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Localization and Function of M-Line-Bound Creatine Kinase

M-BAND MODEL AND CREATINE PHOSPHATE SHUTTLE

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1. Structure of the M Band

1.1. M-Band Structure

The electron-opaque M line or M band that transverses the center of the A band is one of the striking features of cross-striated muscle myofibrils seen with the electron microscope (Figs. 1–5). It appears to be the only myofibrillar structure that connects thick filaments directly to each other. After *in situ* fixation of skeletal muscle, dehydration, and standard embedding for electron microscopic examination, the M-band structure appears as a complex structure made up of several transverse elements connecting the thick filaments through the bare zone region and giving rise to the typical hexagonal thick-filament lattice (Franzini-Armstrong and Porter, 1964; Knappeis and Carlsen, 1968; Pepe, 1971) (see Fig. 1). High-resolution electron microscopy in combination with image analysis of ultrathin transverse sections of muscle fiber bundles shows a hexagonal lattice of thick filaments interconnected by primary m-bridge structures (nomenclature according to Sjöström and Squire, 1977a,b) (Fig. 1, M4) often seen to have a circular thickening in the middle (Luther and Squire, 1978; Luther *et al.*, 1981). At a different level in transverse sections, Y-shaped secondary m bridges (M3) connecting the nodular enlargements (MF) are observed as well (Luther and Squire, 1978;

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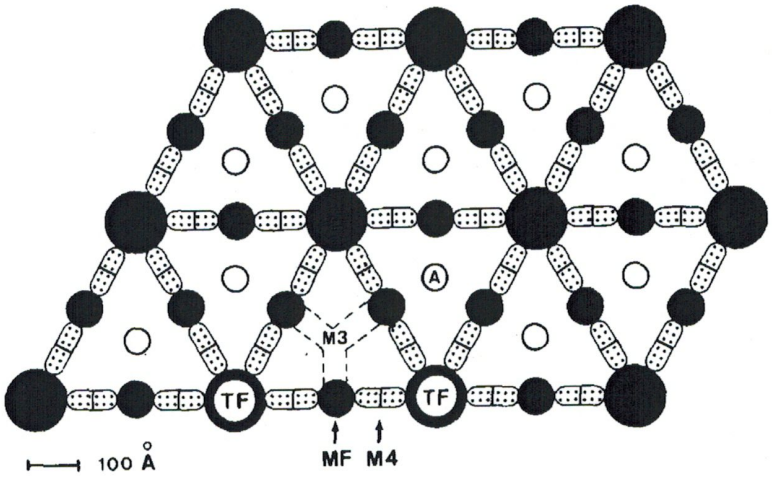
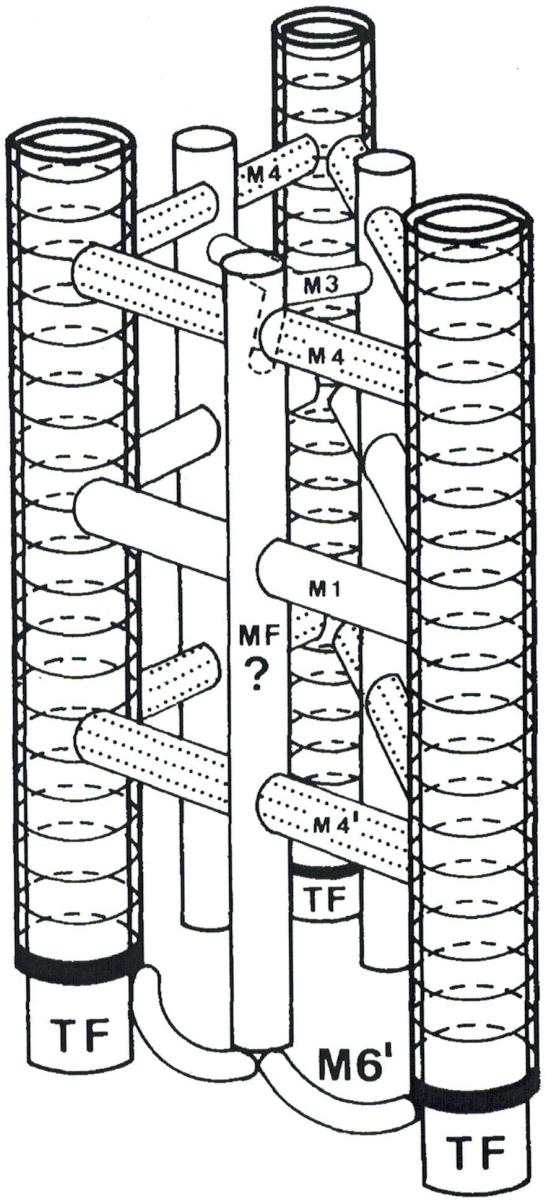


Figure 1. Model of the transverse structure of the M band. Model updated from earlier models by Knappeis and Carlsen (1968), Wallimann *et al.* (1975), and Luther and Squire (1978) of a cross section through the M-band region of skeletal muscle at the level of the M4 m-bridge arrays (see also Fig. 2). The hexagonal lattice of the myosin is shown, containing thick filaments (TF) interconnected by the primary m-bridge structures (M4, representing one half-m-bridge). The typical circular or nodular enlargements between each half-m-bridge seen in the EM (Knappeis and Carlsen, 1968; Luther and Squire, 1978) are thought to be m-filaments (MF) connecting the half-m-bridges (M4) and to run parallel to the thick filaments throughout the M band (see also Fig. 2). Additional secondary m-bridges (M3) connecting the m-filaments are situated at a different level than the primary m-bridges (see also Fig. 2), as seen in extremely thin cross sections through the appropriate level of the M band (Luther and Squire, 1978). Each of the half-primary M4 m-bridges is thought to be made up of one MM-CK dimer (Wallimann *et al.*, 1975, 1983a). The projections of the actin-containing thin filaments at the trigonal points between the thick filaments are indicated by open circles (A).

Luther *et al.*, 1981) (Fig. 1). Ultrathin frozen sections of myofibers cut longitudinally recently showed an even more detailed substructure for the M band (Sjöström and Squire, 1977a,b; Strehler *et al.*, 1983; Thornell and Carlsson, 1984). Besides the three or five main transverse structures (depending on the fiber type) making up the M1, the M4 and M4′, and the M6 and M6′ m-bridge arrays (nomenclature according to Sjöström and Squire, 1977a,b) (Fig. 2), other less prevalent substructures were found, such as the M3 and M3′ m-bridges, which are probably related to the secondary m-bridges seen in transverse sections (Figs. 1 and 2, M3). These additional minor substructures,

Figure 2. Three-dimensional model of the M band. Model updated from earlier models by Knappeis and Carlsen (1968), Wallimann *et al.*, (1975), Luther and Squire (1978), and Strehler *et al.* (1983) of a longitudinal section through the M band of skeletal muscle. A segment of the proposed three-dimensional substructure derived from ultrathin longitudinal frozen section (Sjöström and Squire, 1977b) and ultrathin conventional cross sections (Luther and Squire, 1978) is shown. Three main types of transverse connection between the thick filaments are shown: the three primary m-bridge arrays M1, M4, and M4′, as well as the somewhat thinner M6 transverse bridges (for clarity, only one (M6′) of the two symmetrically placed sets is shown). In addition, also for clarity, only one of the symmetrical sets of secondary M-bridges (M3) placed slightly below the level of M4 is shown. The structure displays bilateral symmetry with the most promi-



nent of the transverse elements, the M1 bridge, as the center. The spacing between M1, M4, or M4′ is some 22 nm. Between M6 and M6′, the thick filaments look somewhat thickened due to "ensheathed" material throughout the M-band region ( ), indicating extra M-band proteins. MM-CK is thought to reside in or make up for the M4 and M4′ m-bridges (one MM-CK dimer, representing one half-m-bridge) and to possibly reside together with other(s) as yet unidentified component(s) within the M1 bridges (Wallimann *et al.*, 1975, 1983a; Strehler *et al.*, 1983). Although cross sections consistently show circular thickenings or nodular enlargements between the half-m-bridges at the points where m-filaments meet with M4, M1, or M4′ m-bridges, the existence of m-filaments (MF) thought to connect these thickenings longitudinally by running parallel to the thick filaments (TF) along a distance of some 75 nm (Knappeis and Carlsen, 1968) is questionable. The possible location for myomesin, the M-band protein of 185,000 M_r (Grove *et al.*, 1984) is the ensheathment around the thick filaments ( ); for the M protein of 165,000 M_r (Masaki and Takaiti, 1974) it is the ensheathment and possibly the M6 and M6′ bridges as well. (L.-E. Thornell, personal communication.)

