LOCALIZATION OF CREATINE KINASE ISOENZYMES IN MYOFIBRILS

II. Chicken Heart Muscle

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ABSTRACT

Chicken heart muscle contains almost exclusively the BB isoenzyme of creatine kinase (CK), its myofibrils, moreover, lack an M-line. This tissue thus provides an interesting contrast to skeletal muscle, in which some of the MM-CK present as predominant CK isoenzyme is bound at the myofibrillar M-line. Approx. 2% of the total CK activity in a chicken heart homogenate remains bound to the myofibrillar fraction after repeated washing cycles; both the fraction and the absolute amount of CK bound are about threefold lower than in skeletal muscle. Almost all of the bound enzyme is located within the Z-line region of each sarcomere, as revealed by indirect fluorescent-antibody staining with antiserum against purified chicken BB-CK. After incubation with exogenous purified MM-CK, positive immunofluorescent staining for M-type CK at the H-region of heart myofibrils was observed, along with weaker fluorescence in the Z-line region. Chicken heart myofibrils may thus possess binding sites for both M and B forms of CK.

In the preceding paper (23) we presented considerable evidence that a small fraction of the MM isoenzyme of creatine kinase (MM-CK) in chicken skeletal muscle is specifically bound at the middle of the H-zone of each sarcomere, where it contributes to the structure of the M-line. We also examined the specificity of binding of exogenous CK isoenzymes (MM and BB) to defined regions of both native skeletal muscle myofibrils and those from which the M-lines had previously been extracted. The MM isoenzyme bound preferentially at the M-line regions of skeletal myofibrils. Weaker binding of BB-CK was also observed, however, with an apparent preference for the Z-lines at the middle of each I-band.

In chicken heart, unlike chicken skeletal muscle (4, 5) and unlike both skeletal and cardiac muscle of mammals (6), BB-CK is the predominant CK isoenzyme throughout the life of the organism (3). In addition, the myofibrils of chicken cardiac muscle differ from those of most other higher vertebrate striated muscles in that their H-zones generally lack an M-line discernible in the electron microscope (18).

Chicken heart muscle therefore suggested itself as a source of myofibrils for further studies of the

specificity (with respect both to location and isoenzyme type) of the interactions of creatine kinases with myofibrils.

MATERIALS AND METHODS

All antisera and samples of purified CK isoenzymes were the same ones used in the preceding paper (23). Except as expressly noted, the procedures described for skeletal muscle in the preceding paper were followed exactly in the preparation and subsequent treatment of myofibrils from fresh, whole, well-cleaned, adult chicken hearts.

RESULTS

Quantitation and Localization of BB-CK Bound to Native Chicken Heart Myofibrils

After repeated washing with "Solution A" (0.1 M KCl, 1 mM ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetate (EGTA), 1 mM dithiothreitol (DTT), 5 mM EDTA; pH 7.0), chicken heart myofibrils contain significant amounts of bound CK activity, amounting to 2–3% of the activity measured in the crude heart homogenate from which the myofibrils were prepared (Fig. 1). Since chicken heart contains no MM-CK and since our chicken heart myofibrils, as expected (see introductory paragraph), lacked M-lines (21), the detection of appreciable amounts of firmly bound CK in heart myofibrils came as a surprise. The total CK activity of the heart homogenate ws only one-third that of a comparable skeletal muscle extract. Since the specific activity of BB-CK, the predominant isoenzyme in heart, is only about half that of MM-CK (23), it could then be calculated that one milligram of CK was bound to each gram of myofibrils, about a third the amount present in skeletal muscle myofibrils.

Like the MM-CK bound at the M-lines of skeletal myofibrils (21, 22, 23), the CK activity firmly bound to heart myofibrils was released by extraction with low ionic strength "Solution C" (1 mM DTT, 5 mM Tris/HCl buffer; pH 7.7), although prolonged multiple extraction of up to 200 min was required for complete removal (Fig. 1).

Immunofluorescent staining with anti-BB-CK serum, employed in an attempt to localize the bound CK, revealed a regular fluorescent banding pattern (Fig. 2*a*). The fluorescent striations were not removed by extensive washing with Solution A (Fig. 2*c*) or with Solution A containing 0.5% Triton X-100 (Fig. 2*e*). After low ionic strength extraction, however, the fluorescence was entirely lacking (Fig. 2*g*) or was reduced to a background fluorescence comparable to that of myofibrils incubated with preimmune control serum (Fig. 2*i*). The myofibrillar BB-CK thus detected is, as can be seen by comparing Fig. 2*a* and *b*, located not in the H-region but at the middle of the I-band, at the Z-line.

