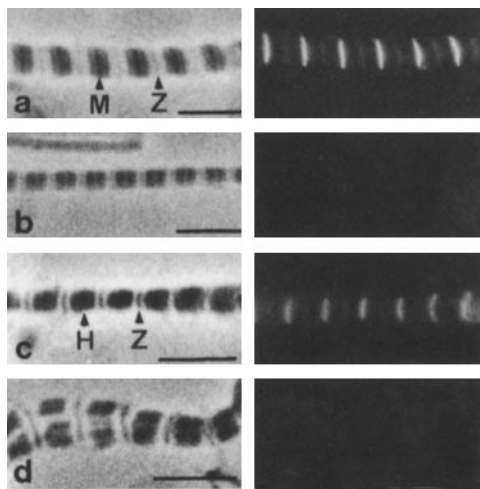

FIGURE 4 Localization of M-protein in isolated chicken breast (a and b) and heart (c and d) muscle myofibrils. a and c, incubation (1 h) with anti-M-protein IgG (10 μg/ml). b and d, incubation (1 h) with preimmune IgG (20 μg/ml). The exposures on the left show phase-contrast and on the right fluorescence micrographs of the same myofibrils. M, M-line; Z, Z-line; H, H-zone. Bar, 5 μm.
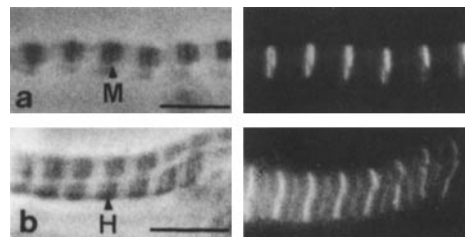

FIGURE 5 Localization of M-protein in previously extracted chicken breast (a) and heart (b) muscle myofibrils. Extraction of myofibrils was performed for 72 h in low ionic strength buffer before incuba-tion with anti-M-protein serum (diluted 1:100 in washing buffer). M, M-line; H, H-zone. Bar, 5 μm.
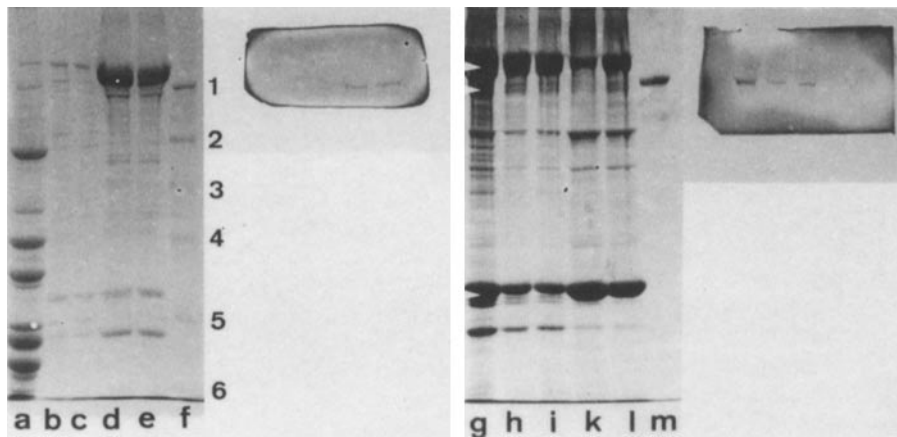
FIGURE 6   NaDodSO₄-polyacrylamide (8%) slab gel electrophoresis of the supernates (lanes *a-e*) and the pellets (lanes *g-l*) of incubations with different solutions of chicken breast muscle myofibrils and identification of M-protein by immunoreplication. Lanes *a-e*: supernates after incubation of myofibrils with washing solution (first wash, 5 min) (*a*); low ionic strength buffer (1 h) (*b*); low ionic strength buffer (72 h) (*c*); high ionic strength buffer (1 h) (*d*); high ionic strength buffer (72 h) (*e*). Lane *f*: M_r marker proteins (approximate values: *1*, chicken debranching enzyme, 165,000; *2*, rabbit muscle phosphorylase *b*, 94,000; *3*, bovine serum albumin, 68,000; *4*, rabbit muscle pyruvate kinase, 57,000; *5*, chicken MM-CK, 40,000; *6*, bovine chymotrypsinogen A, 25,000). Lanes *g-l*: Corresponding pellets after incubation of myofibrils with the solutions mentioned above under *a-e*, respectively. Lane *m*: chicken debranching enzyme (M_r 165,000) as marker protein. Arrowheads indicate the position of myosin heavy chain, M-protein, and actin (from top to bottom). Immunoreplica overlay gels containing anti-M-protein serum and duplicating a part of lanes *a-e* and *g-l* of the polyacrylamide slab gels are shown on the right.
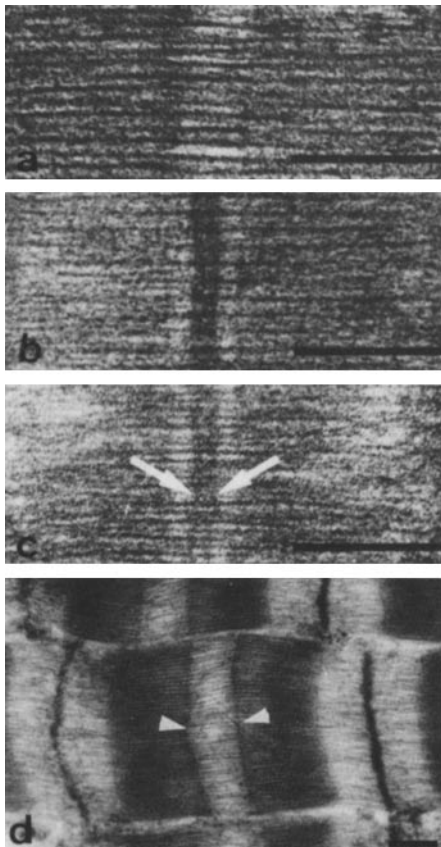