leading to a total of nine numbered transverse elements (M1–M9 and M1–M9′), were described by Sjöström and Squire (1977a,b). The M4 and M4′ m-bridges were inferred to be crucial for the generation of the thick-filament superlattice and thus for defining the symmetry of the A band, whereas the M1 bridges are thought to provide a secondary role in this respect (Luther *et al.*, 1981). A simplified, updated model of the M band, showing the most prominent transverse elements, is presented in Fig. 2. A more detailed description of the rather complex M-band structure as well as the polymorphous forms of M bands is presented in the Addendum to this chapter, where it is shown that the M-band structure differs markedly between different fiber types and different species or even changes during development (Thornell and Carlsson, 1984). For the moment, a simplified, updated model of the M band, showing the most prominent transverse elements, is described (see Fig. 2).

The existence of transverse connections between the thick filaments at the M1, the M4 and M4′, and the M6 and M6′ levels (Fig. 2) that give rise to three or five main cross-striations in longitudinal sections, depending on the type of muscle, is widely accepted. The only evidence for the existence of m-filaments, however, is the appearance of circular thickenings (Fig. 1, MF) in the middle of the main m-bridges seen in transverse sections. Although Knappeis and Carlsen (1968) first postulated the existence of m-filaments as seen in their longitudinal sections, the presence of m-filaments has not been demonstrated with clarity and reproducibility even in ultrathin longitudinal cryosections, which are technically superior to conventional methods. There is general agreement on the existence of the circular thickenings or nodular enlargements between the half-m-bridges (Fig. 1). These thickenings at the points at which m-filaments meet with M4, M1, or M4′ m-bridges (Figs. 1 and 2) seem to connect the half-m-bridges, to form complete m-bridges, and represent additional mass that may contain additional M-band protein(s). However, the question of whether these thickenings at the different m-bridge levels are longitudinally connected by filamentous structures such as the postulated m-filaments (Fig. 2, MF) remains to be clarified. Unless proved otherwise, the existence of m-filaments should therefore be seriously questioned (Fig. 2, indicated by a questionmark).

As pointed out in earlier studies (Wallimann, 1975; *et al.*, 1975, 1977a), the M-band structure cannot be rigid, for the distance between thick filaments increases as the sarcomere shortens during contraction. How this "breathing" of the M band as a function of sarcomere length is accomplished at the molecular level is unclear. Hinge or springlike mechanisms may be envisioned, or a dynamic rearrangement of the structure may be postulated. Another complication concerning M-band structure is foreseen by the fact that the projections of the thin filaments at the trigonal positions between adjacent thick filaments are directed exactly toward the central part of the Y-shaped secondary m-bridges (Fig. 1). Thus, in supercontracting muscle, where the thin filaments slide from both sides through the M band, a collision of thin filaments with M-band structures, e.g., secondary m-bridges (Luther

and Squire, 1978), is to be expected. Clearly, more information is needed on the ultrastructural details of the M band in resting muscle and on the dynamic structural changes that occur during a contraction cycle.

1.2. Protein Constituents of the M Band

Over the past decade, some of the main concerns in the field of myofibril-associated minor proteins have been the identification and characterization of M-band proteins and the elucidation of their function, as well as the assignment of these proteins to specific substructural elements of the M band (Kundrat and Pepe, 1971; Eaton and Pepe, 1972). So far, three M-band proteins have been found and described. The first M-band protein discovered as M protein, consisting of a single polypeptide chain of 165,000 M_r (Masaki *et al.*, 1968; Masaki and Takaiti, 1972, 1974). This protein has been isolated and characterized although, as it turned out later (Grove *et al.*, 1984) it was probably never completely homogeneous. Its localization has been confirmed by a number of investigators (Landon and Oriol, 1975; Palmer, 1975; Etlinger *et al.*, 1976; Trinick and Lowey, 1977; Dhanarajan and Atkinson, 1980; Strehler *et al.*, 1980; Eppenberger *et al.*, 1981). The second M-band protein found was a dimeric polypeptide with a subunit M_r of 43,000 (Morimoto and Harrington, 1972), identified soon afterward as the muscle isoenzyme of creatine kinase (MM-CK) by Turner *et al.* (1973) and subsequently localized in the M band by immunohistochemical and immunoelectron microscopic methods (Wallimann, 1975; Wallimann *et al.*, 1977a,b, 1978, 1983a). Most recently, a protein previously seen on sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) of myofibrils (Etlinger *et al.*, 1976; Porzio *et al.*, 1979) and on myosin preparations (Offer, 1972) has been identified as a third M-band protein during the course of production of monoclonal antibodies against M protein. This 185,000-M_r M-band protein has been named myomesin (Grove *et al.*, 1984). No function has yet been attributed to the two high-molecular-weight M-band proteins, M protein and myomesin.

1.3. MM-Creatine Kinase as an M-Band Protein

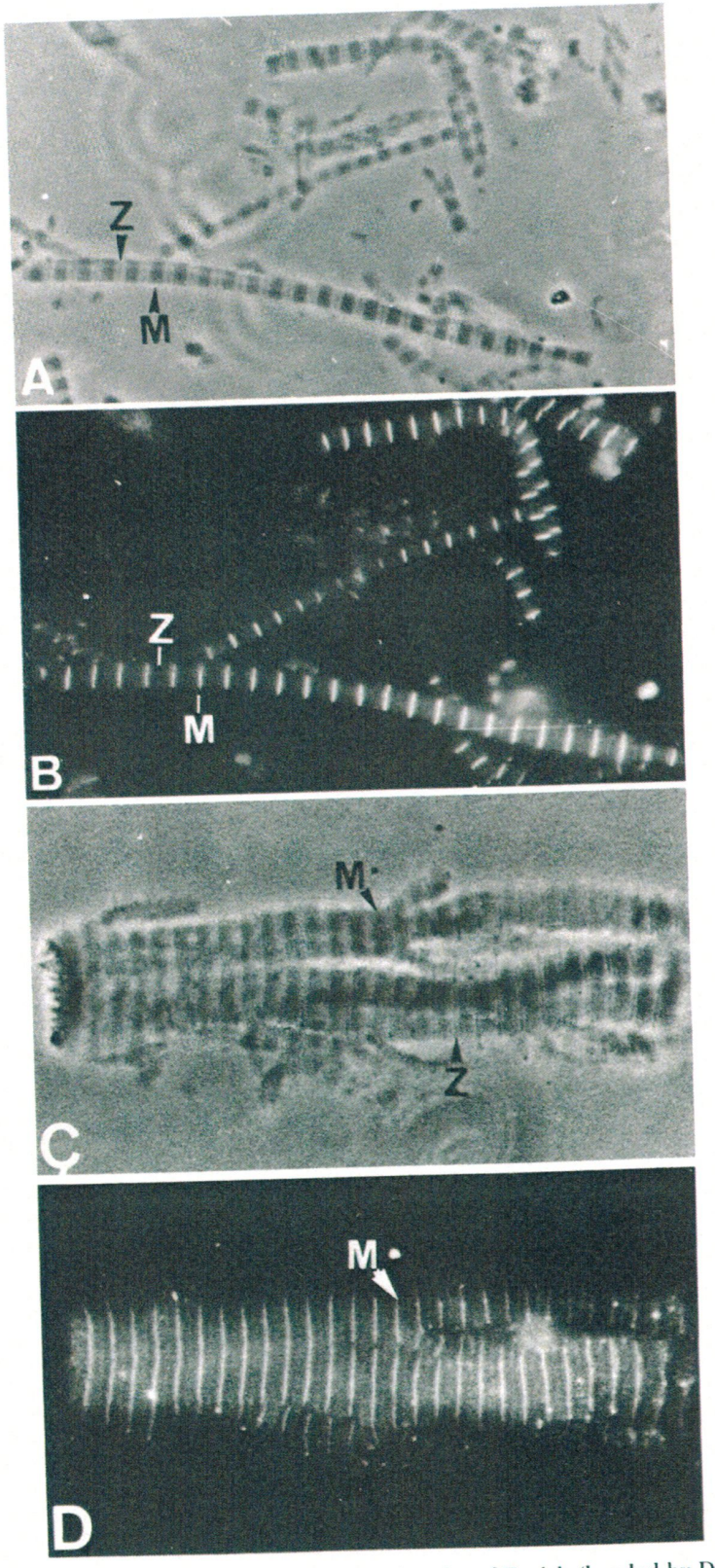
Considering that the bulk of MM-CK is present in soluble form and is known to have an enzymatic function, it was long doubted that MM-CK could be an integral, or a structural, component of the M band. However, considerable evidence that MM-CK represents an integral component of the M-band structure has accumulated over the past few years and can be summarized as follows:

1. Longitudinal cryosections of unfixed chicken pectoralis muscle stained histochemically for CK activity show a repetitive banding pattern of formazan deposits along the myofibrils (Wallimann *et al.*, 1977a).
2. Myofibrils washed under physiological ionic strength and pH condi-

tions (0.1 M KCl, pH 7.0) reproducibly retain a small but significant amount of MM-CK (0.8 EU of CK activity/mg of myofibrils), corresponding to a minimum of 5% of the total CK present in skeletal muscle (Wallimann *et al.*, 1977a, 1982, 1984).

3. Indirect immunofluorescence staining using monospecific anti-M-CK antibodies localizes the myofibril-bound MM-CK in the middle of the A band, at the M band (Turner *et al.*, 1973; Wallimann *et al.*, 1977a, 1978 (see Fig. 3).
4. The binding of CK to the M band is isoenzyme specific, i.e., only the MM isoenzyme, and not the BB or MB forms, binds to the M band (Wallimann *et al.*, 1977a,b). This specificity has also been demonstrated in myogenic cell cultures in myotubes containing simultaneously all three isoforms of CK (Wallimann *et al.*, 1983b).
5. M-line-bound CK is released by incubation of myofibrils with low-ionic-strength buffer with concomitant removal of the electron-opaque M-band structure (Morimoto and Harrington, 1972; Eaton and Pepe, 1972; Turner *et al.*, 1973; Wallimann *et al.*, 1977a).
6. Incubation of glycerinated washed muscle fiber bundles with excess anti-MM-CK IgG leads to heavy labeling of the entire M band as seen in the electron microscope (Wallimann *et al.*, 1978, 1983a) (see Fig. 4B).
7. Bound anti-M-CK IgG at the M band prevents the extraction of electron density and CK activity from the M band by low-salt buffer (Morimoto and Harrington, 1972; Wallimann *et al.*, 1977a, 1978).
8. Incubation with an excess of monovalent anti-M-CK Fab fragments leads to the specific removal of most of the electron density of the M band (Wallimann *et al.*, 1978, 1983a) (see Fig. 5A). The loss in electron density of the M band is accompanied by a release of M-line-bound CK into the supernatant, which can be demonstrated by electrophoresis and immunological methods (Wallimann *et al.*, 1978, 1983b).
9. By contrast, incubation with lower concentrations of monovalent anti-M-CK Fab often give rise to a distinct double-line staining pattern within the M band (Wallimann *et al.*, 1983b (see Fig. 5B), the two lines being spaced axially 42–44 nm apart, corresponding to the two off-center M4 and M4′ m-bridges (Wallimann *et al.*, 1983a). The same pattern at even higher resolution was observed after incubation with low concentrations of divalent anti-M-CK IgG in ultrathin frozen sections (Strehler *et al.*, 1983).
10. The presence of MM-CK at the M band and the existence of an electron-dense M-band structure coincide; e.g., neither an electron-

Figure 3. Localization of M-line-bound creatine kinase in myofibrils from human skeletal and heart muscle. Indirect immunofluorescence staining of human skeletal muscle (A, B) and heart muscle (C, D) myofibrils incubated with anti-human MM-CK antibody (Merck & Co., Darmstadt, BRD) followed by FITC-conjugated goat anti-rabbit IgG. Fluorescence (B,D) and phase-contrast pictures (A,C). M, M band; Z, Z band. Myofibrils from human tissues (3–5 hr postmortem),



kindly provided by the Institute of Pathology, University of Zurich (headed by Dr. J. Ruettner), were prepared and stained for immunofluorescence as described earlier (Wallimann *et al.*, 1977a).

