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A Protein That Binds Specifically to the M-Line of Skeletal Muscle Is Identified as the Muscle Form of Creatine Kinase

(myofibril/M-line protein/localization/isoenzymes)

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ABSTRACT **Published information on the properties of two proteins from chicken muscle, creatine kinase (MM-creatine kinase) and an M-line protein, suggested that they might be identical molecules. Different published procedures were used to purify the two proteins to homogeneity, and the properties of the two preparations were compared. Creatine kinase specific activity increased during purification of M-line protein, reaching a value comparable to that of purified MM-creatine kinase. The two proteins migrated identically in two electrophoretic systems and, after electrophoresis, both could be stained for creatine kinase activity. Double immunodiffusion tests with antibody prepared against MM-creatine kinase established the serological identity of the two protein preparations. Immunofluorescent studies showed that antiserum against MM-creatine kinase was bound in a regular pattern at the centers of the A-band regions of isolated myofibrils. These data show conclusively that the M-line protein and MM-creatine kinase are identical.**

The major proteins of cross-striated skeletal muscle myofibrils (e.g., myosin, actin, tropomyosin) have been extensively investigated in recent years; their known interactions form the basis of our understanding of muscle contraction. Lately, attention has turned increasingly to the "minor" components of the contractile apparatus, those remaining proteins that may have structural, regulatory, and/or catalytic functions.

One approach has been to focus on proteins that regulate activities associated with the contractile mechanism (e.g., Mg^{+2}-stimulated adenosine triphosphatase) and to resolve, isolate, and characterize these factors (1, 2). Another approach has been to try to localize known proteins within myofibrils, by selective extraction, immunofluorescence, and histochemistry. As an example, there is evidence that some of the creatine kinase within muscle cells may be bound to myofibrils (3–7), though most of the creatine kinase appears to be soluble, located in the intermyofibrillar space (8, 9). A third approach has involved the localization, within the myofibrillar structure, of proteins for which no biological activity is yet known. Among the proteins studied in this way is one that evidently is localized at the M-line within the A-band region of each sarcomere (10, 11).

Starting with a low ionic strength extraction of chicken skeletal muscle, Morimoto and Harrington (11) were able to purify this M-line protein to apparent homogeneity. These authors showed that the purified protein is a dimeric molecule of molecular weight 88,000, and they also determined its aminoacid composition.

Comparison of these published data for M-line protein with the known data on molecular weight, subunit structure, extractability in low ionic strength, and aminoacid composition of the muscle (MM) form of creatine kinase (Table 1) suggested to us that these two muscle proteins might be identical. We have, therefore, purified M-line protein by the method of Morimoto and Harrington (11) and compared it with MM-creatine kinase purified by a very different procedure (13, 14).

In this communication we present the following evidence that M-line protein and MM-creatine kinase are identical molecules. (*i*) During the purification of M-line protein from an extract of washed muscle mince, there is a 4.4-fold increase in creatine kinase specific activity. (*ii*) M-line protein and MM-creatine kinase are indistinguishable in their migration in polyacrylamide and cellulose polyacetate electrophoresis and, after electrophoresis, both can be specifically stained for creatine kinase activity. (*iii*) In Ouchterlony (15) double-diffusion tests, M-line protein and MM-creatine kinase react identically with antibody prepared against purified MM-creatine kinase. (*iv*) An antigen localized at the A-bands of the myofibril and capable of reacting with antiserum prepared against MM-creatine kinase can be detected by immunofluorescence.

METHODS

Isolation of M-Line Protein. The procedure used (11) involves: (*i*) exhaustive washing of minced chicken muscle tissue with relatively high ionic strength buffer [0.1 M KCl in 0.02 M phosphate buffer (pH 7.0)]; (*ii*) extraction of the mince at low ionic strength [5 mM Tris·HCl (pH 7.7)]; (*iii*) removal of contaminants from the extract by acid precipitation (0.2 M acetic acid to pH 5.0); followed by (*iv*) batch treatment with DEAE–cellulose (Whatman DE-52). Part of the supernatant solution after acid precipitation was removed before addition of DEAE–cellulose and neutralized separately by dialysis against 50 mM Tris·HCl (pH 8.6).