FIGURE 7   Localization of M-protein in low ionic strength extracted breast muscle fibers. *a*, Incubation with preimmune (control) IgG; *b* and *d*, incubation with anti-M-protein IgG; and *c*, incubation with Fab-fragments of anti-M-protein IgG. Arrows, antibody deposit in two stripes within the M-line region. Arrowheads, laterally displaced antibody deposit in fibers with disrupted M-line region. Bars, 0.3 μm.

Frequently in these experiments myofibrils were found whose M-line region was disrupted. The antibody deposit was laterally displaced so that indeed two stripes along the edges of the H-zone became visible (Fig. 7*d*). Possibly the middle part of the M-band sometimes becomes weakened and loses its resistance to mechanical load upon treatment of muscle fibers with low ionic strength buffer.

Incubation of Myofibrils with Monovalent Antibodies against M-Protein

Incubation experiments with monovalent antibodies against MM-CK were originally undertaken to obtain a better resolution for the localization of this protein within the M-line region. Surprisingly, this procedure resulted in the specific and quantitative release of MM-CK and the concomitant disappearance of electron-dense material from the M-line of breast muscle myofibrils (43). Here a similar experiment was performed with monovalent antibodies against M-protein. Incubation of breast or cardiac myofibrils with the Fab-fragments of antibodies against M-protein and then with fluorescence-labeled goat anti-rabbit IgG resulted in a fluorescence pattern (Fig. 8*a* and *c*) similar to the one observed with anti-M-protein IgG (Fig. 4*a* and *c*) showing the specific binding of the anti-M-protein Fab to the middle of the H-zone of the myofibrils. The intensity of the fluorescence after Fab incubation was usually somewhat lower than after incubation with anti-M-protein IgG, possibly because of the smaller number of binding sites of the Fab-fragments for the fluorescence-labeled second antibody. On electron micrographs the antibody deposit was comparable to the staining pattern obtained with the whole IgG molecules, i.e., the resolution of the antigenic structure was not improved after the incubation with the smaller Fab-fragments. Prolonged incubation of the myofibrils with anti-M-protein Fab (up to three days) did not lead to the specific extraction of M-protein and the concomitant loss of fluorescence. The intensity of the
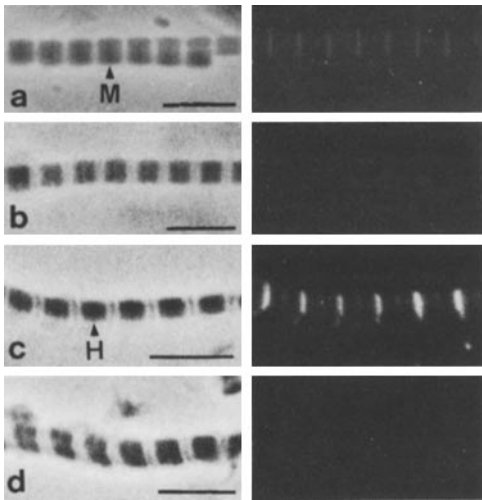
FIGURE 8   Immunofluorescent staining of chicken breast (a and b) and heart muscle (c and d) myofibrils. a and c, Incubation with anti-M-protein Fab-fragments (20 μg/ml, 1 h); b and d, incubation with Fab-fragments of preimmune IgG (20 μg/ml, 1 h). M, M-line; H, H-zone. Bar, 5 μm.