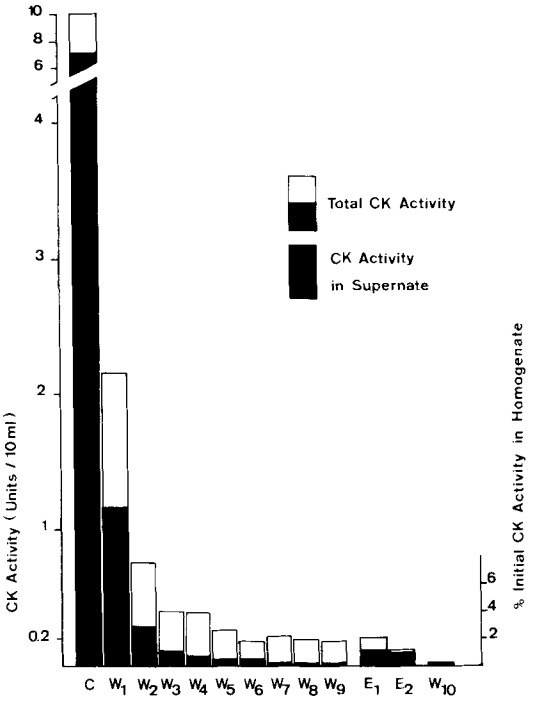

FIGURE 1 Effect of washing with intermediate ionic strength Solution A and extraction with low ionic strength Solution C on the binding of CK to a low-speed (800 *g*) pellet (mainly myofibrils) from adult chicken heart muscle. Total CK activity in the suspension (black plus white bars) was measured (with creatine as substrate) before, and CK activity released into the supernate (black bars) was measured after, each centrifugation. *C*: crude extract ($^1/_{100}$ wt/vol) in Solution A. W_1-W_9: Successive washings in Solution A; about 3% of the total activity present in the original extract is tightly bound. E_1-E_2: Successive extractions (twice 45 min in this case) in Solution C; bound CK is completely released from myofibrils. W_{10}: final wash in Solution A.

In immunofluorescent staining experiments with anti-BB-CK serum (Fig. 3*a*), occasional myofibrils were seen to fluoresce in the H-zone, i.e., where the M-line would be (if there were one present). Fluorescent antibody staining of heart myofibrils with anti-MM-CK serum also resulted in occasional H-zone fluorescence (Fig. 3*b*). Taken together, these results suggest that a small fraction of heart myofibrils contain MB-CK, the heterodimeric CK isoenzyme known to react with

