Proc. Natl. Acad. Sci. USA
Vol. 75, No. 9, pp. 4296–4300, September 1978
Biochemistry

Monovalent antibodies against MM-creatine kinase remove the M line from myofibrils

(Fab' fragments/M bridges/immunofluorescence/electron microscopy/immunoreplica)

THEO WALLIMANN*, GUDRUN PELLONI†, DAVID C. TURNER†, AND HANS M. EPPENBERGER†

* Department of Biology, Brandeis University, Waltham, Massachusetts 02154; and †Institute of Cell Biology, Swiss Federal Institute of Technology, Hoenggerberg, CH-8093 Zurich, Switzerland

Communicated by William F. Harrington, June 6, 1978

ABSTRACT **Column-purified antibodies against creatine kinase (EC 2.7.3.2) from chicken skeletal muscle (the homodimeric isoenzyme designated MM-CK) bind specifically to the M lines of chicken pectoral muscle myofibrils. Incubation of myofibrils with monovalent Fab' fragments of these antibodies solubilizes most of the myofibril-bound creatine kinase, concomitantly removing most of the electron-dense material from the M lines. This strongly indicates that MM-CK is an integral part of the M-line structure and is consistent with the suggestion that MM-CK molecules form the M bridges that are responsible for the principle M-line substriations.**

The M line in vertebrate striated muscle runs transversely through the middle of the A band. Electron microscopy has revealed considerable structural complexity in this part of each sarcomere. Cross sections through the M line show a network of transverse elements, called (primary) M bridges (1, 2). Each of the three (or five, depending on fiber type) principal substriations seen in longitudinal sections corresponds to an array of M bridges (1–3,‡). M bridges apparently do not interconnect thick filaments directly but instead link each filament to six longitudinal elements called M filaments (1, ‡). Each M filament is apparently joined to four other M filaments by secondary M bridges, and there is evidence for still other transverse elements.‡ The protein composition of these different M-line elements is unknown.

Low ionic strength buffers extract much, if not all, of the electron-dense interfilament material from the M line (4, 5). Two of the proteins found in low ionic strength extracts are glycogen phosphorylase (subunit M_r 100,000) and glycogen debranching enzyme (subunit M_r 165,000) (6, 7); contrary to earlier suggestions (6), it now appears that neither of these proteins is a structural component of the M line (7). Another protein with M_r 165,000, probably identical to the "M protein" purified from high-salt extracts (7), has been detected in low ionic strength extracts (8). Antibodies (IgG or Fab') to M protein have been shown to bind to the M line and it is thought that M protein is a constituent of the M line (7). A fourth protein found in low ionic strength extracts is a dimer composed of identical subunits of M_r 40,000 (5). Preincubation of myofibrils with antibodies against this protein prevents M-line extraction and leads to a thickening of the M line (5). Morimoto and Harrington (5) noted that the apparent dimensions of this protein fit reasonably well with those of the M bridges. We have shown (9, 10) that this smallest of the M-line proteins possesses creatine kinase (CK; ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) activity and that, by a number of criteria, this myofibrillar CK is indistinguishable from the bulk "soluble" muscle isoenzyme, the CK homodimer composed of two M subunits (MM-CK). The number of CK molecules firmly bound to myofibrils appears to be roughly equal to the number of M bridges (10). Indirect immunofluorescent staining with specific antiserum elicited against purified "soluble" MM-CK confirmed that MM-CK is specifically located in the H zone (10).

In this paper we show that incubation of fiber bundles with Fab' fragments of monospecific anti-MM-CK IgG leads to the specific removal of both the greater part of the electron-dense material in the M line and almost all myofibril-associated CK activity. This is a strong indication that MM-CK is an integral M-line component—i.e., one essential to the structure of the M-bridge arrays that are responsible for the principal M-line substriations.

MATERIALS AND METHODS

Monospecific Antibody. The rabbit anti-chicken MM-CK antiserum used was the one described previously (10). IgG fractions of both rabbit preimmune serum (control) and specific anti-MM-CK serum were prepared by ammonium sulfate fractionation and DEAE-cellulose chromatography (11). The anti-MM-CK IgG fraction was further purified by antigen-affinity chromatography (12) to yield monospecific IgG.