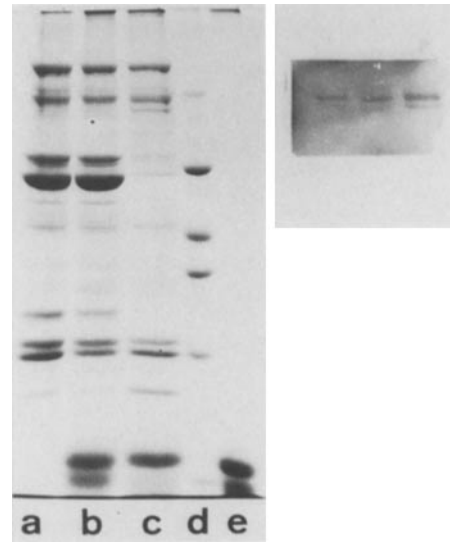

FIGURE 9   NaDodSO₄-polyacrylamide (8%) slab gel electrophoresis of proteins released from breast muscle myofibrils upon different incubations and identification of M-protein by immunoreplication. Lanes a–c, supernates after incubation of myofibrils for 72 h with washing solution (a), Fab-fragments of preimmune IgG (0.1 mg/ml) (b), and anti-M-protein Fab (0.1 mg/ml) (c). d, M_r marker proteins (approximate values, see legend to Fig. 6 f). e, Anti-M-protein Fab-fragments (10 μg). An immunoreplica overlay gel containing anti-M-protein serum and duplicating a part of lanes a–c of the polyacrylamide gel is shown on the right. Multiple bands in the immune overlay result from progressive degradation of M-protein.

staining was, however, further reduced after lengthy incubation with anti-M-protein Fab. Prolonged exposure to washing solution clearly gives rise to some myofibrillar damage and subsequently to the release of protein from the myofibrils. It is, however, also possible that a certain amount of M-protein is specifically solubilized by anti-M-protein Fab when myofibrils are incubated for several hours or days. This is demonstrated in Fig. 9, which shows the release of M-protein from skeletal muscle myofibrils to be slightly weaker after prolonged treatment with washing solution or control Fab than after incubation with anti-M-protein Fab. Quite in contrast, the amount of MM-CK released after 24 h of incubation of breast myofibrils with anti-MM-CK Fab was much higher than that released after incubation with control Fab (43).

Binding of Anti-M-Protein IgG to Myofibrils from Which MM-CK Has Been Selectively Removed

It has been suggested earlier that selective removal of MM-CK from breast muscle myofibrils upon incubation with anti-MM-CK Fab-fragments does not lead to a coextraction of significant amounts of M-protein (43). To prove these findings, skeletal muscle myofibrils were incubated first with anti-MM-CK Fab (which extracts the myofibrillar MM-CK) and then with anti-M-protein IgG. As demonstrated by the indirect immunofluorescence technique as well as by electron micrographs, there was still a perfect binding of the antibody to the M-line region after this pretreatment (Fig. 10). The pattern of antibody staining does not differ from that obtained with unextracted muscle: the whole M-band is uniformly marked and no double line (as found after incubation of low-ionic-strength-treated muscle fibers with anti-M-protein IgG) is recognizable.