Isolation of MM-Creatine Kinase. Creatine kinase from ground chicken muscle was purified according to Eppenberger *et al.* (13): (*i*) extraction at low ionic strength (10 mM KCl); (*ii*) ethanol precipitation; (*iii*) precipitation of the enzyme with $MgSO_4$; and (*iv*) fractionation by column chromatography on DEAE–cellulose (gradient elution).

Preparation of Antibody. MM-creatine kinase from chicken (0.42 mg) was dissolved in 0.5 ml of 50 mM Tris·HCl (pH 8.0), mixed with 0.5 ml of Freund's adjuvant, and injected into the foot pad of a rabbit. A second injection was made

after 3 weeks, and at 4 weeks blood was removed from an ear vein. After it remained for 3 hr at room temperature and 12 hr at 4°, the blood was centrifuged to remove the clot, and the supernatant fluid was taken as antibody preparation.

Creatine Kinase Assays. Creatine kinase activity was measured spectrophotometrically by the method of Eppenberger *et al.* (13). One unit of creatine kinase is defined as the amount necessary to catalyze formation of 1 μmol of ATP per min. Samples were diluted as necessary to keep the amount of enzyme in each assay below 0.005 unit, and correction was made for any ATP production in the absence of creatine phosphate.

Immunofluorescent Localization. Myofibrils were prepared in the same buffer [0.1 M NaCl in 0.02 M phosphate (pH 7.0)] used for the initial washes in the purification of M-line protein (11). Fresh chicken leg muscle was minced, suspended in 10 volumes of buffer, disrupted briefly in a Teflon–glass homogenizer, and centrifuged for 15 min at 10,000 × *g*. The pellet was resuspended in 20 volumes of buffer, homogenized at half-speed for 30 sec in a Sorvall Omnimixer, and again centrifuged. The pellet, containing myofibrils, was then washed twice more with 30 volumes of buffer before use. A small amount of this preparation was incubated at room temperature for 30 min with a 1:40 dilution of antiserum against MM-creatine kinase in phosphate-buffered saline (pH 7.0), washed twice with 30 volumes of phosphate-buffered saline, incubated with a 1:70 dilution in phosphate-buffered saline of fluorescein isothiocyanate-labeled antiserum against rabbit IgG (Central Laboratories of the Netherlands Red Cross, Postbus 9190, Amsterdam), and washed twice more with 30 volumes of phosphate-buffered saline. The stained preparations were finally mounted in a 4:1 mixture of glycerine and 0.1 M NaCl in 0.1 M glycine (pH 9.0).

TABLE 1. *Comparison of published data on the properties of creatine kinase and M-line protein from chicken skeletal muscle.*

	MM-creatine kinase	M-line protein
Molecular weight	83,700 (12)	88,000 (11)
Oligomeric composition	2 Identical subunits (12)	2 Identical subunits (11)
Extractability	Extractable at low ionic strength (7, 13)	Extractable at low ionic strength (11)
Myofibrillar localization	At least some of the CPK extractable at low ionic strength binds tightly to myofibril (7)	Specific binding to M-lines of myofibril (11)
Aminoacid composition*		
Lys	80.3†	73.5‡
His	42.0	36.7
Arg	49.4	40.1
Asp	90.1	93.7
Thr	35.8	33.2
Ser	39.5	44.2
Glu	102.5	96.0
Pro	50.6	44.7
Gly	74.1	68.7
Ala	40.7	41.8
Val	60.5	59.5
Met	24.7	21.1
Ile	29.6	36.2
Leu	87.6	81.3
Tyr	19.8	30.0
Phe	40.8	40.4

Reference nos. are given in parentheses. CPK = creatine kinase.

* No. of residues per 10^5 g of protein.

† Hydrolysis for 24 hr, uncorrected values (13).

‡ Hydrolysis for 20 hr, uncorrected values (11).