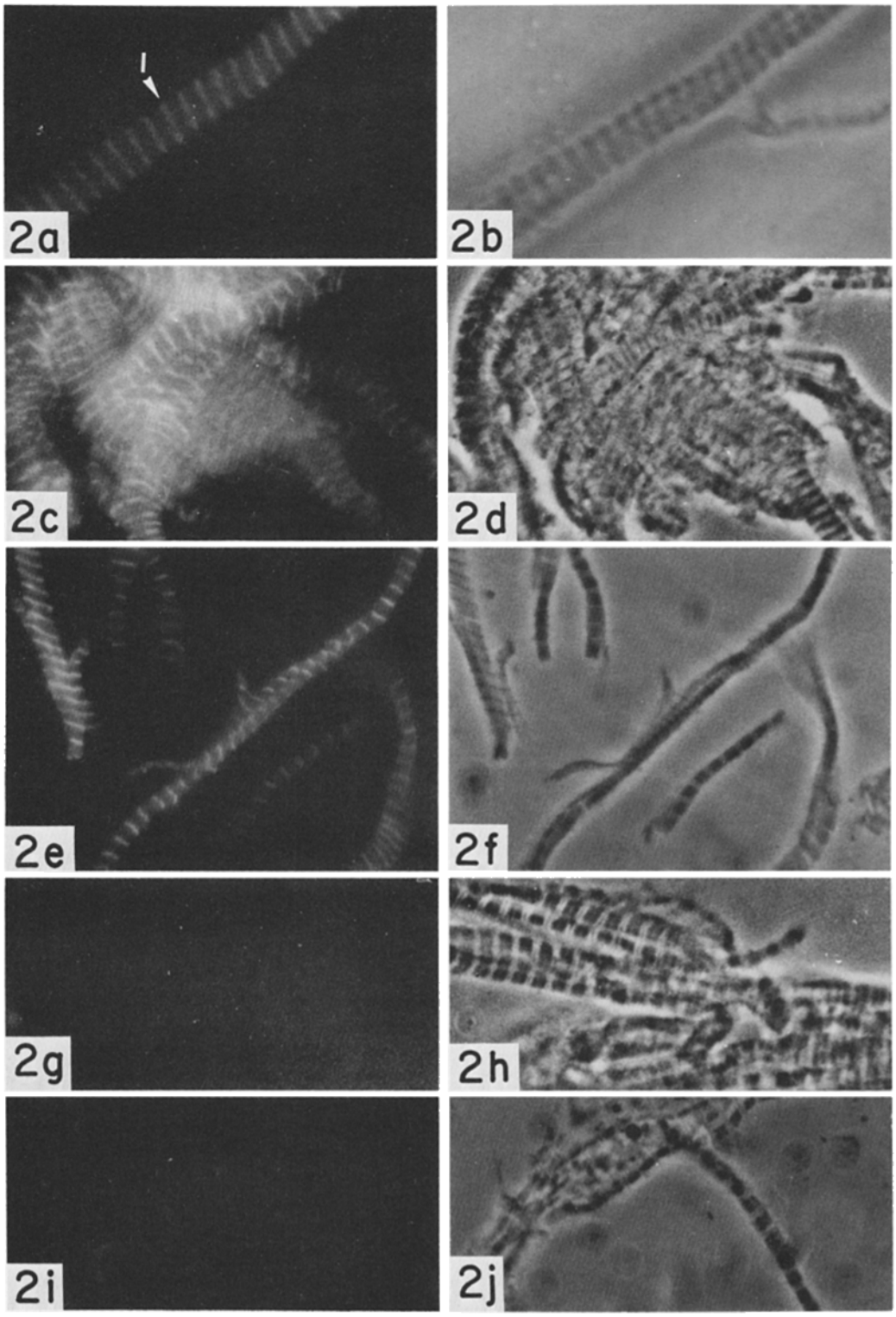

FIGURE 2 Localization of B-type CK by the indirect immunofluorescence technique (23) in chicken heart myofibrils. Fluorescence is shown on the left and the corresponding phase-contrast photograph on the right. Pretreatment of myofibrils: (*a* and *b*) washed once with Solution A; (*c* and *d*) washed nine times with Solution A; and (*e* and *f*) washed once with Solution A containing 0.5% Triton X-100; (*g* and *h*) extracted twice with Solution C (total extraction time: 200 min); (*i* and *j*) washed once with Solution A and incubated with preimmune control serum. (*I*) I-band of sarcomere. × 1,700.

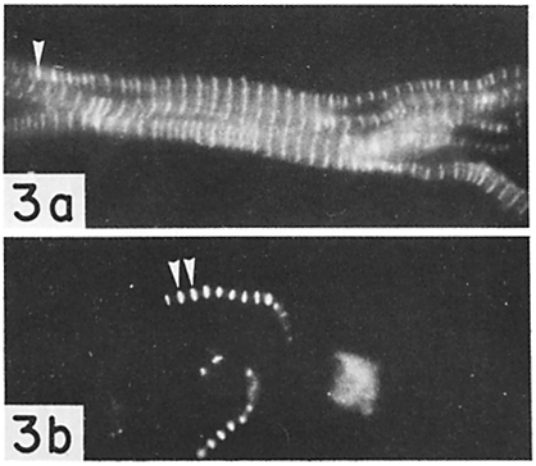

FIGURE 3 Immunofluorescent staining occasionally seen in the H-region of native heart myofibrils. (*a*) washed with Solution A and stained with anti-BB-CK serum; (*b*) washed with Solution A and stained with anti-MM-CK serum. The fluorescence infrequently seen in the H region possibly indicates a reaction with MB-CK; it is thought that these myofibrils may be derived from a minority cell type (see text). (*a*) × 1,700; (*b*) × 1,200.

both of the antisera used, specifically bound at the H-zone. Small amounts of MB-CK have indeed been detected by means of electrophoresis and specific enzymatic staining in extracts of adult chicken heart (4). It is possible that the MB-CK so detected and the myofibrils containing H-zone-bound antigens capable of reacting with antisera against either of the CK homodimers, are derived from a distinct cell type. In this connection it is worth noting that a weak M-line has been observed in the Purkinje cells of chicken heart tissue (17). Although no M-line structures were detected when our heart myofibril preparation was examined by electron microscopy (21), the number of myofibrils examined by immunofluorescence was considerably greater.