Sequential Incubations of Myofibrils with Monovalent Antibodies against MM-CK and M-Protein

That Fab-fragments of anti-M-protein IgG do not remove

their respective antigens (in contrast to anti-MM-CK Fab [43]) might be explained by suggesting that the M-protein is less accessible to the antibody than MM-CK. One could assume that by specific removal of MM-CK the remaining M-protein is more exposed so that it can be more easily extracted by the anti-M-protein Fab-fragments. A sequential treatment of muscle fibers with anti-MM-CK Fab followed by anti-M-protein Fab did not, however, lead to a quantitative removal of the M-protein; there was still a clearly visible antibody deposit within the M-line region (Fig. 10c).

This result was confirmed by immunofluorescence staining of isolated myofibrils treated in the same way. Even when myofibrils were incubated first with low ionic strength buffer (which extracts, in addition to MM-CK, some other myofibrillar proteins [2, 17, 38]), a fraction of the M-protein still remained attached to the M-line region upon incubation with anti-M-protein Fab (data not shown).

NaDodSO₄-polyacrylamide gel electrophoresis in combination with the immunoreplica technique showed, however, that the release of M-protein from myofibrils previously treated with anti-MM-CK Fab was somewhat higher after incubation with anti-M-protein Fab than after treatment with control Fab.

When the sequence of incubations was changed, i.e., when skeletal myofibrils were first incubated with anti-M-protein IgG or Fab and then with anti-MM-CK Fab, the enzyme was still specifically extracted (Fig. 11). The bulky anti-M-protein IgG molecules clearly reduce the accessibility of the structure-bound MM-CK for the anti-MM-CK Fab-fragments, but the amount of MM-CK extracted is still considerably higher than that released after incubation with control Fab (Fig. 11 c and d). In contrast, binding of anti-MM-CK IgG molecules to their respective antigen, MM-CK, strongly reduces the specific extractability of MM-CK by anti-MM-CK Fab (Fig. 11).
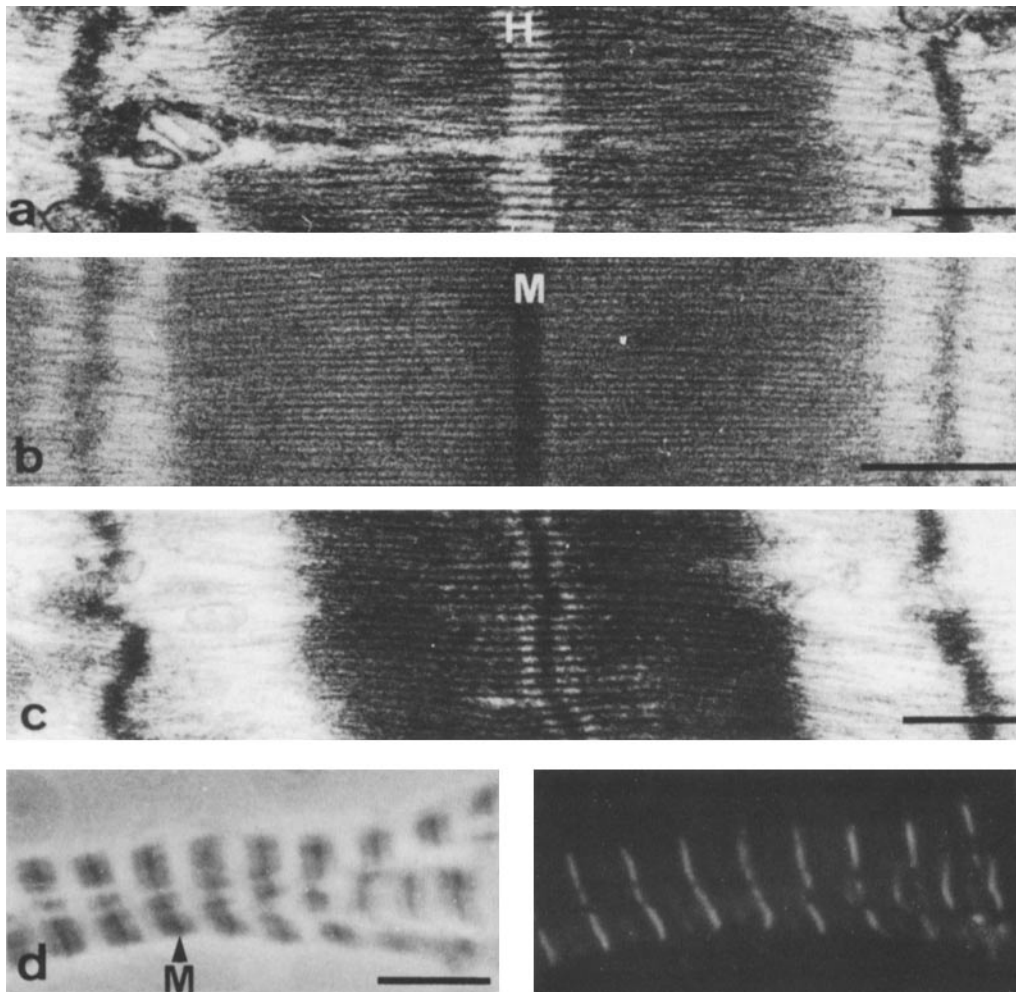
FIGURE 10 Localization of M-protein in chicken breast muscle fibers from which MM-CK has been previously extracted by incubation with anti-MM-CK Fab. a, Extracted fiber; b, incubation with anti-M-protein IgG; c, incubation with Fab-fragments of anti-M-protein IgG; d, immunofluorescence localization of M-protein in isolated myofibrils extracted with anti-MM-CK Fab (24 h, 0.1 mg/ml) followed by incubation for 1 h with anti-M-protein IgG (5 μg/ml). H, H-zone; M, M-line. Bars: a-c, 0.3 μm; d, 5 μm.

