LOCALIZATION OF CREATINE KINASE ISOENZYMES IN MYOFIBRILS

I. Chicken Skeletal Muscle

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ABSTRACT

Purified, repeatedly washed, skeletal muscle myofibrils contain approx. 0.2 U of creatine kinase (CK) activity (equivalent to 2.5 μg CK) per milligram dry weight; this firmly bound CK activity is estimated to represent 3-5% of the total cellular CK. It had been shown previously that the myofibrillar CK, which can be quantitatively extracted at low ionic strength and purified to homogeneity, is very similar, if not identical, to the bulk MM-CK. It is shown that the two protein preparations also have (a) the same peptide pattern after cyanogen bromide fractionation and (b) very similar specific activities, confirming their identity. The earlier demonstration that the bound CK is specifically located at the M-lines of isolated myofibrils has been confirmed by immunofluorescence. Antibodies directed against purified MM- and BB-CK were used in the indirect fluorescent antibody technique to study the specificity of myofibril binding sites for different forms of CK. With myofibrils from adult muscle, which has only MM-CK, as well as from early developmental stages in which BB-CK is the predominant isoenzyme, M-type CK was localized exclusively at the M-line, while greater or lesser amounts of B-type CK were found at the Z-line. The data provide strong evidence that the MM-CK at the M-lines in skeletal myofibrils is not adventitiously bound but is rather an integral element in the M-line structure. The amount of CK bound is (a) reasonably consistent with the earlier proposal that the CK molecules might be the transverse M-bridges and (b) appears to be sufficient to regenerate all of the ATP hydrolyzed during muscle contraction.

KEY WORDS: creatine kinase · isoenzymes · M-line protein · myofibrils

Several accessory components are associated with the two major filament-forming proteins actin and myosin in the myofibrils of skeletal muscle. Troponyosin and the proteins of the troponin-complex (TNC, TNT, and TNI) are associated with actin along the entire length of the thin filament and serve to regulate the interactions between actin and myosin in muscle contraction. The accessory proteins known to be associated with the thick myosin filament, namely the so-called C-proteins (49) and the M-line-proteins (see below),
bind at specific locations and not along its entire length. The functions of the thick filament-associated proteins are not clear.

The M-line, the characteristic electron-dense region which traverses the myofibril in the center of the anisotropic A-zone, has attracted considerable attention during the last few years. As early as 1966, Pepe (53) had shown that M-line protein was different from actin, myosin, and tropomyosin. Kundrat and Pepe (35) extracted protein from the M-line and demonstrated that the extract could specifically absorb M-line labeling antibodies from antibody which labeled both the I-band and the M-line.

In subsequent attempts to selectively extract and characterize the proteins of the M-line, at least three proteins (with estimated subunit mol wt of 40,000, 100,000, and 160,000) have been isolated (12, 18, 19, 36, 40, 41, 51).

For each of these protein species, there is evidence, in addition to mere coincidence of extraction with the disappearance of the M-line, that it actually contributes to the M-line structure (12, 18, 19, 41, 46, 51). However, after it had been shown (16, 63, 64, 66) that the smallest of the three putative M-line proteins was the MM-creatine kinase (CK), it was argued (13) that this protein could not be a structural component of the M-line inasmuch as it is an enzyme known not to be exclusively located in myofibrils. Recent studies have led to the identification of another of the putative M-line components, the one with a subunit mol wt of 90,000-100,000, as glycogen phosphorylase (8, 25, 41, 60, 61), but have also cast doubt that this protein is a structural element of the myofibril (60).

There has been a tendency (13) to assume that the M-line must contain a single "true" M-line protein, i.e., an exclusively structural component lacking enzymic activity. The alternative suggestion is that, like myosin, one or more M-line proteins may serve dual functions as enzymes and as structural elements in the contractile apparatus. Our investigations concern one of the putative M-line proteins, MM-CK. Does it function both as an enzyme and as an integral, specifically bound, structural element? Or does a small, adventitiously bound, fraction of an otherwise soluble enzyme tend to "contaminate" skeletal muscle myofibrils at the M-line, as has been suggested by Etlinger et al. in a recent paper (19). Our approach has been to examine the localization of CK-isoenzymes within two types of striated muscle cells: (a) chicken skeletal muscle, the myofibrils of which possess an M-line (this paper) and (b) chicken heart muscle, the myofibrils of which lack an M-line (the following paper, reference 65). In particular, we have examined the specificity of the interactions of the different isoenzymes with defined regions of the contractile apparatus.

In this paper, we report on the following: (a) additional evidence that the small fraction of the total cellular CK activity reproducibly bound to skeletal muscle myofibrils is identical with the bulk MM-CK present in the sarcoplasm. (b) Experiments on the quantitation of the MM-CK bound to myofibrils, and on conditions for its release. (c) A demonstration that MM-CK specifically bound at the M-line can be detected even in cells that contain little of the MM-isoenzyme. (d) Attempts to reconstitute M-lines by incubating extracted myofibrils with MM-CK.

In the Discussion, we reconsider the working model for the M-line structure published earlier (66), taking into account not only the data reported in this paper but other recently published data on M-line proteins, as well. We show that the amount of CK bound to myofibrils fits reasonably well with the proposed model, further strengthening the argument that MM-CK is truly a myofibrillar protein.

MATERIALS AND METHODS

Purification of Creatine Kinase

The MM and BB isoenzymes of CK were purified from the soluble fractions of extracts of chicken breast muscle and heart, respectively, employing established procedures (11, 34). Using the very different purification scheme of Morimoto and Harrington (46), the "M-line protein" (subunit mol wt ca. 40,000) known to possess CK activity and thought to be identical to MM-CK (63) was also purified. All three preparations appeared homogeneous as judged by polyacrylamide gel electrophoresis (see Results).

Protein Determination

Protein was determined by the method of Lowry et al. (39), using bovine serum albumin as standard.