Fab' Fragment Preparation. Samples of monospecific IgG and control IgG were subjected to papain digestion in the presence of 10 mM cysteine (13). Digestion was stopped by addition of 20 mM iodoacetamide. After dialysis against solution A (0.1 M KCl/1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid/1 mM dithiothreitol/5 mM EDTA, pH 7.0) and removal of a small precipitate of denatured IgG, the Fab' preparation was tested for antibody specificity and stored at −25°.

Electrophoresis and Immunoreplica Technique. Sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide slab gel electrophoresis was carried out according to Laemmli (14). Gels were either stained with Coomassie brilliant blue R or used in the immunoreplica technique (15), in which a 0.6% agarose gel containing anti-MM-CK antiserum (diluted 1:6) was poured on the unfixed slab gel (M. Caravatti and J.-C. Perriard, personal communication). After precipitin lines had formed at sites of antibody–antigen reaction, the overlay gels were washed with 0.01 M P_i/0.15 M NaCl, pH 7.0 and stained with Coomassie brilliant blue.

Preparation of Fiber Bundles from Chicken Breast Muscle. Young chickens were killed by cervical dislocation and their breast muscles were immediately removed and placed on ice.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: CK, creatine kinase (EC 2.7.3.2); MM-CK, and MB-CK, homodimeric (two M subunits) and heterodimeric (one M and one B subunit) isoenzymes of CK, respectively; $NaDodSO_4$, sodium dodecyl sulfate.

‡ Luther, P. & Squire, J. (1977) *Proceedings of the 6th Meeting, European Muscle Club*, p. 61 (abstr.).

Small fiber bundles (0.5–1.5 mm in diameter, 2–5 cm long) were dissected out at 4°, tied to a thin rod, and placed in cold solution A containing 50% (vol/vol) glycerol. Rapid rupturing of the sarcoplasmic reticulum was achieved by alternating solution A plus glycerol with solution A alone every 3 hr for 24 hr. These rapidly glycerinated fiber bundles were most often used directly (after removing the glycerol with three changes of solution A over a 3-hr period). However, they could be stored at −25° for several weeks with no apparent structural damage. For one experiment, nonglycerinated fiber bundles were examined after incubation in solution A containing 0.5% Triton X-100 for 48 hr at 0°.

Incubation of Muscle Fiber Bundles with Antibody. After removal of glycerol by washing with solution A, the fiber bundles were incubated for 24 hr with either specific or control IgG (or Fab′) at the concentrations given in the text. Then the fiber bundles were washed for 2 hr in solution A to remove unbound antibody. Incubations with specific and control antibodies were done on different fragments of the same fiber bundle.

Myofibrils. These were prepared as described by Kundrat and Pepe (4). Additional precautions were taken to avoid contraction (16). Before use, myofibrils were washed five or six times at 4°. For each wash cycle, myofibrils were pelleted at 1500 × *g* for 15 min, resuspended with the aid of a vortex mixer in 10 vol of solution A, and allowed to stand for 10 min.

Other Methods. Enzymatic activity of CK was measured spectrophotometrically (17). Total myofibrillar protein was determined according to the Lowry procedure after dissolution of myofibril pellets in 2 M NaOH. CK within isolated myofibrils was localized by the indirect immunofluorescence technique as described (10).

Electron Microscopy. The fiber bundle preparations were prefixed in P_i/NaCl containing 1.5% glutaraldehyde and 1.5% acrolein at 0° for 5–10 hr. Fixation in P_i/NaCl containing 0.5% osmium tetroxide for 2–10 hr at 0° was followed by dehydration in ethanol and embedding in low-viscosity Spurr medium (E. Fullham, Schenectady, NY). Longitudinal sections (50–100 nm) were poststained (18) with 5% uranyl acetate in 75% ethanol for 1–3 hr, followed by lead citrate. A Philips EM 300 microscope was used at 60–80 kV.