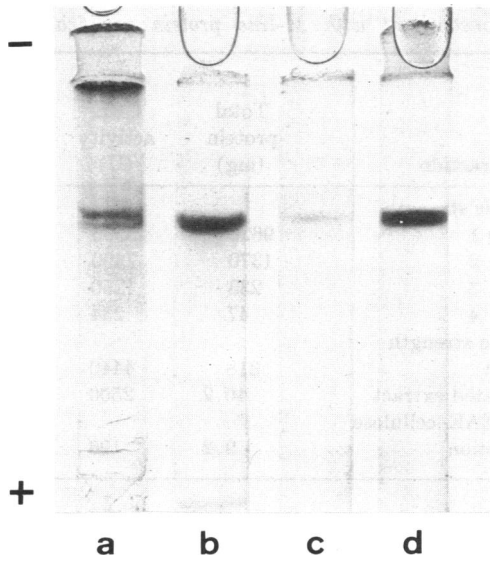

FIG. 1. Disc gel electrophoresis of M-line protein at various stages of purification (*a–c*) and of purified MM-creatine kinase (*d*). (*a*) Low ionic strength mucle extract (80 μg); (*b*) neutralized supernatant after acid precipitation (50 μg); (*c*) supernatant after batch treatment with DEAE–cellulose (15 μg); (*d*) MM-creatine kinase (50 μg). Electrophoresis in 7.5% polyacrylamide gels at pH 9.5 in 0.37 M Tris–glycine buffer was performed for 3 hr at 4° and at a current of 3 mA per tube. Gels were stained for 1 hr with 1% Amido schwarz in 7% acetic acid.

Protein was determined by the method of Lowry *et al.* (16).

RESULTS

Creatine Kinase in Purified M-Line Fractions. Disc gel electrophoresis (Fig. 1*a–c*) at various stages in the purification of M-line protein showed that a single protein species had been obtained after the acid precipitation step. Because the DEAE–cellulose adsorption step resulted in variable high losses of protein from the supernatant, this step was omitted and acid-treated extract was used as M-line protein in these studies. Creatine kinase activity in various fractions was monitored during purification of M-line protein (Table 2). More than 76% of the total extractable creatine kinase was removed during the exhaustive washing at relatively high ionic strength; only 24% was recovered in the low ionic strength extract of muscle. Purification of M-line protein from this extract by acid treatment resulted in a 4.4-fold increase in creatine kinase specific activity. The final specific activity of 62 U/mg is somewhat higher than that (41 U/mg) obtained for the sample of purified MM-creatine kinase used in this work. The creatine kinase activity in the dilute prepa-

TABLE 2. *Purification of creatine kinase (CPK) activity concomitant with M-line protein purification*

Fraction	Total protein (mg)	Total CPK activity (U)	CPK specific activity
High ionic strength washes 1	9620	5500	0.58
2	1370	7490	5.47
3	233	1250	5.36
4	47	234	4.98
Low ionic strength extract	318	4440	14.0
Acid-treated extract	40.2	2500	62.2
After DEAE–cellulose adsorption	9.2	196	21.3

ration obtained after DEAE–cellulose adsorption was relatively unstable, which presumably accounts for the lower specific activity of this fraction (Table 2).

Electrophoretic Evidence for the Identity of M-Line Protein and MM-Creatine Kinase. Purified MM-creatine kinase has the same mobility in disc gel electrophoresis as M-line protein (Fig. 1). The two proteins also have identical mobilities in cellulose polyacetate electrophoresis (Fig. 2). After electrophoresis, M-line protein showed a single band when stained for protein (Figs. 1 and 2), and the mobility of this band coincides with the creatine kinase activity of both MM-creatine kinase and M-line protein as made visible with a specific histochemical stain (Fig. 2).

Immunological Evidence for the Identity of M-line Protein and MM-Creatine Kinase. Both protein preparations were tested in Ouchterlony (15) double-immunodiffusion tests for their ability to react with antibody prepared against MM-creatine kinase (Fig. 3). The precipitin bands formed between antiserum against MM-creatine kinase and M-line protein fuse completely with those formed between antiserum against