Enzyme Activities

Unless otherwise indicated, CK enzymatic activity with phosphocreatine as substrate was measured spectrophotometrically (15); 1 enzyme unit (EU) was defined as the amount of CK necessary to catalyze formation of 1 μmol ATP/min. Samples were diluted as necessary to keep the amount of enzyme in each assay below 0.005
Electrophoresis was done under native or denaturing conditions. Native proteins were run in 7.5% gels (0.37 M Tris-glycine buffer, pH 9.5) according to Davis (9). Denaturing conditions were obtained by employing 7.5-10% gels containing 0.1% sodium dodecyl sulfate (SDS) according to Moore et al. (44). Protein bands were detected by staining with Coomassie Brilliant Blue R (Sigma Chemical Co., St. Louis, Mo.).

Cyanogen Bromide Fragmentation of MM-CK and M-Line Protein

Purified samples (2 mg each) of MM-CK and M-line protein (the latter purified according to reference 46) were precipitated by 6% TCA and subsequently redissolved in 4 ml of 70% formic acid containing 1% phenol. After addition of 30 mg of cyanogen bromide, samples were incubated under N₂ for 18 h in the dark at room temperature, diluted with distilled water to a total volume of 50 ml, and lyophilized. Fragments were separated in the electrophoresis system (10% acrylamide, 4 M urea, 0.1% SDS) described by McGillivray et al. (42).

Preparation of Myofibrils from Chicken Muscle Tissues

Myofibrils were prepared (all steps at 4°C) according to a procedure modified from that described by Kundrat and Pepe (35). Adult white Leghorn chickens were decapitated, and the muscles (various leg muscles, M. pectoralis major, M. anterior latissimus dorsi, M. posterior latissimus dorsi) were immediately cut out, cleaned, minced, and immersed in 20 vol of the contraction-inhibiting "Solution A" (0.1 M KCl, 1 mM DTT, 20 mM phosphate buffer; pH 7.0), pelleting of the myofibrils by centrifugation for 15 min at 1,500 g, and decantation of the washing solution; this was often repeated for many cycles. Washing was sometimes performed with Solution A containing 0.5% Triton X-100 (19); these cases are indicated in the Results. The myofibrils so treated are referred to as "Triton X-100-washed." "Extraction" of previously washed myofibrils involved treatment (100 vol/wt) at low ionic strength ("Solution C": 1 mM DTT, 5 mM Tris/HCl buffer; pH 7.7), pelleting (10 min at 5,000 g) and decantation of the extraction solution.

Specific Activity of CK in Myofibrils

Myofibrils were prepared from three samples of adult chicken breast muscle according to the procedure of Kundrat and Pepe (35), without modifications, and then washed repeatedly with Solution A. After five and nine washes, aliquots were taken for determination of CK activity, for total protein determination according to the Lowry procedure (after incubation for 1 h in 1 N NaOH), and for dry weight determination (after dialysis against 0.2 M ammonium carbonate and lyophilization). Specific activities were then expressed (±SD) either as units per milligram protein or as units per milligram dry weight.

Supplementation of Native Myofibrils with Exogenous CK Isoenzymes

"Supplementation" refers to the treatment of washed (but not extracted) myofibrils with purified CK isoenzymes. Myofibrils, either freshly washed or glycerinated, were added to a solution (0.1 or 1.0 mg/ml in Solution B) of MM-CK or BB-CK and stirred in 1-ml centrifuge tubes for 1-12 h at 4°C. Control myofibrils were incubated in Solution B without CK isoenzymes. This incubation step was followed by several washings to remove nonspecifically bound CK, after which bound CK isoenzymes were localized by immunofluorescence (see below).
"Attempted reconstitution," on the other hand, refers to the treatment of extracted myofibrils with purified CK isoenzymes. freshly prepared myofibrils were extracted and either used immediately or stored in 50% glycerol in Solution A. We distinguish three modes of attempted reconstitution: (a) long-time incubation, 12 h at high concentration (1 mg/ml) of a single CK isozyme; (b) short-time incubation, 1 h at low concentration (0.1 mg/ml) of a single CK isozyme; and (c) short-time competition incubation 1 h with both CK-isoenzymes at low concentration (0.1 mg/ml). Other conditions were as for supplementation (see above). Nonspecifically bound CK was again removed by repeated washings before immunofluorescence localization. Controls were done under identical conditions but without CK isoenzymes.

Preparation of Antibody

After pre-immune serum was removed, rabbits were given two injections of either MM- or BB-CK that had been purified to apparent homogeneity, and that, inasmuch as they had been prepared from the soluble fractions of cell extracts (see above), would in any case not be expected to contain any contaminating myofibrillar proteins. A solution of 0.004 M of enzyme protein in 0.5 ml of 0.05 M Tris/HCl buffer, pH 8.0, was emulsified with 0.5 ml of complete Freund's adjuvant and injected subcutaneously into the foot pad. The second injection was given 3 wk later. 10 days after the second injection, the rabbits were bled from an ear vein. The centrifuged serum (all serum and antibody dilutions in Solution A) was kept frozen without further purification as control sera. Freshly prepared myofibrils were extracted with 0.1 M glycine buffer (pH 9.0) containing 0.1 M KCl, 1 mM EGTA, and 25% glycerol and mounted under a glass cover slip for fluorescence microscopy. The instrument used consisted of a Zeiss Standard Model 18 microscope equipped with Neofluor objectives (Carl Zeiss, Inc., New York, N.Y.), a 50 W HBO mercury lamp, a IV/F condenser, and the following filter combination: excitation LP 455; KP 490, KP 500; emission, Rf 510, LP 520. For fluorescence photography, Ilford HP4 film was used with Diaphane developer to increase the effective film speed.

Electron Microscopy

After extensive washing or washing and extraction, a myofibril pellet (0.1 ml) was taken up in Solution B containing 3% bovine serum albumin. After low speed centrifugation, the pellet was fixed (12 h at 4°C) in glutaraldehyde (1.5%) and acrolein (1.5%) and rinsed twice in Solution B (without DTT). The myofibrils embedded in serum albumin were treated twice for 1 h with 0.1% osmium tetroxide dissolved in 0.1 M phosphate buffer, pH 7.0. The fixed material was dehydrated in ethanol and acetone, embedded in Epon/Araldite, incubated for 12 h at 20°C and then for 12 h at 60°C. Thin sections were cut on a LKB 4800 A ultratome (LKB Instruments, Rockville, Md.), poststained with 5% uranyl acetate (15 min), followed by 2.6% lead citrate (5 min). Electron microscope examination was performed with a Siemens Elmiskop 1.