The results (presented here and in the preceding paper [23]) of localization experiments with native, washed myofibrils from both heart and skeletal muscle are consistent with the conclusion that binding sites for the B type of CK subunit are located at the Z-lines, while binding sites for M subunits are restricted to the center of the H-zone. The nature of the binding at the two locations must be presumed to be different: whereas MM-CK at the M-line is thought to bind directly to the myosin molecules of the thick filaments present there (13), no myosin filaments are present at the Z-lines where BB-CK is bound in heart myofibrils.

Supplementation of Native Heart Myofibrils with Purified CK Isoenzymes

To confirm that binding sites for MM-CK do exist at the middle of the H-zone of heart myofibrils, purified MM-CK was incubated with native, washed heart myofibrils, followed by immunofluorescent staining with homologous antiserum (Table I). Almost all myofibrils showed medium-to-strong fluorescence at the middle of the H-zones as well as weaker fluorescence at the Z-lines. Immunofluorescent staining with heterologous antiserum, i.e., serum elicited against BB-CK purified from the soluble fraction of a heart homogenate, showed Z-line fluorescence comparable to that of unsupplemented control fibrils; excess MM-CK is thus unable to displace BB-CK from its binding sites at the Z-lines. An increase in electron-dense material in the middle of the H-zone was also observed after incubation of heart myofibrils with MM-CK,[1] as in the case of attempted reconstitution of extracted skeletal muscle M-lines with exogenous MM-CK (preceding paper, [23]). As in the previous case, the added bound material appeared diffuse and did not resemble an intact M-line.

Similar supplementation experiments with purified BB-CK led to an enhancement of Z-line fluorescence and to weak H-zone fluorescence (Table I).

Supplementation with excess purified enzyme of either type thus reveals a lack of binding specificity not seen with native, unsupplemented myofibrils; to some extent, BB-CK can bind in the H-zone and MM-CK can bind at the Z-line. Similar findings were reported in the preceding paper (23) for skeletal muscle myofibrils.

Attempted Reconstitution: Incubation of Extracted Heart Myofibrils with Purified CK Isoenzymes

Myofibrils extracted for 90 min with low ionic strength Solution C to remove bound CK, as completely as possible i.e., all BB and any traces of MG, were incubated with exogenous BB- and/or MM-CK as described in the preceding paper for extracted skeletal myofibrils (23). Fig. 4*a–d* show

[1] T. Wallimann, unpublished observations.

TABLE I

Localization of CK by the Indirect Immunofluorescence Technique after Supplementation of Native Heart Myofibrils with Purified CK Isoenzymes

	H-region*		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)	−	+	−	+++

* Adult chicken heart myofibrils show no M-line structure (18).

‡ Antisera used are specific either for M- or B-subunits; thus a reaction with the hybrid isoenzyme, MB-CK, if present, is expected with both antisera. Staining intensity shown by the great majority of myofibrils in each preparation is indicated as follows: −, none; +, weak; ++, moderate; and +++, intense.

§ Infrequently appearing moderate fluorescence, possibly MB-CK (see Fig. 4); most myofibrils show no staining.