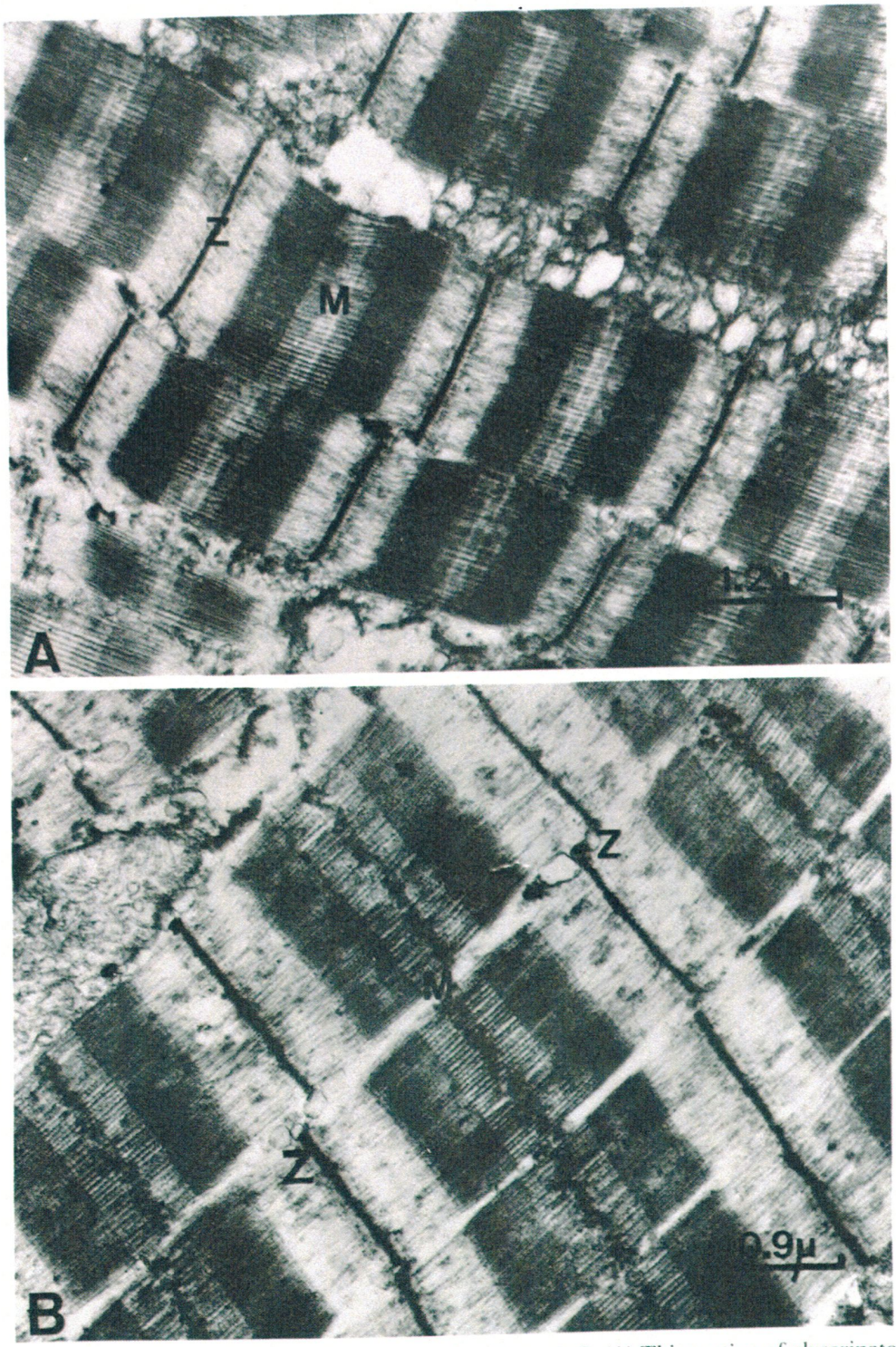


Figure 4. Decoration of the M-band by anti-creatine kinase IgG. (A) Thin section of glycerinated washed chicken muscle fiber bundles (pectoralis major) after incubation with 2 mg/ml of control IgG. (B) Fiber bundle after incubation of monospecific, affinity-purified rabbit anti-chicken M-CK IgG. Note heavy labeling of the entire M band by the antibody (Wallimann *et al.*, 1977a, 1978, 1983a). M, M band; Z, Z band.

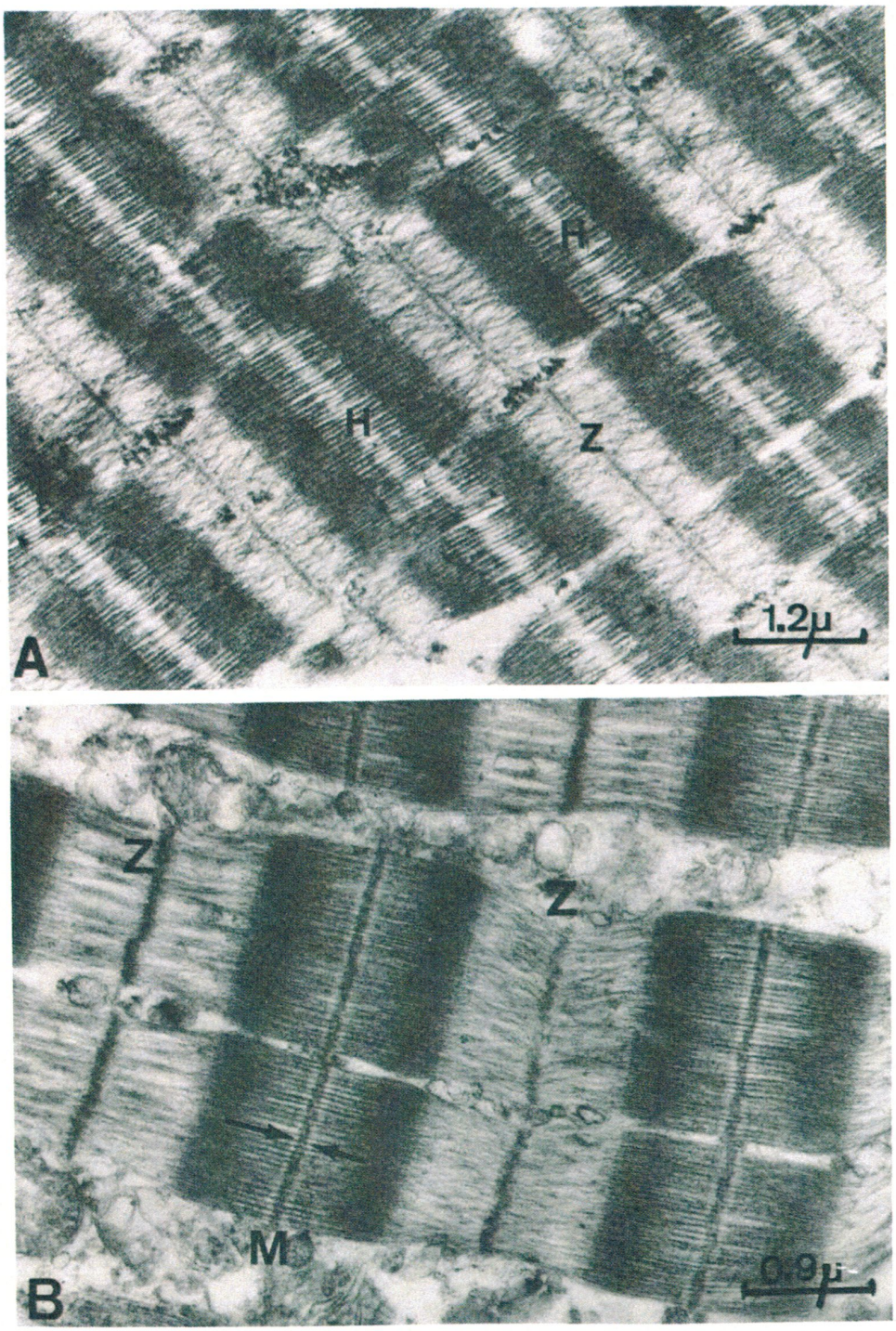


Figure 5. Extraction of M band by excess anti-CK Fab and double-line decoration by low concentrations of anti-CK Fab. Thin section of glycerinated washed chicken skeletal muscle fiber (pectoralis major) after incubation with a low concentration (0.1 mg/ml) of monovalent anti-M-CK Fab fragments. (A) Fiber bundle after incubation with a higher concentration (3 mg/ml) of monovalent anti-M-CK Fab. Note removal of electron-dense material from the M band by excess of monovalent antibody. (Wallimann *et al.*, 1978). (B) Note the appearance of a double-line staining pattern with the lines spaced axially apart by a distance of some 42–44 nm, corresponding to the two off-center M4 and M4′ primary m-bridge stripes (Wallimann *et al.*, 1983a; Strehler *et al.*, 1983). H, H zone; M, M band; Z, Z band.

opaque M band (Sommer and Johnson, 1969) nor MM-CK is found in chicken heart muscle (Eppenberger *et al.*, 1964; Wallimann *et al.*, 1977b). In addition, heart myofibrils of some mammals (e.g., rat) showed delayed incorporation of CK into the M band and the concomitantly delayed appearance of an electron-dense M-band structure until some days after birth, when both appear simultaneously (Carlsson *et al.*, 1982).

11. Very recently, it was shown that commercially available anti-MM-CK antibodies (Merck, Darmstadt, BRD) and monoclonal antibodies against MM-CK (L. Cerny, personal communication) also bind specifically to the M band of myofibrils from human muscle (see Fig. 3) and chicken muscle, respectively.