RESULTS

Washed myofibrils incubated first with monospecific IgG from rabbit and then with fluorescein-labeled anti-rabbit-IgG showed strong fluorescence in the middle of the H zone (Fig. 1 *a* and *b*). Much weaker fluorescence was also evident in the Z-band regions as was also observed with specific anti-MM-CK antiserum (10). It is not clear whether this staining is due to MB-CK bound to the Z band or to nonspecific binding of MM-CK (10). Controls with preimmune IgG in the first incubation were negative (Fig. 1 *c* and *d*). Fiber bundles incubated with monospecific IgG (2.5 mg/ml) showed a heavy deposit of electron-dense material within the M line (Fig. 2*a*). At higher magnification, much of this material appeared to lie between the thick filaments (Fig. 2*b*). There was no thickening of the M line in fiber bundles incubated with control IgG, even at the high concentration of 5 mg/ml (Fig. 2 *c* and *d*).

Similar experiments were then performed with monovalent antibody fragments (Fab′) in order to avoid formation of complex immunoprecipitates. We hoped to achieve better antibody penetration and better resolution of the pattern of antibody binding. To our surprise, when myofibrils were allowed to react first with anti-MM-CK Fab′ and then with fluorescein-labeled anti-rabbit-IgG, they did not fluoresce (Fig. 1 *e* and *f*). The same sequence of reagents gave positive cytoplasmic staining in muscle cell cultures (for method, see ref. 19), demonstrating that the labeled antibody used was capable of reacting with Fab′ fragments (not shown). Experiments with control Fab′ showed that the failure to stain could not be attributed to destruction of antigen by residual papain activity in the Fab′ preparations: There was no specific binding of labeled antibody after incubation with control Fab′ (not shown) but, when myofibrils were allowed to react after having been incubated first with control Fab′ and then with anti-MM-CK IgG, normal M-line staining was observed (Fig. 1 *g* and *h*). By contrast, sequential incubation with anti-MM-CK Fab′, anti-MM-CK IgG, and labeled antibody produced, at most, faint striations in the M region (Fig. 1 *i* and *j*).

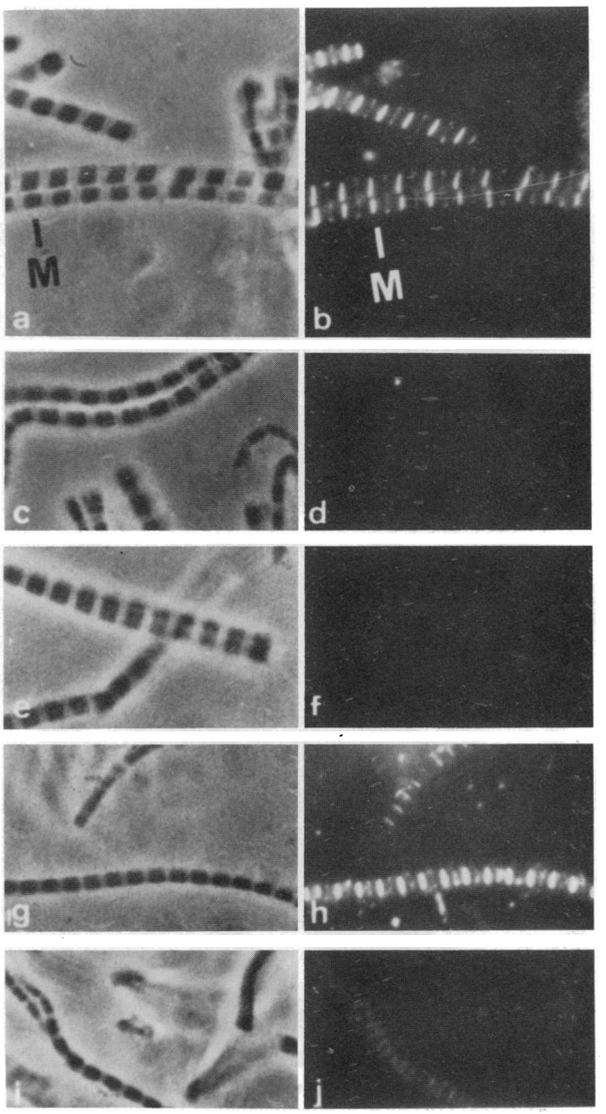

FIG. 1. Localization of MM-CK by indirect immunofluorescence in relaxed skeletal myofibrils. Fluorescence is shown on the right and the corresponding phase-contrast photograph on the left. First incubation(s) (with 20 μg of protein per ml in each case): monospecific anti-MM-CK IgG (*a,b*); control IgG (*c,d*); monospecific anti-MM-CK Fab′ (*e,f*); control Fab′ followed by monospecific anti-MM-CK IgG (*g,h*); monospecific anti-MM-CK Fab′ followed by monospecific anti-MM-CK IgG (*i,j*). Second incubation: fluorescein-labeled anti-rabbit-IgG. M, M line. (×1500.)