DISCUSSION

M-Protein Is an Integral Component of the M-Line Region

In support of previous findings (37, 40, 43), this analysis of the so-called M-line proteins revealed that only two proteins, namely MM-CK and an M_r 165,000 (5S) protein (M-protein) can be considered as "true" M-line components at the present. Recent studies showed that two of the copurified proteins are in fact phosphorylase b (8, 40) and glycogen-debranching enzyme (9, 40), which are, though weakly, bound to the I-band region rather than to the M-line region of skeletal muscle myofibrils (40). In contrast to breast muscle myofibrils, chicken cardiac myofibrils (known to lack a visible M-line [35, 42]) are devoid of MM-CK (the creatine kinase form in chicken heart myofibrils, BB-CK (3), is bound predominantly to the I-band [6, 42]). M-protein, however, could be also detected in chicken heart myofibrils (37). A possible explanation of this fact may well be that the electron-dense M-line, when present, is mostly (if not exclusively) composed of MM-CK. The question of whether M-protein is found in all types of muscle, and in nonmuscular tissue as well where it could be part of intrinsic contractile systems, is under current investigation in our labo-

ratory. Recent evidence obtained from immunofluorescence studies revealed a synchronous appearance of M-protein and other myofibrillar proteins such as myosin and actin in myogenic cell cultures (36). This can be expected for a structural component of the myofibril assumed to be necessary for a proper functioning of muscle contraction. Although M-protein can be localized in chicken gizzard cells, no evidence for its presence in the fibroblasts contaminating the primary skeletal muscle cell cultures can be given.[1] This is in contrast to earlier observations by J. Schollmeier et al. (30).

It has been postulated earlier (6, 44) that MM-CK may have a mixed enzymatic and structural function in certain types of muscles. Only a small fraction of the total cellular MM-CK (~5% [44]) is bound to the myofibrillar structure. In contrast, most, if not all, of the M-protein seems to be structure-bound and not present in the soluble fraction.