Since the removal of M-line-bound CK by low-ionic-strength extraction and by specific anti-M-CK Fab leads to the loss of M-band structure, CK must be an integral component of the M-band structure. Our interpretation (Strehler *et al.*, 1983; Wallimann *et al.*, 1983b) is that individual MM-CK molecules are part of the M4 and M4′ m bridges or in fact make up the actual M4 and M4′ m-bridges, one dimeric MM-CK molecule representing one-half the length of an m-bridge, as indicated in Figs. 1 and 2. This interpretation is supported by the fact that the amount of CK activity, and hence the number of MM-CK molecules extracted from the M band, corresponds to the number of half-m-bridges of both M4 and M4′ substriations (one CK molecule or one half-m-bridge for roughly 40 myosin molecules) (Wallimann *et al.*, 1984). In addition, the dimensions of the dimeric MM-CK molecule as measured by hydrodynamic methods (Morimoto and Harrington, 1972) correspond well to the dimensions required for each half-m-bridge (13 × 4 nm) (Knappeis and Carlsen, 1968; Luther and Squire, 1978; Wallimann *et al.*, 1975b, 1977a, 1983a).

1.4. Interactions between M-Band Proteins

Studies of the interactions among M protein, probably containing as an impurity a slightly degraded form of myomesin as well (Grove *et al.*, 1984), MM-CK, and myosin, and fragments thereof have provided somewhat conflicting results. Using various biochemical and biophysical techniques, some investigators have demonstrated the interactions required by the structural model (Fig. 2), e.g., interaction of M protein with myosin rod and with MM-CK as well as interaction of MM-CK with myosin (Botts and Stone, 1968; Morimoto and Harrington, 1972; Houk and Putnam, 1973; Botts *et al.*, 1975; Mani and Kay, 1976, 1978a,b, 1981; Mani *et al.*, 1980; Arps and Harrington, 1982). Others, however, have either been unable to confirm these findings or have found only weak interactions among the above proteins *in vitro* (Woodhead and Lowey, 1983). From the detailed study by the latter investigators, one can infer that only weak interactions of both MM-CK and M-protein occur with myosin and that no interaction takes place between purified M

protein and MM-CK with each other. Attempts to reconstitute myofibrillar M bands from isolated M-band proteins have been unsuccessful, but partial reconstitution of M-band electron density was reported after incubation of M-band-extracted myofibrils with crude M-band protein extracts (Stromer *et al.*, 1969). Our interpretation of these results is that while the biochemical data obtained by *in vitro* studies on the interaction of the M-band proteins with various fragments of myosin and with each other must be fitted to the structural model with reservations, additional M-band proteins (e.g., myomesin) do exist and may have important structural or possibly enzymatic functions. Thus, all these protein components and more may be needed for proper interaction and reconstruction. In addition, the state of polymerization of myosin and proper initiation of packaging may be important for the M-band proteins to interact with myosin. Experiments by Niederman and Peters (1982), who used bare zone assemblages of native thick filaments that still contain the high-molecular-weight M-band proteins (M. Bähler, personal communication) as nucleation centers for myosin filament assembly, point out the importance of M proteins for the assembly of thick filaments from individual myosin molecules (M. Bähler, personal communication). More recently, experiments with reversible phorbol ester treatment of myogenic cell cultures also indicate that the M band, hence some of the M-band proteins, are responsible for the structural integrity of A segments (Doetschman and Eppenberger, 1984). Even though electron microscopic studies emphasize the important structural role of M4 and M4′ m-bridges (which we believe consist mainly of CK) for thick-filament superlattice formation and A-segment symmetry, it is obvious that both M protein and myomesin are bound more tightly at the M band than is MM-CK, which is dissociated from this structure by prolonged incubation with buffers of low ionic strength or by excess of monovalent antibodies against MM-CK. Thus, M protein and myomesin alike are more likely to play important roles in the assembly of thick filaments from myosin molecules, in the structural stabilization of thick filaments, and in the proper alignment of thick-filament arrays into hexagonal lattices.

1.5. M Band and Cytoskeleton

Some of the M-band proteins may be directly involved in the anchorage of the M-band structure to the network of myofibrillar cage proteins, such as titin, nebulin (Wang *et al.*, 1979; Wang and Williamson, 1980; Wang, 1982), or connectin (Maruyama *et al.*, 1977), or to the cytoskeletal network of transverse and longitudinal intermediate filaments (Pierobon-Bormioli, 1981; Wang and Ramirez-Mitchell, 1983). Such intramyofibrillar connections at the M-band level (Street, 1983), besides the well-documented Z to Z-band connections (Breckler and Lazarides, 1982; Street, 1983; and others) and the anchorage of myofibrils to the plasma membrane via "costameric" networks, possibly also involving vinculin and spectrin (Pardo *et al.*, 1983; Wang, 1983), would guarantee the structural alignment, stability, and anchorage of the myofibrillar structures. This type of a harness would lend elasticity to the

muscle fiber, prevent breakage, and permit lateral transmission of tension (Street, 1983), with the M band as well as the Z band representing the major structures that provide myofibrillar anchorage (Street, 1983).

1.6. Conclusion

Besides the two high-molecular-weight proteins, M protein and myomesin, which are located in the M band, MM-CK also has to be considered as an integral M-band protein. MM-CK has been shown to be associated with or making up the M4 and M4′ m-bridges and possibly to contribute as well, together with other as yet unidentified components, to some of the electron density of the M1 m-bridges, which are structurally different from the M4 and M4′ m-bridges (Strehler *et al.*, 1983; Wallimann *et al.*, 1983a). The assignment of the 165,000-M_r M protein to the ensheathment shown in Fig. 2 and the 185,000-M_r myomesin to the same structure as well as to the M6 and M6′ m-bridges is tentatively based on recent immunolabeling studies of ultrathin frozen sections of muscle with monoclonal antibodies against the two proteins (L. E. Thornell, personal communication).

More information is needed for the unambiguous assignment of the individual M-band components to the structural elements of the M band. Since the number of distinctly different structural M-band elements found by electron microscopic study is greater than the number of described M-band proteins, it is highly probable that other, hitherto unknown, M-band components will be discovered in the future. These additional components may provide the missing links in correlating the biochemical data on the interaction of the M-band components with myosin and each other with the structural details of the M-band model. It will be a challenging task to attribute functions to all M-band components, especially those of high molecular weight. So far, the only M-band protein with a known function is MM-CK. Since the M-line-bound MM-CK most likely serves a dual structural and enzymatic role, its further investigation should lead to important information concerning the study of muscle function in general.

2. Function of M-Line-Bound MM-CK

2.1. Introduction

Since, of the three main protein constituents so far found to reside within the M band, only CK has a known enzymatic function, it is worth going into some detail concerning the possible physiological significance of this enzyme, which is associated with this myofibrillar structure.

Upon activation of muscle, phosphorylcreatine (CP), representing a storage and transport form of energy, is efficiently transphosphorylated by creatine kinase (CK) (EC 2.7.3.2), to yield ATP as the actual source of energy for contraction. Creatine kinase is involved in maintaining proper intra-

cellular ATP : ADP ratios and CP pool sizes and is therefore a key enzyme in muscle energetics (for review, see Carlson and Wilkie, 1974).