Histochemical Localization of CK

Freshly prepared chicken breast muscle was cut into cubes 3–5 mm on a side and rapidly frozen by immersion in Freon cooled by liquid nitrogen. Frozen slices of 5 μm thickness were overlaid with a 10% gelatin gel containing all reactants for specific histochemical staining of CK (15, 32) and incubated for 15 min at 30°C. Overlay gels from which creatine phosphate had been omitted were used for control sections. After incubation, the gels were removed and the stained slices observed with bright field microscopy.

RESULTS

Purification and Characterization of Antigens and Antisera

After purification through the final Sephadex G-100 gel filtration step (11), preparations of MM- and BB-CK were free of contaminating pro-
tein, as demonstrated by polyacrylamide gel electrophoresis (Fig. 1A and B). The specific activities of the purified proteins determined in the reaction with creatine as substrate (83 EU/mg for MM-CK and 40 EU/mg for BB-CK) compared favorably with previously reported values (11). The antisera obtained against MM- and BB-CK both gave single precipitin lines when tested against homologous antigen in the double-diffusion procedure (Fig. 2).

The specificities of the two antisera were further tested by immuno-electrophoresis (Fig. 3). Crude extracts of skeletal muscle or heart, and purified samples of MM- or BB-CK, were run for 2 h at 125 V and reacted with 1:1 diluted antiserum. Single precipitin lines were obtained when anti-MM-CK was reacted with purified MM-CK or with the skeletal muscle extract; no cross-reaction with BB-CK or with proteins in the heart extract was seen. Conversely, anti-BB-CK gave a single precipitin line when reacted with heart extract or purified BB-CK, but showed no reaction with MM-CK or with the skeletal muscle extract. When the gels were subsequently reacted with a histochemical staining mixture (11) specific for CK activity, the staining coincided exactly with the

FIGURE 2 Double immunodiffusion test (50) showing specificities of anti-CK sera. (a) Center well: anti M-CK serum, 1:1 diluted. Outer wells: (1) MM-CK (50 μg); (2) skeletal muscle extract (25%); (3) MM-CK (50 μg); (4) skeletal muscle extract (12.5%); (5) BB-CK (50 μg); and (6) heart extract (25%). (b) Center well: anti B-CK serum, 1:1 diluted. Outer wells: (1) BB-CK (50 μg); (2) heart extract (25%); (3) BB-CK (100 μg); (4) heart extract (12.5%); (5) MM-CK (50 μg); and (6) skeletal muscle extract (25%).

FIGURE 1 (A) Comparison of the electrophoretic mobilities of conventionally purified MM- and BB-CK isoenzymes with M-line protein purified according to Morimoto and Harrington (46). Discontinuous polyacrylamide gel electrophoresis (7.5%) of native preparations: (1) M-line protein (30 μg); (2) MM-CK (50 μg); (3) l + 2; (4) BB-CK (25 μg); (5) BB-CK (25 μg) + M-line protein (20 μg); and (6) 4 + 5. Protein staining with 1% Amido black. (B) Polyacrylamide gel electrophoresis under denaturing conditions (7.5% gel + 0.1% SDS): (1) MM-CK (60 μg); (2) M-line protein (60 μg); and (3) l + 2. Protein staining with Coomassie Brilliant Blue R.
Figure 3 Immunoelectrophoresis of CK isoenzymes. 1.5% agarose gel in 0.05 M veronal buffer, pH 8.6. Migration time: 2 h at 125 V. Protein stain (a–d); 0.1% Amido black. Detection of CK activity (e and f) with an overlay gel procedure (15). (a) Antiserum: anti M-CK (1:1 dil.); (1) MM-CK (100 µg); and (2) skeletal muscle extract (25%). (b) Antiserum: anti M-CK (1:1 dil.); (1) BB-CK (100 µg); and (2) heart extract (25%). (c) Antiserum: anti B-CK (1:1 dil.); (1) BB-CK (100 µg); and (2) heart extract (25%). (d) Antiserum: anti B-CK (1:1 dil.); (1) MM-CK (100 µg); and (2) skeletal muscle extract (25%). (e) Antiserum: anti M-CK (1:1 dil.); (1) MM-CK (100 µg); and (2) skeletal muscle extract (25%). (f) Antiserum: anti B-CK (1:1 dil.); (1) BB-CK (100 µg); and (2) heart extract (25%).

This demonstrates that in each case the only precipitating antigen-antibody reaction was that between CK and anti-CK. Immunotitration of CK activity in purified MM-CK preparations and in crude breast muscle extracts with anti-MM-CK serum was performed. All CK activity was quantitatively eliminated from the supernate by this procedure (not shown). Titration of purified BB-CK with anti-MM-CK serum failed to eliminate CK activity from the supernate. In addition, in a microcomplement fixation test (37), neither antiserum showed any cross reactivity with the heterologous homodimeric isoenzyme, whereas both cross-reacted with the heterodimeric isoenzyme, MB-CK (62), confirming similar earlier observations (15).

Further Evidence That the Bound CK Activity Is Identical to MM-CK

We had shown (16, 63) that some of the CK activity in chicken breast muscle was tightly bound to myofibrils at the M-line and that this bound CK was indistinguishable from the bulk of cytoplasmic MM-CK by several criteria, including molecular weight, subunit number, amino acid composition, immunological identity, and electrophoretic mobility. As noted above (see introductory paragraph), however, it has been questioned whether the binding of CK at the M-line is specific and physiological. If it could be demonstrated that the molecules so bound were structurally distinct from MM-CK (perhaps partially degraded or otherwise altered forms of the enzyme), this might provide an explanation for the binding of part of the cellular CK activity to myofibril. We therefore undertook to develop additional evidence that M-line protein is identical to MM-CK. In our original paper (63) advancing evidence that the M-line protein of Morimoto and Harrington (46) was MM-CK, much of the CK activity in the M-line protein preparation was lost in the final (DEAE-cellulose) purification step and the purified protein had a lower specific activity than conventionally purified MM-CK. These disquieting features,
which might suggest the presence of modified enzyme molecules, can be avoided by addition (to 1 mM) of β-mercaptoethanol or DTT to the buffer during purification. Furthermore, when M-line protein and MM-CK are co-electrophoresed (Fig. 1A and B) they are indistinguishable. As shown in Fig. 4, the two protein preparations also give identical peptide patterns after cyanogen bromide fragmentation.