‖ Supplementation refers to a procedure wherein myofibrils, either freshly washed or glycerinated, were added to a solution containing either MM-CK or BB-CK in Solution B stirred for 1–12 h at 4°C.

such fibrils incubated under long-time incubation conditions with either BB- or MM-CK and subsequently stained with the homologous antiserum. The main conclusion to be drawn from the fluorescent banding patterns of Fig. 4*a* and *c* is that, after extraction, both the Z-line region and the region corresponding to the M-line of skeletal myofibrils possess binding sites for both CK isoenzymes. Again, there is a parallel to the situation with skeletal myofibrils, which also exhibited lessened tendency after extraction for the two myofibrillar regions to interact with only one of the isoenzymes (see preceding paper [23]). It should be noted, however, that interaction between isoenzymes and myofibrils remains restricted to two distinct zones of the sarcomere; extracted myofibrils do not simply bind exogenous CK nonspecifically along their entire length. As in similar experiments with extracted skeletal myofibrils, a generally higher binding affinity of BB-CK towards the Z-line and of MM-CK towards the H-region of extracted myofibrils was nevertheless observed in short-time incubation experiments at lower enzyme concentrations.[1]

DISCUSSION

In chicken heart, as in chicken skeletal muscle (see preceding paper), a small fraction of the total cellular CK is firmly bound to the contractile apparatus, even after repeated washings. In both cases, also, the bound enzyme is located at specific sites along the myofibril. For the MM-CK bound at the M-lines of skeletal myofibrils, it was also possible to show (22, 23) that the number of bound CK molecules corresponds well to the number expected form published models of the M-line structure.

There is as yet no comparable model of the Z-line structure incorporating both ultrastructural and compositional information, with which the quantity of Z-line-bound CK might be matched. Several models of the Z-line structure have been proposed, mainly on the basis of electron microscope evidence (11, 12, 16). These models differ strikingly in the extent to which the Z-line is thought to be composed of thin filaments as opposed to other proteins, reflecting the fact that the protein composition of the Z-line has not been clearly established. The available evidence (9, 15) does suggest, however, that the composition may be more complex than that of the M-line. Whether the considerable variation in the thickness of the Z-lines of different muscle types (16) is a reflection of differences in protein composition remains to be elucidated also. In view of these uncertainties, the possibility must be considered that CK (and perhaps other enzymes, as well) are integrated into the Z-line structure, at least in some tissues. In heart, the amount of CK firmly bound to myofibrils is appreciable: relative to other heart enzmes. CK is present at high concentration (10), and the 2% of this CK that is bound to myofibrils amounts to approx. one molecule for every 100 myosin molecules (10). There is other evidence that in heart as in skeletal myofibrils the binding of CK is not adventitious: (*a*) the inability of excess exogenous MM-CK to compete for the myofibrillar binding sites occupied by BB-CK; (*b*) the presence of BB-CK throughout the entire thickness of the myofibril, revealed in preliminary immunofer-