Creatine kinase (80,000 M_r) is a dimeric enzyme known to exist as isoproteins. Three isoenzymes formed by combination of either two homologous subunits (MM for muscle and BB for brain CK) or heterologous subunits (MB-CK) have been described (Eppenberger *et al.*, 1964; for reviews, see Eppenberger *et al.*, 1983; and Caplan *et al.*, 1983). The ubiquitous form of creatine kinase, BB-CK, found in brain, smooth muscle, and heart is also the predominant form in embryonic skeletal muscle. During muscle cell differentiation, MM-CK synthesis and accumulation is induced, and MM-CK becomes the predominant form of CK, both *in vivo* (Eppenberger *et al.*, 1964; Perriard *et al.*, 1978a) and *in vitro* (Turner *et al.*, 1974, 1976a,b). Since the two subunits of CK are at times simultaneously synthesized within myogenic cells, the transition from BB-CK to MM-CK proceeds via the transitory MB-CK hybrid; thus, at intermediate stages of development, all three isoenzymes of CK are found within a single cell (Turner *et al.*, 1976a; Perriard *et al.*, 1978a). As shown by *in vitro* translation experiments (Perriard *et al.*, 1978b) and protein turnover studies (Caravatti and Perriard, 1981), the transition is principally regulated by differential rates of synthesis of B and M subunits (Perriard, 1979; Caravatti *et al.*, 1979), which is further supported by the regulated accumulation of M-CK mRNA (Rosenberg *et al.*, 1982). Consequently, the predominant form of CK in fully differentiated chicken skeletal muscle is the MM type, which is present at high levels (~5 mg of CK per gram wet weight of muscle), while MB-CK or BB-CK are undetectable (Eppenberger *et al.*, 1964; Wallimann *et al.*, 1977a,b). For a long time, these CK isoenzymes were considered strictly cytoplasmic and therefore soluble; rapid ATP synthesis (by glycolytic enzymes) and ATP regeneration (CK-catalyzed transphosphorylation of CP) were both thought to occur in the sarcoplasm. However, with the development of more sensitive techniques, it became apparent that CK as an "ambiquitous" enzyme (a term coined by Wilson, 1978) is present not only in the sarcoplasm, but is specifically bound at strategically important locations as well (Ottaway, 1967). For example, 10–30% of total CK activity, depending on muscle type, is located within the mitochondria, where a fourth isoprotein of CK, the mitochondrial isoenzyme of CK (MiMi-CK), is bound to the outer side of the inner mitochondrial membrane (Jacobs *et al.*, 1964; Scholte, 1973; Scholte *et al.*, 1973; Jacobus and Lehninger, 1973; Iyengar and Iyengar, 1980; Roberts and Grace, 1980). The mitochondrial CK is intimately coupled to the ADP/ATP translocase (reviewed by Klingenberg, 1979), forming a microcompartment that permits efficient CP synthesis from mitochondrial matrix-generated ATP (Vial *et al.*, 1972; Jacobus and Lehninger, 1973; Saks *et al.*, 1975, 1980; Yang *et al.*, 1977; Moreadith and Jacobus, 1982; Erickson-Viitanen *et al.*, 1982a,b; Gellerich and Saks, 1982).

In addition, relatively small amounts of CK have been reported to be bound to the sarcoplasmic reticulum membrane (Baskin and Deamer, 1970; Khan *et al.*, 1971), where a close spatial proximity of CK with the Ca^{2+}-dependent ATPase has been suggested (Levitsky *et al.*, 1977), and to the plasma membrane of heart cells (Sharov *et al.*, 1977; Saks *et al.*, 1977), where a

kinetic coupling of this enzyme with the (Na^+, K^+)ATPase has been proposed (Grosse *et al.*, 1980).

Furthermore, a small but significant amount of MM-CK, about 5% of the total CK activity present in skeletal muscle, is located within the myofibrillar apparatus at the M band of the sarcomere (Turner *et al.*, 1973; Wallimann, 1975; Wallimann *et al.*, 1975, 1977a, 1978, 1983a). These observations, when taken together with the molecular dimensions of CK and the amount of CK extractable from the M band, led to the conclusion that CK is the principal component of the M4 and M4′ m-bridges and is therefore a myofibrillar structural protein (Wallimann *et al.*, 1975, 1983a). The presence of MM-CK at a specific location within the contractile apparatus suggests a possible catalytic function for the bound enzyme in addition to its structural role (Turner and Eppenberger, 1974; Wallimann, 1975; Saks *et al.*, 1976a; Bessman and Geiger, 1981). Although an association of CK with myosin was suggested earlier by Yagi and Mase (1962) and Botts and Stone (1968), there was no direct experimental evidence that the myofibrillar M-line-bound CK had an enzymatic function. Recent results (Wallimann *et al.*, 1982, 1984) provide experimental evidence of the physiological significance of myofibrillar CK by demonstrating that the bound CK is enzymatically active and that it acts as a potent intramyofibrillar ATP regenerating system (see Section 2.3). These findings support a functional coupling, within the contractile apparatus, of the M-line-bound CK with the myofibrillar actin-activated Mg^{2+}-ATPase. Experimental evidence for the involvement of the M-line-bound CK in a CP shuttle (Wallimann, 1975; Bessman and Geiger, 1981; Wallimann *et al.*, 1982) is presented. The ATP regeneration potential of M-line-bound CK seems to have a capacity that may account, at least in muscles with an M-band structure and M-line-bound CK, for the intramyofibrillar regeneration of most or all of the ATP hydrolyzed by the myofibrillar ATPase during muscle contraction. Taking into account the intracellular compartmentalization and the isoenzyme-specific localization of CK, the physiological significance of the M-line-bound CK as an ATP-regenerating system located within the contractile apparatus will now be discussed and placed within the context of the CP shuttle.

2.2. Content of Total and M-Line-Bound CK in Skeletal Muscle

The predominantly white, fast-twitch glycolytic pectoralis major from chicken contains ~2200 EU of CK per gram of wet weight, as measured by the direct pH stat assay after homogenization and sonification of the tissue. Assuming a maximal specific activity of 400 EU/mg of purified chicken MM-CK as measured by the same assay, the total amount of CK represents ~5 mg of CK per gram of wet weight. After extensive washing of myofibrils, the amount of M-line-bound CK was 0.8 EU/mg of myofibrillar protein. Since the protein content of chicken pectoral muscle is 18% of its wet weight (T. Wallimann, unpublished observations), and 69% of the total muscle protein represents myofibrillar protein (Huxley, 1972), 1 g of wet muscle tissue contains

some 124 mg of myofibrils. Thus, the percentage of myofibrillar M-line-bound CK (0.8 EU/mg myofibrils) represents ~4.5% of the total CK present in chicken pectoralis muscle (2200 EU/g wet weight). This relative value of M-line-bound CK, determined by the direct CK assay in the pH-stat and then calculated by using the myofibrillar protein content of the muscle tissue, is very similar to the value reported earlier (3–5% of the total CK) (Wallimann *et al.*, 1977a), which was obtained by different means. However, taking into account the specific activity for CK of 400 EU/mg, the absolute amount of M-line-bound CK per myosin on a molar basis is now calculated to be ~1 CK per 40 myosins. (For details of assumptions, e.g., number of myosin molecules per thick filament, see Wallimann *et al.*, 1977a.) This value would correspond quite well to the number of half-m-bridges per myosin (1 : 35) (Knappeis and Carlsen, 1968; Wallimann *et al.*, 1977a), if only two out of the three main m-bridge arrays (M-4, M-4′) were to consist of CK dimers.