Amount of MM-CK Bound to Intact Skeletal Myofibrils

Since we obtained further proof that CK associated with the particulate fraction during repeated washings at intermediate ionic strength but extractable at low ionic strength was identical to the bulk soluble MM-CK of muscle cells, it was important to quantitate the amount of CK actually bound to myofibrils.

We had earlier reported (63) that 24% of the CK activity ultimately recovered had remained bound to the minced breast muscle pellet after exhaustive washing at intermediate ionic strength according to the procedure of Morimoto and Harrington (46). Later experiments have shown this value to be spuriously high, most likely due to loss of CK activity in the supernates (which had not been supplemented with a sulfhydryl reagent and which had all been assayed at the conclusion of the washings). Numerous subsequent experiments (approx. 10 over a 3-yr period) have been performed in order to determine the fraction of total cellular CK which is firmly bound to myofibrils. Fig. 5 shows one of these experiments; in this case, 7% of the CK activity measured in the initial suspension was found to be firmly bound to a low-speed pellet consisting primarily of myofibrils. In other experiments, we have obtained varying, and

FIGURE 5 Effect of washing and extraction on the binding of CK to the low-speed pellet (predominantly myofibrils) from adult chicken breast muscle. Total CK activity in the suspension (black plus white bars) was measured (according to Reference 3) before, and CK activity released into the supernate (black bars) was measured after, each centrifugation. C: Crude homogenate (1/100 wt/vol) in Solution A. W1-W6: Successive washings in Solution A (intermediate ionic strength); little CK activity is released after the first few cycles. In this experiment, about 7-8% of the total activity present in the original suspension was tightly bound. E: Extraction (45 min) in Solution C (low ionic strength); bound CK is completely released from myofibrils. W6+: final wash in Solution A; very little CK remains bound.

FIGURE 4 Polyacrylamide gel electrophoresis of cyanogen bromide fragments of MM-CK and M-line protein. (1) MM-CK (70 μg); and (2) M line protein (70 μg).
generally lower, values for the bound CK fraction; the results of several recent experiments are summarized in Table I. The observed variation reflects differential losses of myofibrils in the course of the many wash cycles, as well as difficulties in measuring CK activity reliably in crude suspensions; it appears not to be related to the source of the muscle, to the degree of contraction of the myofibrils, or to the presence of mitochondrial CK (20, 29, 30) in the low-speed pellet (data not shown). Our best estimate (which must be regarded as a lower limit in view of the losses of myofibrils and in CK activity inevitably suffered in the course of the prolonged series of washes) is that 3-5% of the CK present in skeletal muscle cells is firmly bound to myofibrils.

Because of the difficulties encountered in estimating the fraction of CK bound to myofibrils, we turned to the measurement of the CK specific activity in well-washed, purified myofibrils (see Materials and Methods). After five washes, myofibrils contained 0.21 ± 0.02 EU/mg dry weight and 0.27 ± 0.02 EU/mg protein (as estimated by the Lowry procedure); after nine washes, the corresponding values were 0.16 ± 0.2 and 0.21 ± 0.02, respectively. Even after nine washing cycles, myofibrils retain a clearly discernible M-line structure (Fig. 6a). A single brief extraction with low ionic strength buffer suffices to remove 90% or more of the CK activity from skeletal myofibrils (Fig. 5). Extraction causes the myofibrils to swell considerably and the M-line to disappear (Fig. 6b). We have been unable to observe removal of all or most of the CK activity bound to myofibrils without also observing these gross changes of the normal myofibrillar structure. This, coupled with our finding (Table II) that CK remains bound and the myofibrillar structure remains intact in the presence of various "physiological" salt solutions and in the presence of substrates of the CK reaction, is strong evidence that skeletal myofibrils in situ contain bound CK.

**Localization of MM-CK within Skeletal Myofibrils**

Our first indication of CK localized in myofibrils was obtained by applying the gel overlay method for the specific histochemical staining of CK (see Materials and Methods) in longitudinal sections of adult chicken breast muscle. In accordance with earlier observations (57, 32), most of the enzyme activity was located in the intermyofibrillar space. However, a distinct pattern of cross striations suggested that some CK might be bound to myofibrils in a regular fashion (Fig. 7a). Control slices showed only faint cross striations (Fig. 7b), indicating that the regular pattern of staining was not due to adenylate kinase.

Much more precise localization of the bound CK was obtained with the use of immunocytochemical methods. Fig. 8a shows a regular fluorescent banding pattern in a skeletal myofibril that had been washed once with Solution A and stained for M-CK by the indirect immunofluorescence method. The photograph is a double exposure, with the fluorescence pattern superimposed on the phase-contrast photograph, which is also shown without superimposed fluorescence in Fig. 8b. The arrow (Fig. 8a) shows that the fluorescent striations are located in the lighter central H-regions of the otherwise dark A-bands. Figs. 8c and d show fibrils stained after repeated washing procedures as described in Fig. 5. Clearly, M-CK remained at the M-line even after extensive washing. Myofibrils washed with Triton X-100 at a concentration known to remove all membrane

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**Table I**

*Summary of Experiments Designed to Measure the Fraction of Total Cellular CK Bound to Myofibrils after Repeated Washing*

| Exp | Animal | Homogenate | CK bound after nine washes as percentage of activity in homogenate |
|-----|--------|------------|------------------------------------------------------------------|
| 1   | A      | 1          | 2.5                                                              |
|     |        | 2          | 5.0                                                              |
|     |        | 3          | 3.4                                                              |
|     |        | 4          | 4.7                                                              |
| 2   | A      | 1          | 2.9                                                              |
|     |        | 2          | 3.8                                                              |
|     | B      | 3          | 3.4                                                              |
|     |        | 4          | 2.7                                                              |
|     | C      | 5          | 6.6                                                              |
| 3†  | A      | 1          | 3.9                                                              |
|     |        | 2          | 3.1                                                              |
|     |        | 3          | 3.3                                                              |
|     | B      | 4          | 3.4                                                              |
|     |        | 5          | 3.9                                                              |
|     |        | 6          | 3.4                                                              |

* In all cases the design of the experiments followed that given in Fig. 5. The activity released by extracting with low ionic strength Solution C invariably exceeded by a factor of 10 or more the activity released in the wash immediately preceding.