Electron micrographs of glycerinated fiber bundles incubated with monovalent antibody (1 mg/ml) revealed that, whereas exposure to control Fab′ leaves the M line intact (Fig. 3 *a* and *c*), treatment with anti-MM-CK Fab′ results in a striking

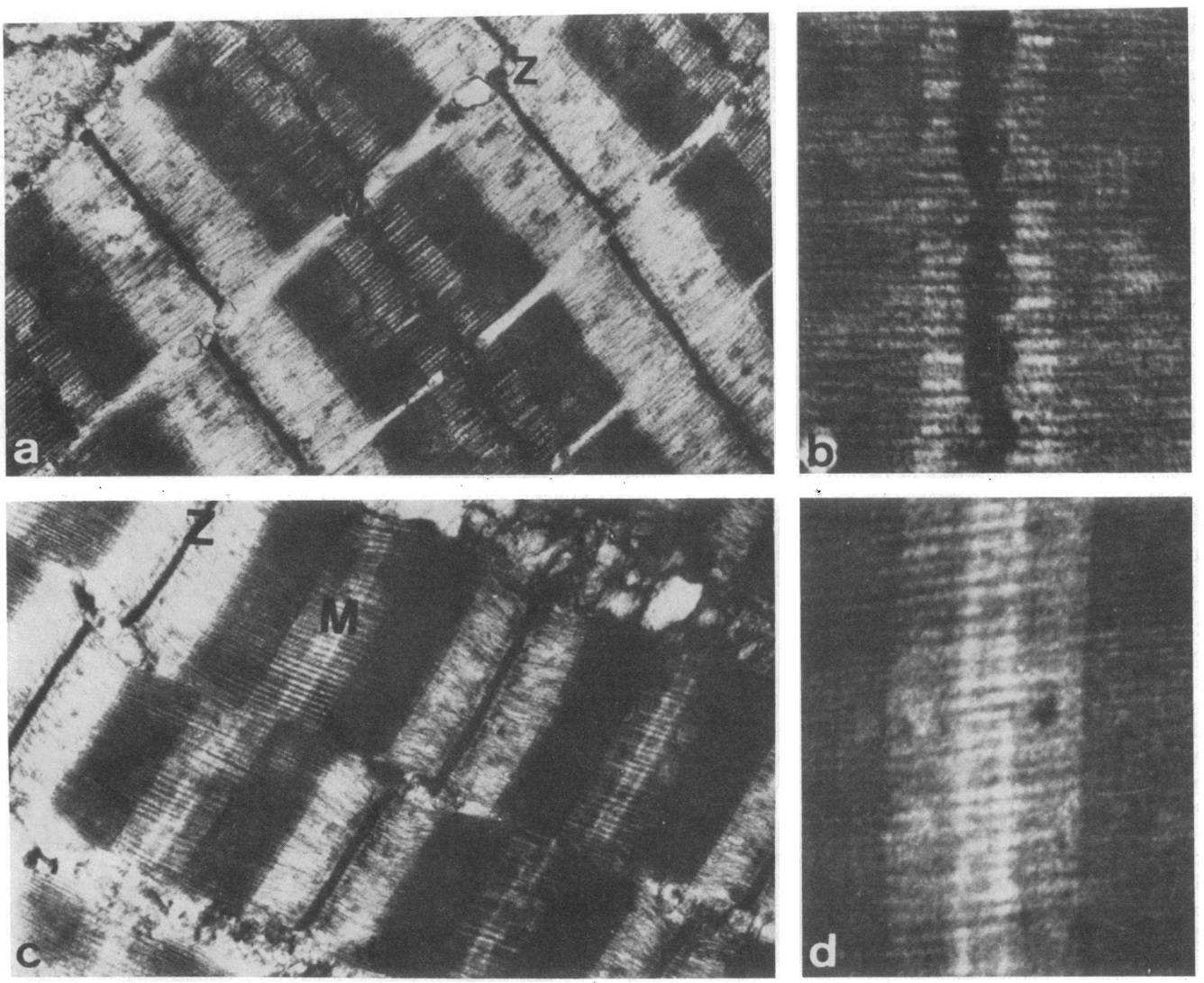

FIG. 2. Thin sections of glycerinated skeletal fiber bundles. (*a*) Heavy staining of M line by monospecific anti-MM-CK IgG (2.5 mg/ml; 24 hr; 0°). (×24,000.) (*b*) As in *a*, enlargement of H region, showing accumulation of monospecific IgG between thick filaments. (×60,000.) (*c*) Incubation with preimmune (control) IgG (5 mg/ml; 24 hr; 0°). M line appears normal. (×24,000.) (*d*) As in *c*, with enlarged H region showing unstained M line after incubation with control IgG. (×60,000.) M, M line; Z, Z line.

loss of electron-dense material from the M line (Fig. 3 *b* and *d*). Identical results were obtained with nonglycerinated fiber bundles pretreated with Triton X-100 (not shown). The removal of the M line appears to be highly specific. There is no evidence for loss of integrity of other myofibrillar structures. As a further control for the inactivation of papain, a mock Fab′ was prepared in the absence of antibody and incubated with fiber bundles for 24 hr exactly as in the Fab′ experiments. There was no evidence of M-line removal (not shown).

As demonstrated by $NaDodSO_4$/polyacrylamide electrophoresis (Fig. 4), incubation of myofibrils for 24 hr with control Fab′ (lane 6) or solution A (lane 7) released several proteins into the supernate; in lane 6 there are, besides the Fab′ bands (see lane 2), nine readily detectable bands. Of these, one comigrated with MM-CK (marker d, lanes 3 and 8). This band was much more prominent after a 24-hr incubation with