2.3. ATP Regeneration Potential of Washed Myofibrils

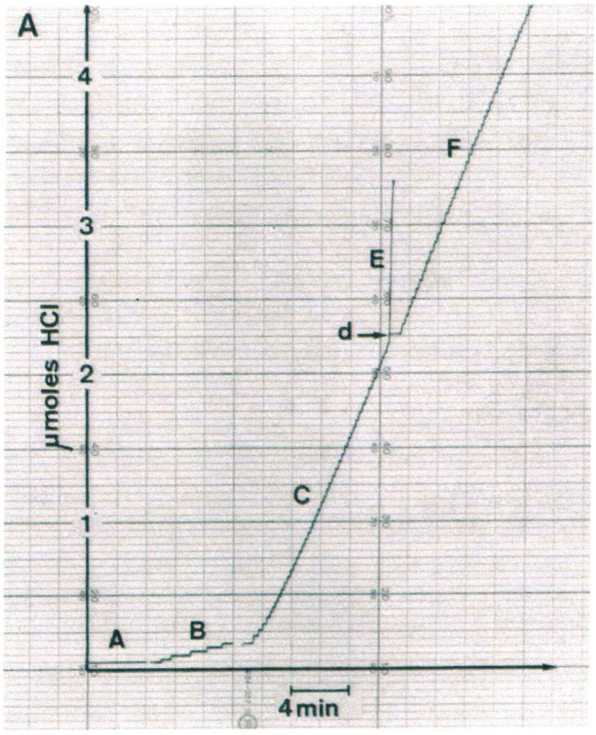
Isolated chicken myofibrils, which are freed of soluble mitochondria and sarcoplasmic reticulum by differential centrifugation in conjunction with several wash cycles, contain 0.8 EU of CK activity per mg of protein, and the CK is exclusively located at the M band, which is not shown here (Wallimann *et al.*, 1984). The ATP regeneration potential of washed myofibrils can be directly measured by a combined pH stat assay (Fig. 6). This assay permits a continuous nondestructive survey of the combined CK/ATPase reactions and accurate determination of steady-state rates (Fig. 7A,B). The assay has been optimized for both the CK and the myofibrillar ATPase activities, the conditions of which are given in Fig. 7A,B (for details, see Wallimann *et al.*, 1984). Provided that CK (endogenous M-line-bound or exogenously added) is pre-

$$1)\ \text{myofs.} + \text{ATP} \xrightarrow[\text{Mg}^{2+}\text{-ATPase } (+\ \text{Ca}^{2+})]{\text{actin-activated}} + \text{ADP} + \text{P}_i + \text{aH}^+$$

$$2)\ \text{ADP} + \text{xCP} + \text{bH}^+ \xrightarrow{\text{creatine kinase}} \text{ATP} + (x-1)\ \text{CP} + [x-(x-1)]\ \text{C}$$

$$\text{xCP} + (\text{bH}^+ - \text{aH}^+) = (x-1)\text{CP} + [x-(x-1)]\ \text{C} + [x-(x-1)]\text{P}_i$$

Figure 6. pH-stat assay system of the coupled myofibrillar CK and myofibrillar ATPase reactions. The combined CK/actin-activated Mg^{2+}-ATPase reaction of myofibrils is measured in the presence of ATP, CP, and Ca^{2+} as indicated. Hydrolysis of ATP by the actin-activated Mg^{2+}-ATPase leads to a production of protons and ADP. If CK (endogenous M-line-bound or exogenously added) is present in excess, the ADP is rephosphorylated via CP by CK. This latter reaction is consuming protons (Eisenberg and Moos, 1970). At pH 7.0, the net consumption of protons per CP hydrolyzed is 0.33 (bH^+-aH^+ = 0.33). Thus, the steady-state rate of the myofibrillar actin-activated Mg^{2+}-ATPase is measured, provided that CK is present in excess (Wallimann *et al.*, 1984). C and CP represent creatine and phosphorylcreatine; H^+ are protons; a and b are molar fractions of protons liberated per hydrolyzed ATP and protons consumed per hydrolyzed CP, respectively; X is the initial number of CP molecules present. (For details see Wallimann *et al.*, 1984.)



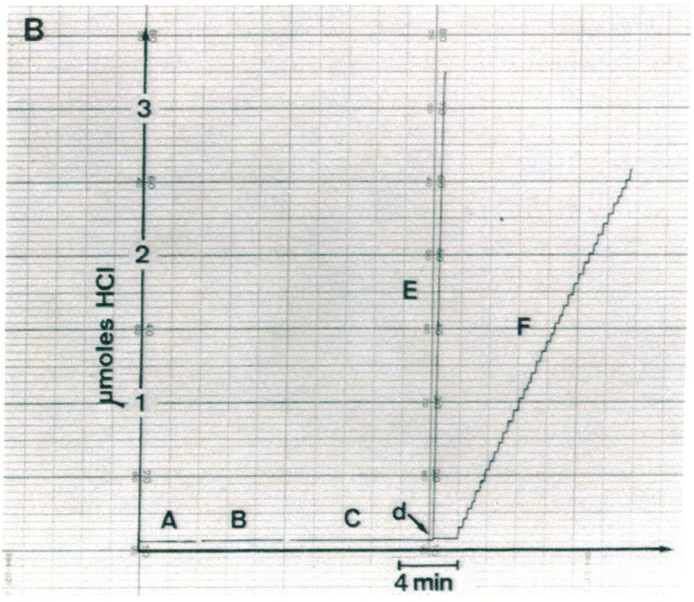


Figure 7. (A) *ATP regeneration potential of CK bound at the M-band of washed pectoralis myofibrils.* Copy of original pH-stat tracing showing the actin-activated Mg^{2+}-ATPase activity measured by the combined CK/ATPase assay with CP and ATP as substrates. Protons are consumed as CP is hydrolyzed. The myofibrillar ATPase is supported first by the endogenous, M-line-bound CK only (at C) and then by endogenous plus excess of exogenously, added CK (at F). Blank reaction of assay mix (75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, 4 mM ATP, and 10 mM CP at pH 7.0) (at A). Addition of 2 mg of washed myofibrils giving rise to some myofibrillar ATPase activity in the absence of Ca^{2+} (at B). Steady-state rate after the addition of 0.2 mM Ca^{2+} of the actin-activated Mg^{2+}-ATPase activity supported only by the endogenous, M-line-bound CK (at C).

sent in excess, the ADP produced by the actin-activated Mg^{2+}-ATPase is rephosphorylated by CK via CP, resulting in a net hydrolysis of CP, with a concomitant consumption of protons that can be monitored by the pH stat (Fig. 6).

When skeletal myofibrils prepared and washed as described were assayed by the combined CK/ATPase reaction in the presence of Mg-ATP, CP, and EGTA, but without addition of exogeneous CK, the myofibrillar actin-activated Mg^{2+}-ATPase activity as measured by CP hydrolysis was very small in the absence of Ca^{2+} (Fig. 7a, phase B) and only slightly above the background obtained in the absence of myofibrils (Fig. 7A, phase A). However, upon addition of Ca^{2+}, a linear steady-state activity was observed that was maintained by the endogenous CK (Fig. 7A, phase C) bound to the M band. The small interruption of the original pH stat tracing between phase B and C in Fig. 7A indicates the time for recovery from the slight drop in pH caused by proton liberation from EGTA upon addition of Ca^{2+}. After addition of excess exogenous CK, a fast reaction took place that was attributable to recharging of most of the ADP present during phase C, which was necessary to turn on the M-line-bound CK (Fig. 7a, phase E). The steady state level of ADP remained constant during phase C according to parallel experiments in which exogenous CK was added at different time points during extended C phases (not shown). Upon establishing a new, lower steady-state level of ADP, dictated by the excess of added CK, a linear steady-state rate of activity was observed (Fig. 7A, phase F) that was identical to that obtained in the presence of endogenous M-line-bound CK only (Fig. 7A, phase C). This indicates that after a certain steady-state level of ADP was established, the endogenous, M-