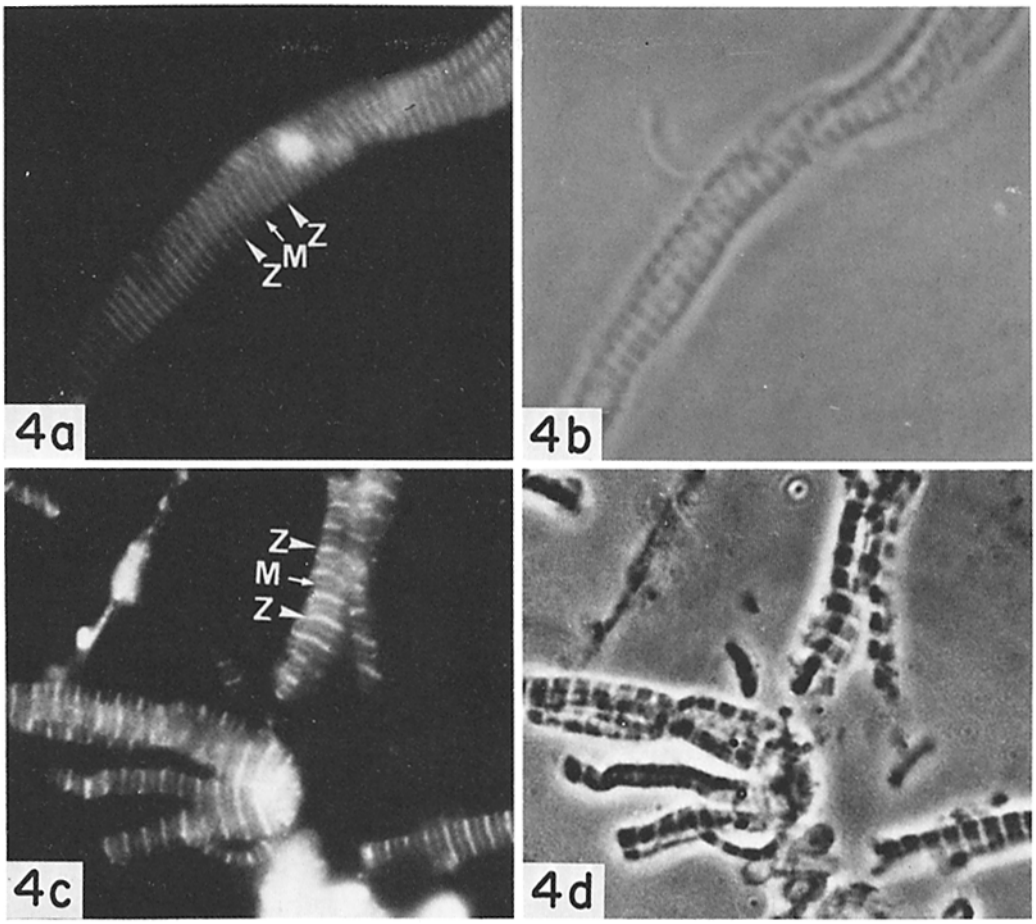

FIGURE 4 Supplementation of extracted heart myofibrils with CK isoenzymes. For low ionic strength extraction conditions, see legend to Fig. 1. Fluorescence is shown on the left and the corresponding phase-contrast photograph on the right. (*a* and *b*) Long-time incubation (1 mg MM-CK/ml for 12 h) of freshly extracted heart myofibrils, staining with anti-MM-CK serum; equal fluorescence in M- and Z-line regions (*M* and *Z*). (The same staining pattern was seen with myofibrils that had been washed but not extracted [Table I].) (*c* and *d*) long-time incubation (1 mg BB-CK/ml for 12 h) of freshly extracted heart myofibrils, staining with anti-BB-CK serum; strong fluorescence in the Z-line region, weak fluorescence in the H-zone region. × 1,700.

ritin staining experiments (21).

It is becoming clear that the M-line of skeletal muscle myofibrils contains other enzymes in addition to MM-CK (8, 14, 19). Several laboratories are attempting to elucidate the adaptive significance of such M-line-bound enzymes for the functioning skeletal muscle cell. For the case of MM-CK, we have discussed some of the possibilities (7). Among other points, we have drawn attention to the fact that there are striated muscle cells able to function without M-lines or MM-CK; we have suggested that this fact may be important in allowing us to restrict the potential functions of M-line-bound MM-CK that need be considered. If some myofibrils have M-lines containing MM-CK and others do not, then it seems reasonable to propose that differences in the dynamics of the contractions performed by the two types of myofibril might provide a crucial clue to the understanding of the function(s) of M-line proteins.

The findings reported in this paper introduce an important new aspect into these considerations. They suggest that, in the case of creatine kinase, the fact of most universal importance may be that

all myofibrils possess binding sites for CK molecules. The capacity to bring forth differentiated muscle cells able to contract rapidly, forcefully, and continuously is surely adaptive for the organism; it is conceivable that striated muscle cells are able to perform as they do because particular isoenzymic forms of certain key enzymes are located at fixed positions throughout the myofibrillar organelles themselves. Indeed, as noted before (20), there exists considerable evidence (1, 2) that several glycolytic enzymes, among them fructose diphosphate aldolase A and triosephosphate dehydrogenase, are located in the I-bands, at least in skeletal myofibrils.

In the preceding paper (23), we speculated that myofibrillar CK in particular might function as direct regenerator of the ATP for muscle contraction. This function would not necessarily depend on the precise location, i.e., M- or Z-line, of CK within the myofibrils. A model of this kind is therefore applicable in principle to the myofibrillar CK of chicken heart as well.

The M and B forms of CK, like the multiple forms of many enzymes, appear to be very nearly equivalent in their catalytic properties (5). It was this fact that prompted the suggestion (21) that, in general, the importance of isoenzymes may lie not so much in enzymatic differences as in their differing abilities to interact with other macromolecules in the cell. It is perhaps significant that those cells that contain nonmyofibrillar actomyosin invariably contain BB-CK (20). The preferential binding of different CK isoenzymes to different regions of native myofibrils, demonstrated in this and the preceding paper, is consistent with the hypothesis that only the MM form of CK is able to participate in constructing an M-line.

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