Although purified M-protein is soluble in low salt buffer, it is only partially released from skeletal as well as cardiac muscle in this medium, which suggests a strong interaction of M-protein with other structural components present in the M-region. In contrast, MM-CK is quantitatively released from

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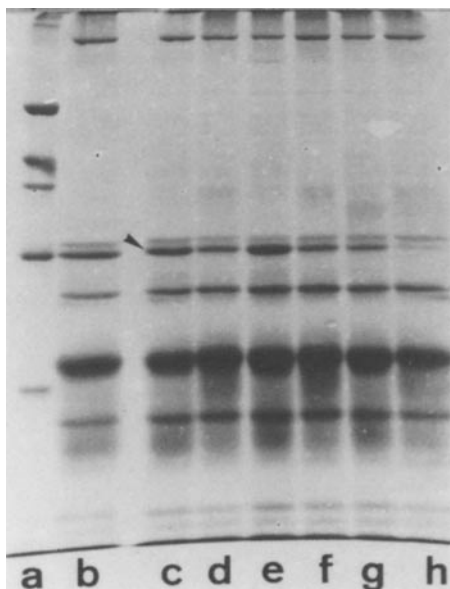[1] H. M. Eppenberger. Manuscript submitted for publication.

FIGURE 11 NaDodSO₄-polyacrylamide (10%) gel electrophoresis. Lane a: M_r marker proteins (approximate values from top to bottom: rabbit muscle phosphorylase b, 94,000; bovine serum albumin, 68,000; rabbit muscle pyruvate kinase, 57,000; chicken MM-CK, 40,000; bovine chymotrypsinogen A, 25,000). Lane b: supernate after incubation of breast myofibrils with anti-MM-CK Fab (24 h, 0.1 mg/ml). Lanes c–h: proteins released from preincubated myofibrils upon treatment with anti-MM-CK Fab (24 h, 0.1 mg/ml) (c, e, and g) and control Fab (24 h, 0.1 mg/ml) (d, f, and h); preincubation (1 h) with anti-M-protein IgG (0.04 mg/ml) (c and d), anti-M-protein Fab (0.1 mg/ml) (e and f), and anti-MM-CK IgG (0.1 mg/ml) (g and h). Arrowhead indicates position of MM-CK.

skeletal muscle myofibrils under the same conditions (44). Moreover, monovalent antibodies against MM-CK cause the specific release of MM-CK and the concomitant disappearance of the electron-dense M-line from myofibrils. This specific extractability of MM-CK is not lost when the myofibrils have been previously incubated with anti-M-protein IgG or Fab, suggesting different binding sites for MM-CK and M-protein within the M-line region. On the other hand, anti-M-protein Fab were not able to solubilize M-protein quantitatively, as there was still antibody staining after this kind of incubation.

The strong attachment of M-protein within the M-line region was further indicated by the finding that the removal of MM-CK and of most electron-dense M-line material did not significantly alter the binding of anti-M-protein antibodies to their respective antigenic sites. It cannot be excluded, however, that the extractability of M-protein is enhanced after removal of the myofibrillar MM-CK.

The data suggest that the removal of a part of the M-region by extracting MM-CK does not affect the interaction of M-protein with the residual myosin filaments or possible other structures in that region of myofibrils. A conformational change of M-protein after the loss of a possible interaction with MM-CK or any other deterioration of the binding sites for M-protein within the M-line region would, however, not be detectable with the methods applied in this work.

The M-line Region in Chicken Breast Muscle

The primary M-bridges lying in register perpendicular to the myosin filaments have been shown to be arranged in three to five vertical arrays on electron micrographs of ultrathin cry-

osections (32, 33) and are believed to make the principal contribution to the electron-dense material forming the M-line. That the visible M-line disappears upon the specific extraction of MM-CK by anti-MM-CK Fab (43) thus gives strong evidence for the association of MM-CK with the primary M-bridge architecture.

After incubation of chicken breast muscle fibers with anti-MM-CK IgG, the deposit of electron-dense material within the M-lines can be clearly seen (43); however, the width of these "stained" M-lines usually does not exceed the corresponding value of untreated fibers, thus further supporting a possible role of MM-CK in building up the primary M-bridges. A third argument for an association of MM-CK with the primary M-bridges comes from independent biophysical studies showing that an interaction between MM-CK and myosin, especially the rod portion, occurs in vitro (1, 12).