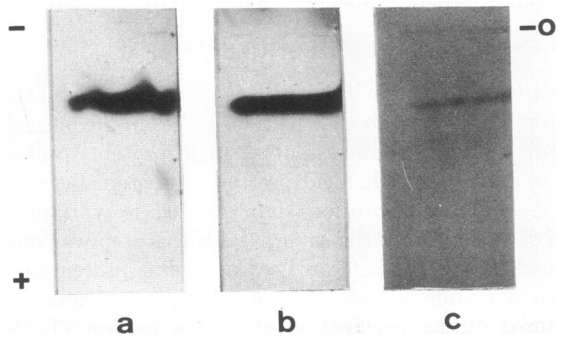

FIG. 2. Cellulose polyacetate electrophoresis. (*a*) 5 μg of MM-creatine kinase stained for creatine kinase activity; (*b*) 2 μg of M-line protein (purified through the acid-precipitation step) stained for creatine kinase activity; (*c*) 2 μg of M-line protein (purified as in *b*) stained for protein with 1% Amido schwarz in 7% acetic acid. Gelman Sepraphore III strips were equilibrated with 60 mM sodium barbital (pH 8.6), containing 0.06% 2-mercaptoethanol. Samples (about 2 μl applied at *0*) were electrophoresed in the cold (4°) for 150 min at 250 V. Zones of creatine kinase were visualized with a specific staining method (14).

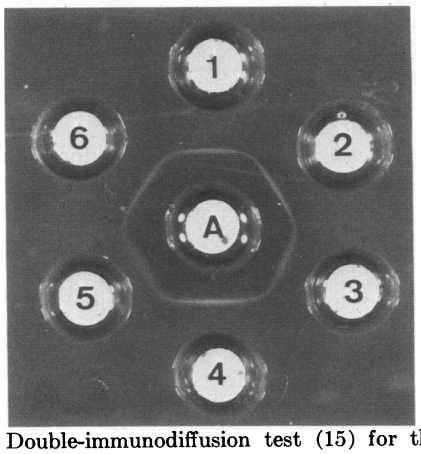

FIG. 3. Double-immunodiffusion test (15) for the identity of M-line protein and MM-creatine kinase. Center well (*A*) contains antibody prepared against MM-creatine kinase. MM-creatine kinase is in outer wells *1* (12 μg), *3* (6 μg), and *5* (3 μg); M-line protein (purified through the acid-precipitation step) is in wells *2*, *4*, and *6* (6 μg each).

MM-creatine kinase and MM-creatine kinase, demonstrating that MM-creatine kinase is serologically identical to M-line protein.

Immunofluorescence. Antibody prepared against M-line protein can be used to show that this protein binds specifically to the M-line (10, 11). If MM-creatine kinase and M-line protein are identical, then antibody prepared against MM-creatine kinase should also bind to the M-line. Fig. 4 shows a regular, cross-striated banding pattern in a myofibril reacted with rabbit antiserum against chicken MM-creatine kinase and then incubated with a fluorescein-labeled horse antiserum against rabbit IgG. Phase-contrast microscopy of the same fibril showed that the bright fluorescent lines were located in the middle of the A-band, i.e., at the M-line. This result demonstrates the presence of an antigen localized in the M-line and capable of reacting specifically with antiserum against MM-creatine kinase.

DISCUSSION

Our results on the parallel purification of MM-creatine kinase and M-line protein, their electrophoretic and immunological identity, and the localization of creatine kinase within the A-band of the myofibril, taken together with previously published information on the properties and composition of the two proteins, provide conclusive evidence for the identity of MM-creatine kinase and M-line protein.

The finding that creatine kinase binds to a specific region of the myofibril may be important to our understanding of muscle contraction. Data have long been available that suggest that creatine kinase interacts with structural components of muscle and that this interaction may be of basic importance in muscle energetics (3–7). Although it was argued (3) that creatine kinase can catalyze a reaction between actin-bound ADP and creatine phosphate, it now appears that MM-creatine kinase does not bind to actin (17). M-line protein (MM-creatine kinase) does bind to myosin (5, 11) and may bind to the actomyosin complex (7). Studies of the interactions of MM-creatine kinase with myosin and myosin fragments may lead to a better understanding of the structure of the M-line. The catalytic activity of creatine