† Homogenates in this experiment were 1/20 (wt/vol).
Figure 6 Thin sections of skeletal myofibrils. (a) Extensively washed (as described in Fig. 5). Electron-dense material is clearly visible in the M-line region (M). Z = Z-line. (b) Extracted for 45 min with low-ionic-strength Solution C (see Materials and Methods). Loss of electron-dense material from the center of the H region is evident. × 35,000.
TABLE II
Attempts to Extract Bound CK from Washed Skeletal Myofibrils by Various Salt Solutions and Substrates of the CK Reaction

| Solutions                                      | CK in supernate % |
|------------------------------------------------|-------------------|
| Solution A, pH 7.0, 4°C                         | 20                |
| Solution B, pH 7.0, 4°C                         | 13                |
| Buffered saline solution (Simms' solution)     | 19                |
| Minimal essential medium (Eagle's MEM)         | 15                |
| 0.1 M KCl, 6.5 mM creatine phosphate, 1.5 mM ADP, 0.1 M triethanolamine-HCl buffer, pH 7.2§ | 30                |
| 0.1 M KCl, 15 mM creatine, 2.1 mM ATP, 0.1 M glycine buffer, pH 7.6§ | 13                |
| Solution C, pH 7.7, 4°C                         | 100               |

* Aliquots of myofibrils washed six times with Solution A were incubated for 90 min with stirring, were centrifuged, and the supernate was tested for activity. No difference was observed when myofibrils were prepared in the test solutions from the beginning.

† Control: a certain amount of bound CK is always liberated, even after six previous washing cycles (see Fig. 4).

§ Standard assay conditions for CK (3, 14).

∥ For formulations, see reference 52.

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Figure 7 (a) Frozen section (5 μm) of adult breast muscle stained for CK activity (see Materials and Methods). (b) Control without creatine phosphate. Both bright field illumination. × 1,200.

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Localization of CK Capable of Reacting with Anti-BB-CK Serum within Skeletal Myofibrils

Myofibrils of adult skeletal muscle incubated with antibody against BB-CK never showed immunofluorescence staining in the M-line region, whereas a weak fluorescence (as can be seen in Fig. 10g) was routinely observed within the Z-line region. Since a weak fluorescence in the Z-line region was sometimes seen in myofibrils incubated with anti-MM-CK serum (see above), it seems possible that small amounts of the heterodimeric isoenzyme MB-CK, which reacts with both antisera, may be present in the region of the Z-line. Electropherograms of extracts of chicken skeletal muscle have sometimes revealed traces of MB-CK (14).

Myofibrillar CK in Skeletal Muscle Development

A transition in the CK isoenzyme pattern takes place during skeletal muscle development in the chicken (14). The only isoenzyme detectable in electropherograms of extracts of the breast and limb muscle anlagen of the 7-day embryo is BB-CK. The embryonic isoenzyme is gradually replaced during the succeeding 2 wk, so that, at the time of hatching, the now overtly differentiated fragments (19) also retain the M-line-bound MM-CK (Figs. 8e and f). This confirms earlier observations (19) that myofibrils treated with Triton X-100 retain proteins which correspond in size with subunits of MM-CK. Low ionic strength extraction of the myofibrils removed all bound CK (Fig. 8g and h), however.

Preincubation of native unwashed myofibrils with anti-MM-CK serum prevented the extraction of the M-line structure visible in the electron microscope (not shown). This confirmed observations made by Morimoto and Harrington (46) and by Palmer (51) with antibodies against their "M-line proteins." It should be noted at this point that (a) weak fluorescence was occasionally observed in the Z-line region of washed skeletal myofibrils using anti-MM-CK serum, and that (b) a staining pattern identical to that shown in Fig. 8a-d for M. pectoralis major, consisting primarily of "fast-twitch" fiber types, could be observed for M. posterior latissimus dorsi (also fast-twitch), leg muscle (slow-twitch), and for M. anterior latissimus dorsi (slow-tonic).
Localization of MM-CK by indirect immunofluorescence in skeletal myofibrils. Fluorescence and phase-contrast photographs have been superimposed in Fig. 8a; otherwise, fluorescence is shown on the left and the corresponding phase-contrast photograph on the right. Pretreatment of myofibrils: (a and b) washed once with Solution A; (c and d) washed nine times with Solution A; (e and f): washed nine times with Solution A containing 0.5% Triton X-100; (g and h) extracted with Solution C (45 min). Fig. 8a shows a bright fluorescent line running through the middle of each H zone (H). (a-f): × 1,700; (g and h): × 1,200.
skeletal muscles contain almost exclusively MM-CK; as in the adult, only faint traces of MB-CK are detectable.

The myofibrils used in the foregoing studies were derived from adult muscle and, consequently, from cells containing MM-CK as the single predominant isoenzyme. By examining the immunofluorescent staining of myofibrils from somites, a tissue in which BB-CK is still the predominant enzyme and in which only traces of MB or MM-CK can be detected electrophoretically, we hoped to be able to learn more about the specificity of the interaction of CK with the M-line region. Somites of 4-day embryos were chosen for this purpose because, in this tissue, terminally differentiated muscle cells containing myofibrils that are indistinguishable from those of adult muscle appear very early (by day 3-4 in the most anterior somites) (26). Myofibrils prepared from somite-derived back muscle at later developmental stages were also stained by indirect immunofluorescences for comparison. Back muscles were chosen because it is not clear that chicken breast muscle, the tissue from which we routinely prepare myofibrils, is entirely derived from the somites (21).