On the other hand, incubation of chicken breast muscle fibers with anti-M-protein IgG results in a significant broadening of the M-lines (average width, ~60 nm). The appearance of the anti-M-protein-stained "M-lines" is very regular, with a sharp borderline on both sides. M-protein has been recently shown to form a complex with subfragment 2 (21), the flexible hinge region of the myosin molecule (11). As the subfragment 2 portions of the myosin molecules are assumed to begin at the edge of the bare zone, because of the antiparallel packing of myosin filaments in the middle of the A-band (13), an interaction of M-protein with myosin beyond the electron-dense M-line is well possible.

Whether M-protein makes some principal contribution to the M-filaments or to the secondary M-bridges is not yet known. An association, however, of M-protein with the secondary M-bridges is unlikely, because these structural features have been suggested to interconnect the M-filaments at a defined region (M3, possibly also M1 [20]) lying inside the visible M-line (20) and are not thought to interact directly with myosin.

An interpretation of the results obtained from localization studies presented here can only be speculation at the present time. Extraction with low ionic strength buffer might remove, besides MM-CK, one (or more) additional true M-line components, e.g., also a certain amount of M-protein. The two distinct lines seen at the edge of the M-band after incubation of low-ionic-strength-extracted myofibrils with anti-M-protein Fab (or even with the larger IgG molecules) would then represent staining of the remaining M-protein still sticking to the myosin filaments on both sides of the extracted M-line. In contrast, extraction with anti-MM-CK Fab removes only MM-CK, so that specific staining for M-protein could still occur throughout the whole M-line region.

The M-Line Region in Chicken Heart Muscle

It has been known for some time that chicken cardiac myofibrils lack a visible electron-dense M-line (35, 42). As yet, however, there has been no detailed ultrastructural investigation of the H-zone of this type of muscle. Biochemical and immunological studies showed that BB-CK, not MM-CK, is found in chicken heart muscle (5); BB-CK is, however, bound to the I-band rather than to the H-zone of chicken cardiac myofibrils (42). The absence of MM-CK from chicken heart myofibrils lacking a visible M-line should probably be expected, as MM-CK seems to contribute to most of the M-line electron density in other types of muscles.

There is evidence from our recent work (37) for the existence

of M-protein at the H-zone of chicken heart muscle myofibrils. Unfortunately it is not known whether these myofibrils contain structural elements resembling "M-bridges" and "M-filaments." From our data one could, as a working hypothesis, postulate the absence of primary M-bridges (because of the lack of electron density [and MM-CK] in chicken hearts), although the existence of structural features such as secondary M-bridges and M-filaments cannot be excluded. Ultrastructural studies on ultrathin sections of untreated chicken cardiac myofibrils are certainly needed to test this hypothesis.

Function of the M-line

After it is recognized that chicken heart muscle does not show an electron-dense M-line but, despite this, contains at least one typical "M-protein" (the M_r 165,000 M-protein) one should modify the meaning of the term "M-line" to some extent. In this paragraph we shall therefore refer to M-line as the total of structural features within the middle of the H-zone, regardless of whether or not these structures are visible as a line in the electron microscope. It has been suggested that holding the myosin filaments in proper register might be the general function of the M-line (6, 10, 21). But what is the special function of each of the structural components of the M-line in different types of muscles? There are muscles probably lacking primary M-bridges (e.g., chicken heart) and muscles differing in the number of primary M-bridge arrays (e.g., type I and II fibers from human m. tibialis anterior [33]). The finding that number, density, and width of the M-bridge lines is a characteristic of species, muscle, and fiber type (32) suggests a connection between the presence of M-bridges and some specific needs of a given muscle type. If, as we propose, MM-CK makes the principal contribution to the primary M-bridges, this would not be unreasonable because, depending on the type of contraction and thus on the mechanism of energy supply (i.e., also on the amount of glycogen and mitochondria), the presence or absence as well as the amount of MM-CK within the M-line could provide an effective mechanism for the regulation of ATP production. It would be of great interest to know whether continuous electrical stimulation of isolated muscle fibers in a defined manner results in a change of the M-line pattern in accordance with the new mode of contraction. Besides this, a structural role for the primary M-bridges must not be excluded; the degree of order required within the H-zone might also depend on the type of muscle.

The function of secondary M-bridges and M-filaments is not clear; if their distribution is ubiquitous in myofibrillar systems, they could well be part of a basic structure responsible for the "proper" arrangement of thick filaments in the H-zone of myofibrils.

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