anti-MM-CK Fab′ (lane 5) but was barely detectable after incubation with anti-MM-CK IgG (lane 4; band too faint to be seen in photograph). The intensity of several other bands was roughly the same after all four treatments; the relative concentrations of still other proteins in the supernates were variable (lanes 4–7). Lane 5 contained no band not also present in lanes 4, 6, and 7. No protein other than the one that comigrated with marker d showed approximately the same intensity in lanes 6 and 7 (control Fab′ and solution A), greatly increased intensity in lane 5, and greatly decreased intensity in lane 4. There is thus no evidence from this experiment that any other protein is specifically released by anti-MM-CK Fab′ and protected from release by anti-MM-CK IgG. It is of interest that no band corresponding to the "M protein" (subunit M_r 165,000) was detectable in lane 5 (supernate after treatment with anti-MM-CK Fab′), although in other experiments (not shown) we demonstrated that M protein penetrates the gel under these electrophoretic conditions.

The myofibrillar protein specifically removed by Fab′ treatment was positively indentified as MM-CK by immunoreplication (Fig. 4, lanes 9–13); the pattern of staining corresponds exactly to that in lanes 4–8. Only a trace amount of myofibrillar MM-CK was released by anti-MM-CK IgG (lane 9), as expected from demonstrations that reaction of myofibrils with antisera against MM-CK prevents subsequent extraction of the M line (5, 10) (Table 1). The immunoreplication technique detected larger amounts of MM-CK released by incubation with control Fab′ or solution A (lanes 11 and 12); this is consistent with our findings that each washing in solution A releases some MM-CK (10) (Table 1). The much more intense