Myofibrils from 4-day somites showed an intense fluorescence restricted to the M-line when stained with anti-MM-CK serum as did myofibrils from back muscle of later embryos (11 or 14 days) or adults (Fig. 9a). Especially in myofibrils from earlier stages, very weak fluorescence was sometimes seen at the Z-line (Fig. 9a and c); this perhaps reflects a higher relative concentration of MB-CK early in muscle differentiation (see above). Washed 4-day somite myofibrils reacted with anti-BB-CK serum showed some indication of banding but fluoresced fairly intensely along their entire length (Fig. 10a). With myofibrils from later embryonic stages, the fluorescence is increasingly restricted to broad bands covering the entire I-region of each sarcomere (Fig. 10b-f). Finally, as also observed for adult breast muscle myofibrils, faint fluorescence restricted to the narrow Z-lines within the I-region is occasionally seen after reaction with anti-BB-CK serum in adult back muscle myofibrils (Fig. 10g and h).

These staining patterns demonstrate that MM-CK (and only MM-CK) is bound at the M-line in cells in which the absolute concentration of MM-CK is much lower than in adult muscle and in which B-subunit-containing forms of CK predominate. The observed patterns of staining suggest that the I-region (at least in myofibrils from earlier developmental stages) possesses binding sites for BB-CK.

Supplementation of Native Skeletal Myofibrils with Purified CK Isoenzymes

The interaction of the two homodimeric CK isoenzymes with defined regions of skeletal muscle myofibrils was further investigated by incubating washed myofibrils from adult breast muscle with either MM- or BB-CK, followed by immunofluorescent staining with homologous antiserum (Table III). Supplementation with exogenous MM-CK enhanced the apparent intensity of M-line fluorescence; in addition, some MM-CK seemed also to be bound to the Z-lines. Incubation with BB-CK resulted in an increased Z-line fluorescence; at most, very faint fluorescence was detected at the M-line. Myofibrils supplemented with large excess of BB-CK and then reacted with anti-MM-CK serum showed no reduction in M-line fluorescence, indicating that BB-CK could not compete effectively with the MM-CK already present for binding sites at the M-line (not shown). In terms of binding specificity, exogenous MM-CK exhibits a preference for the M-line region while exogenous BB-CK binds almost exclusively at the Z-line region.

Attempted Reconstitution of the M-line of Extracted Myofibrils

The foregoing results strongly support our earlier contention (see introductory paragraph) that MM-CK is an integral element of the structure of skeletal myofibrils. To prove this point conclusively, it will be necessary to show that the protein can participate in the reconstitution of the M-line structure in myofibrils from which the M-line has been completely extracted. In this connection, although it appears that several proteins contribute to the definitive M-line structure (see introductory paragraph), it will be important to learn to what extent (if any) MM-CK alone is able to reconstitute the M-line lattice joining adjacent thick filaments. We report here a first series of experiments undertaken to determine whether MM-CK, when incubated with extracted myofibrils, binds specifically to the M-line region. In a second series, now in progress, we are studying the ability of MM-CK to combine with other putative M-line proteins in reconstituting a complete M-line in skeletal myofibrils.

The myofibrils used in these experiments were
Figure 9 Localization of antigen capable of reacting with anti-MM-CK serum in native skeletal myofibrils from different development stages. Fluorescence is shown on the left and the corresponding phase-contrast photograph on the right. All fibrils were washed in solution A. (a and b) Myofibril from somites of a 4-day chick embryo; strong fluorescence in the M-line (M), occasional weak fluorescence in the Z-line. (c and d) Myofibril from back muscle of an 11-day embryo; strong fluorescence in the M-line, very little fluorescence in the Z-line. (e and f) Myofibrils from back muscle of a 14-day embryo; strong fluorescence in the M-line, very little fluorescence in the Z-line. (g and h) Myofibrils from back muscle of an adult chicken; strong fluorescence in the M-line. × 1,700.
Figure 10 Localization of antigen capable of reacting with anti-BB-CK serum in native skeletal myofibrils from the same stages as in Fig. 9. (a and b) Fluorescence dispersed over the whole myofibril. (c and d) Fluorescence dispersed over the whole myofibril, but indication of broad bands in I-region (I). (e and f) Fluorescence restricted to I-region. (g and h) Weak fluorescence restricted to Z-line (Z). (a-f) × 1,700. (g and h) × 1,250.
TABLE III
Localization of CK by the Indirect Immunofluorescence Technique after Supplementation of Native Skeletal Myofibrils with Purified CK Isoenzymes

| Condition                        | M-line | Z-line |
|----------------------------------|--------|--------|
|                                  | anti-MM-CK* | anti-BB-CK* | anti-MM-CK | anti-BB-CK |
| Native myofibril (no supplementation) | ++++‡ | - | ±§ | + |
| Supplementation with pure MM-CK (1 mg/ml) | +++ | + | ± | ++ |
| Supplementation with pure BB-CK (1 mg/ml) | ± | | | |

* Antisera used are specific for either M- or B-subunits; thus, a reaction with the hybrid isoenzyme, MB-CK, if present, is expected with both antisera.
‡ Intensity of fluorescence: ++++, strong; ++, medium; +, weak; ±, trace; and −, none.
§ Possibly MB-CK.

either freshly extracted or stored in glycerol after extraction (see Materials and Methods); in both cases, M-line bound MM-CK was completely removed (Fig. 11 b). Fig. 11 c–h shows the results of attempts to reconstitute the M-line of such myofibrils with CK isoenzymes, using the different incubation procedures described under Materials and Methods. While fluorescent cross striations appeared in all cases, patterns most resembling native myofibrils were obtained (Fig. 11 g and h) with short-time competition incubation of freshly extracted myofibrils (glycerinated fibrils not tested). Under these conditions, MM-CK bound preferentially to the M-line region (only faint fluorescence at the Z-line) while BB-CK bound exclusively to the Z-line region. In this case, as in all the others, however, the fluorescence intensity was generally less than observed with native myofibrils. The binding patterns obtained with long-time incubation were less specific. With freshly extracted myofibrils, neither MM- nor BB-CK bound exclusively to either the M-line or the Z-line region, but the intensity of staining with anti-MM-CK serum at the Z-line region was generally weak and staining for BB-CK was generally more intense at the Z-line than at the M-line region (Fig. 11 c and d). Extracted and subsequently glycerinated myofibrils fluoresced at the M-line after incubation with either isoenzyme, although more strongly with MM-CK than with BB-CK (Fig. 11 e and f). Rearrangement or conformational alteration of the proteins leading to a modification of binding sites (particularly at the Z-line) might be responsible for the anomalous binding pattern seen with glycerinated myofibrils; Pepe (54) has observed a considerably changed reactivity of myofibrillar antigens in glycerinated myofibrils.