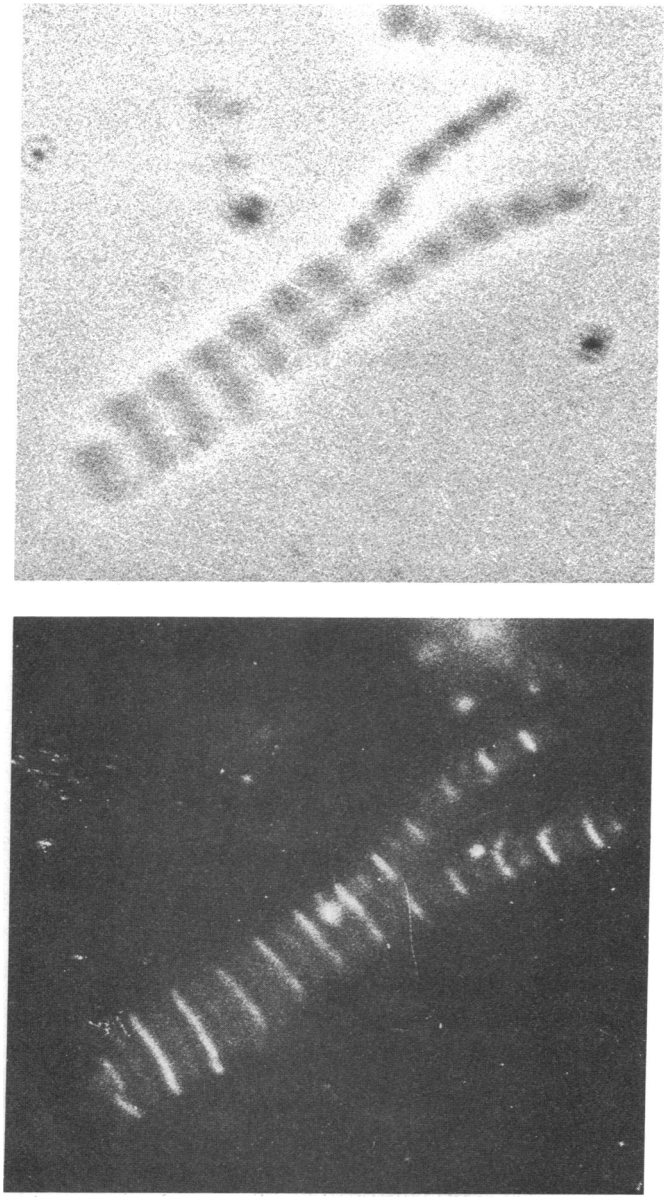

FIG. 4. Binding of antiserum against MM-creatine kinase to myofibrils. *Top*, phase contrast and *bottom*, fluorescence microscopy of the same fibril (×3500). The fibril shown is partially contracted [stage B according to the nomenclature of Tunik and Holtzer (21)], with alternating A-bands (darker, broader) and I-bands (lighter, narrower) seen in phase contrast (*top*). Each bright fluorescent line (*bottom*) runs through the middle of an A-band. A Leitz Orthoplan microscope equipped with an NPL 100x phase-contrast (oil immersion) objective was used. An Osram HBO 200 mercury lamp served as light source for vertical illumination. Excitation filters: UG 1, BG 38; emission filters: K 495, K 510.

kinase may be affected in important ways when it is bound to the myofibril.

Most vertebrates possess three isoenzymic forms of creatine kinase formed by binary association of M- and B-subunits into active dimeric molecules, MM-, MB-, and BB-creatine kinase (18). These enzyme forms are distributed in a tissue-specific manner, and in many cases there is a transition during development of a tissue or organ from one form to another (19).

In chickens and in other birds, both heart and skeletal muscle contain BB-creatine kinase early in embryonic life; in heart BB-creatine kinase remains the predominant form throughout life, while adult skeletal muscle contains only the MM form. Heretofore, comparison of purified MM- and BB-creatine kinases has revealed only relatively minor differences in catalytic parameters (13), and the physiological significance of the different isoenzymes has remained obscure.

It should be noted that the scheme for purification of M-line protein of Morimoto and Harrington (11) is a considerably simpler method for isolation of MM-creatine kinase than those used previously for isolating the chicken enzyme (13, 20). It is possible that one or more of the procedures will be useful in preparing MM-creatine kinase from other sources, or in achieving a simpler purification method for the BB or MB enzyme (14).

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