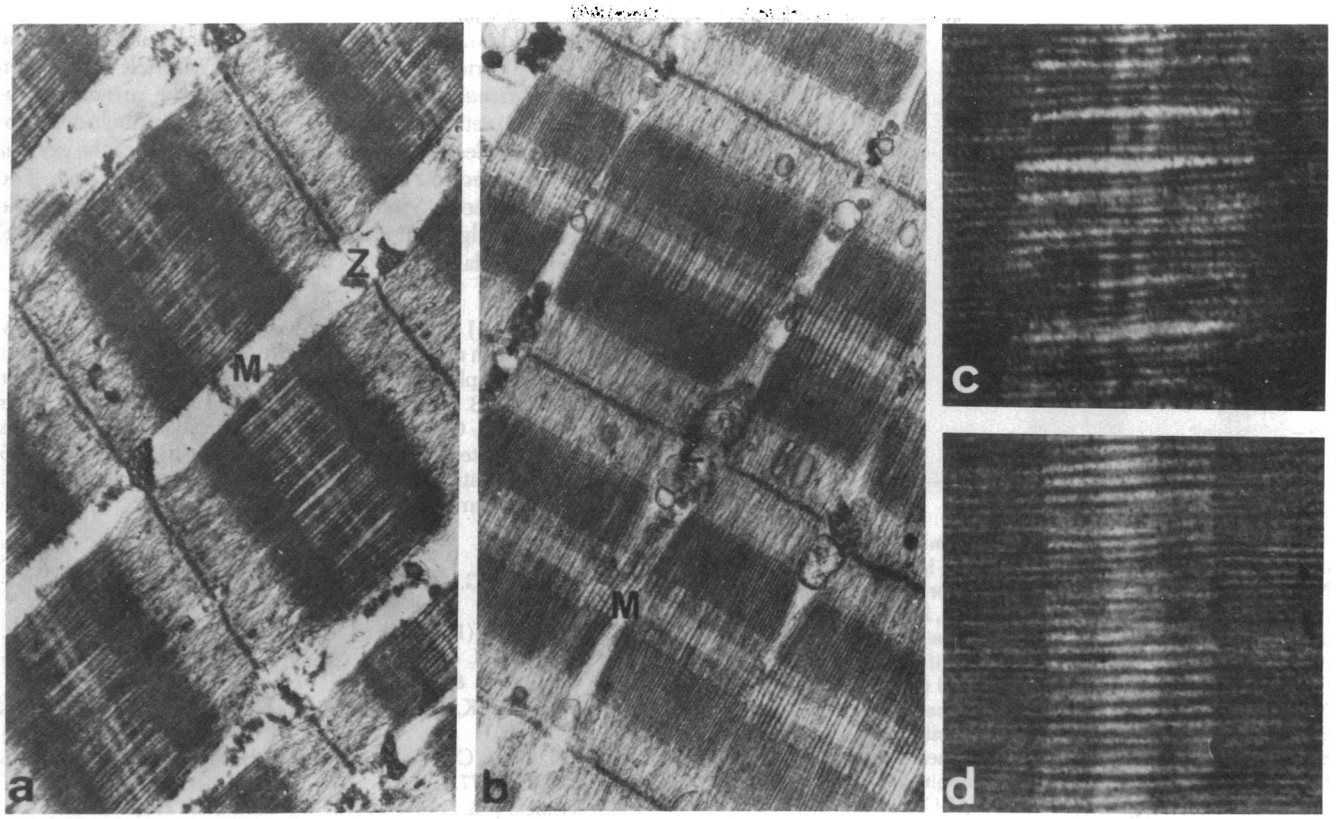

FIG. 3. Thin sections of glycerinated skeletal fiber bundles. (*a*) Previously incubated with Fab′ from preimmune (control) IgG (1 mg/ml; 20 hr; 0°); electron-dense material is clearly visible in the M-line region. (*b*) Previously incubated with anti-MM-CK Fab′ (1 mg/ml; 20 hr; 0°); M-line material appears to be almost totally removed. M, M line; Z, Z line. (*a* and *b*, ×21,000; *c* and *d*, ×55,000.)

staining in lane 10, however, demonstrates that anti-MM-CK Fab′ released an amount of MM-CK greatly exceeding that released nonspecifically.

Table 1 shows that, after treatment with anti-MM-CK Fab′, relatively little CK activity could be released from myofibrils by low ionic strength extraction. Much more activity was releasable from myofibrils pretreated with control Fab′. A straightforward measurement of the CK activity released by specific Fab′ was not possible because of inhibition of the enzymic reaction by Fab′. However, the apparent activity after correction for inhibition by Fab′ is given. After incubation of myofibrils with divalent, affinity-purified IgG, little CK activity was detected in the supernate, and subsequent low ionic strength extraction removed relatively little additional activity. From earlier results with specific antisera (5, 10), it is likely that IgG prevented the extraction of most of the myofibrillar CK activity. From this experiment, however, we cannot exclude the possibility that greater amounts of CK were released but not detected because of inhibition or precipitation of CK by specific IgG.