It has been demonstrated (27) that purified MM-CK binds strongly to isolated myosin rods. In showing that, in the absence of other M-line proteins, MM-CK binds precisely at the M-line of extracted myofibrils, we provide further evidence that MM-CK possesses binding sites for the rod portions of the myosin molecules as they are arrayed at the M-line. The binding patterns just described suggest, moreover, that BB-CK also possesses binding sites capable of interacting, albeit more weakly than MM-CK, with the tail-to-tail arrangement of myosin molecules exposed at the M-line. It has recently been suggested (5) that there is a close association between the thiol groups (presumably at the active center) essential for MM-CK activity and the myosin binding site of this isoenzyme. If this is so, it is perhaps not surprising that BB-CK (which must have an active center similar to that of MM-CK) can also bind to myosin.

Electron microscopy revealed an enhancement of electron-dense material in the M-line region of extracted myofibrils after supplementation with CK, but in no case was anything resembling a complete M-line (not shown) seen.

DISCUSSION
We have presented important additional evidence that the small fraction of skeletal muscle MM-CK which is located in the M-line is a bona fide myofibrillar protein: (a) the identity of MM-CK and the Morimoto-Harrington M-line protein (46) was confirmed by their identical mobility in SDS gels and by their identical peptide patterns after cyanogen bromide fragmentation. (b) By the use of specific antibody prepared against MM-CK, MM-CK was localized within the M-line in two types of experiments: (i) indirect fluorescent antibody staining of myofibrils before and after extraction; and (ii) prevention of M-line extraction by prior incubation of native myofibrils with anti-MM-CK serum (c) MM-CK was detected at the M-line in myofibrils from somites of 4-day-old embryos,
demonstrating a binding specificity in a differentiating muscle tissue containing only minute amounts of the MM-CK isoenzyme. (d) Preferential binding of MM-CK molecules to the M-line region of extracted myofibrils was demonstrated. (e) Regular myofibrillar cross striations were observed in tissue slices stained for CK with a specific histochemical procedure indicating that the binding of CK to the M-lines of isolated myofibrils was not an artifact of myofibril preparation.

Morimoto and Harrington (46) had noted that the dimensions of their "M-line protein," which we now know to be MM-CK, fit well with those required of "M-bridges" in the M-line model proposed earlier by Knapppeis and Carlsen (33) on the basis of electron microscope evidence. This identification of MM-CK with the M-bridges of the Knapppeis-Carlsen model was adopted as an important feature of our recently proposed model of the M-line (64, 66), in which we attempted to integrate the biochemical and ultrastructural data then available. Since it has been shown that MM-CK can bind directly to myosin molecules (5, 27) as well as to myosin filaments (shown by Morimoto and Harrington [46]), at least one important criterion required of the M-bridges is fulfilled by MM-CK. Recently, it has been reported that MM-CK binds more strongly to heavy meromyosin and to myosin subfragment S-1 than it does to light meromyosin (5). This finding, while it does introduce a possible complication, does not of itself invalidate the notion that the M-bridges connecting thick filaments are MM-CK molecules.

On the other hand, our proposal that the "M-filaments" observed by Knapppeis and Carlsen (33) and more recently by others (58) might be composed of the A-protein (subunit mol wt approx. 100,000) described by Eaton and Pepe (12) will have to be revised, if not abandoned altogether. For one thing, it appears possible that the M-filaments seen in thin longitudinal sections are thickenings in the central regions of thick filaments whose axes are in another plane. For another, whereas our proposal had been based on evidence suggesting that A-protein possessed both a tendency to form filamentous aggregates and the ability to bind to MM-CK (16), it has recently become clear (8, 25, 61) that A-protein is actually phosphorylase b, which is not known to self-aggregate into filaments. It has also been questioned, on the basis of immunofluorescence localization studies (60), whether phosphorylase is a component of the M-line at all. Studies recently performed in this laboratory (17, 25) suggest: (a) that it may not have been self-aggregation of Protein A, i.e., of phosphorylase b, which Eaton and Pepe (12) observed, but rather formation of an insoluble complex of phosphorylase b, glycogen and the 160,000-dalton protein; (b) that the 160,000-dalton protein is glycogen debranching enzyme; and (c) that both phosphorylase and glycogen debranching enzyme can be localized in the M-line by immunofluorescence. Work is in progress to determine whether one or both of the latter proteins are components of the M-filaments and to define the interactions of these proteins not only with each other, but also with myosin, MM-CK, and glycogen.

The CK firmly bound to myofibrils (approx. 0.2 EU/mg) amounts to approximately 1 CK for every 38 myosin molecules. The latter estimate is based on the following assumptions: mol wt of 83,000 for CK (10) and 470,000 for myosin (59); 55% myosin content of myofibrils; CK specific activity of 80 EU/mg.

**Figure 11** Attempted reconstitution of M-line-extracted skeletal myofibrils with CK isoenzymes. For details of the different modes of incubation, see Materials and Methods. (a) Native myofibrils washed with Solution A and stained with anti-MM-CK serum to show M-line-bound MM-CK. Strong M-line (M) fluorescence. (b) 45-min extracted myofibrils stained with anti-MM-CK serum demonstrating that no MM-CK remains at the M-line. (c) Long-time incubation of freshly extracted myofibrils with MM-CK, staining with anti-MM-CK serum; fluorescence in the M-line, weak fluorescence in the Z-line (Z). (d) Same as Fig. 11c, but incubation with BB-CK; staining with anti-BB-CK serum; generally stronger fluorescence in the Z-line, relatively little fluorescence in the M-line; occasionally M-line fluorescence alone as seen in one myofibril in the photograph. (e) Long-time incubation of extracted and glycerinated myofibrils with MM-CK, staining with anti-MM-CK serum; strong fluorescence of M-line only. (f) Same as Fig. 11e, but incubation with BB-CK, staining with anti-BB-CK serum; weak fluorescence in the M-line. (g) Short-time incubation of freshly extracted myofibrils with BB- and MM-CK together, staining with anti-MM-CK serum; fluorescence in the M-line, only weak fluorescence in the Z-line. (h) Same as Fig. 11g, but staining with anti-BB-CK serum; generally weak fluorescence of Z-line only. (a-e and e-h) x 1,700; (d) x 2,200.
within a factor of two of the number of CK molecules required by our model (64, 66) in which CK molecules are the transverse M-line elements; dividing the 18 M-bridges per thick filament of the Knappeis-Carlsen (33) model by a value of 384 myosins per thick filament (28) gives an expected ratio of 1 CK for every 21 myosins. The amount of CK present in the M-line would be in even better agreement with Pepe's (54, 55) M-line model, which has somewhat fewer M-bridges per thick filament. In any event, in view of the number of uncertain assumptions involved in such calculations, one can conclude on the present evidence that there is sufficient myofibrillar CK for it to be taken seriously as a candidate for the transverse M-line elements.

The number of MM-CK molecules in myofibrils may also suffice to catalyze the regeneration of ADP produced by the myosin-ATPase reaction during each contraction cycle of skeletal muscle. The enzymatic capacity (as measured by the $V_{\text{max}}$) of purified CK is 25-35 times higher on a molar basis than that of isolated myosin-ATPase measured under identical conditions (Table IV). If similar conditions obtain in vivo, the 18 CK molecules per thick filament would be enough to maintain a constant level of ATP in the intramyofibrillar space (7, 43). Kinetic studies with a coupled system consisting of purified myosin and CK indicate that, although CK binding to myosin may inhibit the myosin-ATPase reaction (4, 67), maximal activity of the coupled system can be obtained at a ratio of less than 1 CK for every 10 heavy meromyosin molecules (68).

The evidence on the intracellular distribution of the substrates of the myosin ATPase and CK reactions (7, 22) is consistent with having most ATP regeneration take place within the myofibrils. Creatine and phosphocreatine diffuse readily into and out of the intramyofibrillar space, whereas adenine nucleotides (which are in any case present in lesser amounts) do not (31, 47, 48). Assuming further that soluble CK does not penetrate freely into the intramyofibrillar space, the CK bound at the M-line would have a crucial function, since ADP produced by myosin-ATPase action could only be rephosphorylated by the CK located in the myofibrils.

These considerations are fully in keeping with models of the "functional compartmentation" of adenine nucleotides and of components of the creatine/phosphocreatine/CK system within the muscle cell (2, 23, 24, 48, 56, 67). According to such models, the ADP produced in muscle contraction does not diffuse to the mitochondria to be converted to ATP. Instead, the creatine produced in the regeneration of ATP diffuses to the mitochondria, where CK catalyzes its reaction with ATP (acceptor reaction). The ADP produced stimulates mitochondrial ATP production (respiratory control), while the phosphocreatine (which, unlike ATP, is a substrate for only one enzyme-catalyzed reaction in the cell) then diffuses to the myofibrils. There, other CK molecules effect the regeneration of ATP for muscle contraction, completing the cycle. Up to now, the major piece of direct evidence favoring such models was the discovery of a distinct form of CK bound to the outer surface of the inner mitochondrial membrane (20, 29, 30). Several groups of workers had suggested the existence of a counterpart-CK bound to myofibrillar proteins (reviewed in reference 16). The data presented in this paper and in the accompanying one (65) on the quantity, isoenzyme type, and localization of CK in myofibrils from two kinds of muscle tissue strongly support the functional compartmentation models.

We are indebted to Mr. H. J. Kuhn for help with some of the early localization experiments. We thank Drs. C. Heizmann, H. Lebherz, F. Pepe, M. Reedy, M. C. Schaub, A. G. Szent-Györgyi, and J. Trinick for helpful discussions and Drs. J. B. Burckhardt, M. Eppenberger,

| Creative kinase* | Myosin-ATPase | CK | Myosin-ATPase |
|------------------|---------------|----|---------------|
| $V_{\text{max}}$ | $V_{\text{max}}$ | $V_{\text{max}}$ (CK) | $V_{\text{max}}$ (Myosin-ATPase) |
| 70,000$^\dagger$ | 2,850$^\dagger$ | 35 | 25 |
| 134,000$^\ddagger$ | 3,943$^\ddagger$ | 25 | 34 |

* Creatine phosphate$^{2-} + MgADP^{2-} + H^+$

† MgATP$^{2-}$

‡ $MgATP^{2-} + MgADP^{2-} + P_i + H^+ + Creatine.$

§ Moles ATP formed/mole CK/minute at 25°C, pH 7.0; pH-stat measurement (28).

¶ Same as $\S$, but at 35°C.

5 Mols ATP hydrolyzed/mole myosin (actin-activated myosin-ATPase)/minute at 25°C, pH 7.0; pH-stat measurement (40).

** Mols ATP hydrolyzed/mole myosin (Mg$^{2+}$-activated myofibrillar myosin-ATPase)/minute at 35°C, pH 7.2; determination of inorganic phosphate released (1).
R. von Fellenberg, and G. Pelloni for their advice and help with the various immunological methods.

This paper was supported by grant no. 3.8640.72 of the Swiss National Science Foundation, by a grant of the Muscular Dystrophy Association of America to H. M. Eppenberger, and by a grant of the Schweizerische Kommission für Molekularbiologie to T. Wallimann.

Parts of this work were presented in preliminary form at the Third International Conference on Isozymes, New Haven, Connecticut in April, 1974 (16), and at the Federation of European Biochemical Societies' Meeting in Budapest, Hungary, in August, 1974 (65).

Received for publication 1 June 1976, and in revised form 15 June 1977.

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