DISCUSSION

The specific labeling of the M line seen with affinity-purified anti-MM-CK IgG (Figs. 1 *a* and *b* and 2 *a* and *b*) confirms earlier results with specific antisera (5, 10). Monovalent antibody derived from the monospecific IgG clearly removes most of the bound CK from myofibrils. We do not know whether the binding of MM-CK to specific Fab′ in soluble complexes is more stable than the binding of MM-CK to its M-line binding sites or whether the binding of Fab′ alters the conformation of MM-CK, interfering with its binding at the M line.

The M line is no longer detectable after treatment with anti-MM-CK Fab′. What this means, almost certainly, is that at least those elements responsible for the principal substriations are no longer present. Whether any M-line elements at all remain is not known for certain. However, because we did not detect M protein in the supernate after treatment of myofibrils with anti-MM-CK Fab′ and because washing with solution A should not release appreciable amounts of M protein (7), we conclude that M protein probably does remain associated with myofibrils.

Table 1. CK activity released from myofibrils incubated under different conditions

Myofibrils incubated for 24 hr with	Activity units per mg of myofibrils: In supernate after incubation	Additionally extracted with solution C (45 min)	Total released
Specific IgG	0.016	0.042	0.058
Control IgG	0.070	0.088	0.157
Specific Fab′	(0.164)	0.032	(0.196)
Control Fab′	0.059	0.104	0.163
Solution A	0.059	0.083	0.142
5 mM Tris·HCl/1 mM dithiothreitol, pH 7.7 (45 min)	0.120	0.020	0.140

Antibodies were dissolved in solution A at 1 mg/ml. Values given in parentheses represent an attempt to correct for the inhibition of activity by monovalent antibody present in the supernate, based on a standard curve established for the same concentration of anti-MM-CK Fab′ in the presence of varying amounts of purified MM-CK.

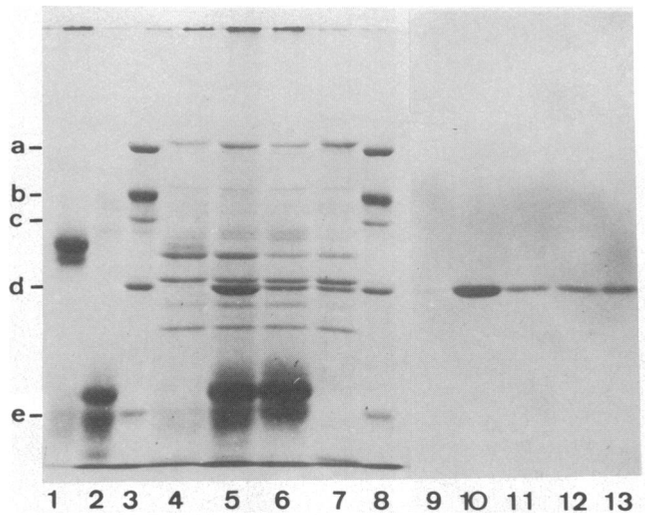

FIG. 4. $NaDodSO_4$/10% polyacrylamide gel electrophoresis of proteins released from myofibrils and identification of released MM-CK by immunoreplication. Myofibril pellets (3.5 mg) were suspended in 500 μl of treatment solution, incubated for 24 hr at 4°, and spun down. Proteins in the supernates were precipitated with 2 ml of acetone (−10°) and then, after centrifugation, were redissolved in 30 μl of sample buffer (14) and applied to the gel. Lanes: 1, anti-MM-CK IgG (9 μg), showing prominent bands of IgG heavy chains; 2, anti-MM-CK Fab′ (10 μg), showing position of Fab′ fragments; 3, 8, and 13, M_r markers (approximate values: a, rabbit muscle phosphorylase b, 94,000; b, bovine serum albumin, 68,000; c, rabbit muscle pyruvate kinase, 57,000; d, chicken MM-CK 40,000; e, bovine chymotrypsinogen A, 25,000); 4–7, supernates after incubation with anti-MM-CK IgG, 0.1 mg/ml (lane 4), anti-MM-CK Fab′, 0.1 mg/ml (lane 5), control Fab′, 0.1 mg/ml (lane 6), solution A (lane 7); 9–13, immunoreplica of a part of the same gel exactly duplicating lanes 4–8 not fixed and stained directly but instead overlaid with an agarose gel containing anti-MM-CK antiserum. Single precipitin lines were found in lanes 9–13 at the position of marker d. The protein specifically released by anti-MM-CK Fab′ (prominent band in lane 5 comigrating with marker d in lanes 3 and 8) was unequivocally demonstrated to be MM-CK by its reaction with antiserum against MM-CK (lane 10).

It must be assumed that the arrays of M bridges and the M filaments make significant (or even the principal) contributions to the electron density observed in the M line. Therefore, if MM-CK were not a true structural element—i.e., if MM-CK were merely associated with thick filaments in the M-line region or with preexisting M bridges and M filaments—one would expect a much less extensive removal of electron-dense material from the M line upon Fab′ treatment. Moreover, because the principal substriations are thought to correspond to the M-bridge arrays, it would seem likely that MM-CK is an integral component of the transverse arrays. The disappearance of the M line would not be expected if MM-CK molecules formed the *secondary* M bridges: selective removal of MM-CK should then leave the *primary* M bridges (and hence the principal substriations) intact. No other protein (M protein included) appeared to be released concomitantly with the loss of the greater part of the electron-dense material from the M line. The working hypothesis (5, 10, 20) that MM-CK molecules form the primary M bridges (and that the M-protein molecules do not) thus remains plausible.

We thank Dr. J.-C. Perriard and Mr. M. Caravatti for generously providing us with affinity-purified antibodies and for introducing us to the immunoreplication procedure. We also thank Dr. R. Craig for advice and Mr. B. Saunders for help with the photography. T.W. is grateful to Dr. A. G. Szent-Györgyi, in whose laboratory part of this work was completed. This research was supported by Grant 3.575-0.75 from the Swiss National Science Foundation and by a grant to H.M.E. from the Muscular Dystrophy Association, Inc.

1. Knappeis, G. G. & Carlsen, F. (1968) *J. Cell Biol.* **38**, 202–211.
2. Pepe, F. A. (1971) *Progr. Biophys. Molec. Biol.* **22**, 77–96.
3. Sjöström, M. & Squire, J. M. (1977) *J. Mol. Biol.* **109**, 49–68.
4. Kundrat, E. & Pepe, F. A. (1971) *J. Cell Biol.* **47**, 340–347.
5. Morimoto, K. & Harrington, W. F. (1972) *J. Biol. Chem.* **247**, 3052–3061.
6. Heizmann, C. W. & Eppenberger, H. M. (1978) *J. Biol. Chem.* **253**, 270–277.
7. Trinick, J. & Lowey, S. (1977) *J. Mol. Biol.* **113**, 343–368.
8. Landon, M.-F. & Oriol, C. (1975) *Biochem. Biophys. Res. Commun.* **62**, 241–245.
9. Turner, D. C., Wallimann, T. & Eppenberger, H. M. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 702–705.
10. Wallimann, T., Turner, D. C. & Eppenberger, H. M. (1977) *J. Cell Biol.* **75**, 297–317.
11. Sober, H. A. & Peterson, E. A. (1958) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **17**, 1116–1126.
12. Perriard, J.-C., Caravatti, M., Perriard, E. R. & Eppenberger, H. M. (1978) *Arch. Biochem. Biophys.*, in press.
13. Porter, R. R. (1959) *Biochem. J.* **73**, 119–127.
14. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
15. Showe, M. K., Isobe, E. & Onorato, L. (1976) *J. Mol. Biol.* **107**, 55–69.
16. Heizmann, C. W., Bläuenstein, I. E. & Eppenberger, H. M. (1978) *Experientia* **34**, 38–40.
17. Eppenberger, H. M., Dawson, D. M. & Kaplan, N. O. (1967) *J. Biol. Chem.* **242**, 204–209.
18. Reynolds, E. S. (1963) *J. Cell Biol.* **17**, 208–212.
19. Turner, D. C., Gmür, R., Lebherz, H. G., Siegrist, M., Wallimann, T. & Eppenberger, H. M. (1976) *Dev. Biol.* **48**, 284–307.
20. Wallimann, T., Turner, D. C. & Eppenberger, H. M. (1975) *FEBS Proc. Meet.* **31**, 119–124.