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Addition of an excess (20 EU) of purified MM-CK (d). Fast-recharging reaction (at E) by the excess of exogenous CK of ADP that was present at a certain steady-state level during the previous reaction phase (at C), leading to a new, lower steady-state level of free ADP during the next phase (at F). New steady-state rate of the actin-activated Mg^{2+}-ATPase supported by the endogenous plus the excess of exogenous CK (at F). Note the identical slopes at C and F. Under above conditions the net consumption of HCl per mole of CP hydrolyzed at pH 7.0 and 25°C was 0.33 (see Fig. 6). (B) *Loss of ATP regeneration potential of myofibrils after treatment with DNFB*. Copy of original pH-stat tracing showing the actin-activated Mg^{2+}-ATPase activity measured by the combined CK/ATPase assay. Protons are consumed as CP is hydrolyzed. The myofibrillar ATPase is supported first by endogenous, M-line-bound CK only (at C) and then by endogenous plus excess of exogenously, added CK (at F). Blank reaction of assay mix (at A). Addition of 2 mg of washed myofibrils that had been treated with 50 μm of DNFB to specifically inactivate M-line-bound CK (at B). Addition of 0.2 mM Ca^{2+} (at the beginning of phase C) and subsequent addition of an excess (20 EU) of purified MM-CK (at d). Recharging of accumulated ADP (at E). New steady-state rate of the actin-activated Mg^{2+}-ATPase activity driven by the endogenous plus excess of exogenous CK (at F). Note the complete loss of the ATP regeneration potential after inactivation by DNFB of the M-line-bound CK (at C). During phase C (no exogenous CK added), hydrolysis of ATP by the actin-activated Mg^{2+}-ATPase, which was not affected by DNFB (see Table 1) led to a continuous drop of the pH value, which was at pH 6.8 at the end of phase C. Since HCl was used as a titrant, this "negative" activity (production of protons by the myofibrillar ATPase) could not be recorded. Similar tracings were obtained with myofibrils, the M-bands, and thus the M-line-bound CK of which, had been extracted by low salt buffer or specific anti-M-CK Fab (see Table 2). From Wallimann *et al.* (1984).

line-bound CK was sufficient to regenerate all ATP hydrolyzed by the myofibrillar actin-activated Mg^{2+}-ATPase. Addition of excess soluble CK lowered the steady-state concentration of ADP but did not increase the ATPase activity of skeletal myofibrils as measured by CP hydrolysis under *in vitro* conditions optimized for both the actin-activated Mg^{2+}-ATPase and CK reactions. Nor did ADP accumulate during phase C beyond a certain steady state level determined by the amount of CK present at the M band. ATP regeneration potential and actin-activated Mg^{2+}-ATPase activity (i.e., the steady-state rates shown in phases C and F in Fig. 7A) were not significantly altered after preincubation of the myofibrils with 1 mM p^1p^5-diadenosine-5′-pentaphosphate (Ap-5-A) and with 200 μM atractyloside (AT) or 50 μM carboxyatractyloside (CAT), inhibitors of myokinase (Cohen *et al.*, 1978), and mitochondrial ATP/ADP translocase activity (Moreadith and Jacobus, 1982), respectively (Wallimann *et al.*, 1984). Sodium azide (5 mM) or KCN (2.5 mM), both blockers of mitochondrial respiration, had no significant effect on the rate of the combined CK/ATPase reaction, nor did an additional washing cycle in which myofibrils were incubated overnight at 4°C with washing solution containing 1% Triton X100. After treatment of washed myofibrils with these agents, the rate of phase C was always identical to that of phase F (as in Fig. 7A; not shown here). Thus, myokinase, with an activity of less than 5% that of M-line-bound CK, and membrane-bound CK cannot have contributed significantly to the observed ATP regeneration. In addition, both rates (phase C and F, as in Fig. 7A), although changing in absolute terms as a function of pH at which the combined CK/ATPase assay was performed, were always identical in relative terms, when measured at any set pH of 6.6–7.4. This finding indicates that the ATP regeneration potential of M-line-bound CK is sufficient to keep up with the myofibrillar ATPase within a broad, physiological pH range (Wallimann *et al.*, 1984). During the combined CK/ATPase assay, the CK activity, and thus the ATP regeneration potential, remained associated with the myofibrillar pellet, obtained upon completion of the assay by centrifugation of the myofibrils, which were mostly supercontracted. No significant amount of CK activity was found in the supernatants.

2.4. ATP Regeneration Potential after Inactivation of M-Line-Bound CK by IAA or DNFB

After treatment of washed myofibrils with reagents that block CK activity (10 mM iodoacetic acid (IAA) or 50 μM dinitrofluorobenzene (DNFB) (Infante and Davies, 1965), the ATP regeneration potential via M-line-bound CK was completely lost (Fig. 7B). In contrast with washed, but untreated myofibrils (Fig. 7A, phase C), no hydrolysis of CP was observed after addition of Ca^{2+} (Fig. 7B, phase C). Thus, although the endogenous myofibrillar CK was still bound at the M band, as demonstrated by indirect immunofluorescence (not shown), it was inactivated and therefore unable to support regeneration of the ATP hydrolyzed by the myofibrillar ATPase (Table 1). Continuous hydrolysis of ATP by the myofibrillar ATPase, which was not affected significantly by IAA and DNFB (Table 1), resulted in a continuous drop in pH

Table 1. ATP Regeneration Potential of M-Line-Bound MM-Creatine Kinase in Myofibrils: Effect of Inactivation or Removal of M-Line-Bound CK on the ATP Regeneration Potential of Myofibrils[a–m]

	Actin-activated Mg^{2+}-ATPase activity measured directly[g] (μmoles ATP/min, mg myofs.)		Calcium sensitivity[h]	Actin-activated Mg^{1+}-ATPase activity, measured by the combined CK/ATPase assay in the presence of Ca^{2+}[i] (μmoles CP/min, mg myofs.)		Amount of M-line-bound active CK[j]	
	EGTA	Ca^{2+}	(%)	*Without* exogenous CK	*With* exogenous CK	μmoles CP / min, mg myofs.	(%)
Untreated[b] *myofibrils*	0.022	0.37	94	0.37	0.37	0.8	100
Myofibrils after[c] 10 mM IAA	0.039	0.33	88	Not measurable	0.33	0.001	0.13
Myofibrils after[d] 50 μm DNFB	—[m]	—	—	Not measurable	0.31	0.02	2.5
Myofibrils after[e] 15 min of 5 mM Tris pH 7.8	0.038	0.33	88	Not measurable	0.33	0.06	7.5
Myofibrils after[f] 45 min of 5 mM Tris pH 7.8	0.040	0.29	86	Not measurable	0.29	0.03	3.8

[a]From Wallimann *et al.* (1984).
[b]*Myofibrils from chicken pectoralis major after extensive washing in buffer of physiological ionic strength, freed by differential centrifugation of soluble CK, mitochondria,* and membrane debris.
[c]Myofibrils after treatment with 10 mM IAA at 4°C, pH 7.0 for 12 hr.
[d]Myofibrils after treatment with 50 μm DNFB at 4°C, pH 7.0 for 3 hr.
[e]Myofibrils after M-band extraction by incubation with low-ionic-strength buffer (5 mM Tris, pH 7.8) for 15 min.
[f]As in *d* but incubated for 45 min.
[g]Myofibrillar actin-activated Mg^{2+}-ATPase activity obtained by direct pH-stat measurements of ATP hydrolyzed in the absence and presence of Ca^{2+} expressed in μmoles of ATP hydrolyzed *per min and mg of myofibrils* (Wallimann and Szent-Györgyi 1981; Wallimann *et al.*, 1984).
[h]Calcium sensitivity of myofibrils (1-(ATPase in EGTA/ATPase in Ca^{2+})×100) expressed in percent (Wallimann and Szent-Györgyi, 1981).
[i]Actin-activated Mg^{2+}-ATPase activity of myofibrils measured by the combined CK/ATPase pH-stat assay in the presence of ATP, CP, and Ca^{2+} before and after addition of exogenous CK is expressed in μmoles of CP hydrolyzed per min and mg of myofibrils (Wallimann *et al.*, 1984).
[j]CK activity of myofibrils measured by direct pH-stat assay expressed in μmoles of hydrolyzed per min and mg myofibrils.
[k]CK content of untreated *myofibrils was taken as 100%.*
[l]Means were taken from four experiments and standard deviations were less than 10%.
[m]Not determined.

during phase C (Fig. 7B). After addition of excess exogenous CK (Fig. 7B, at point d, where the pH had dropped to 6.7), the ADP that had accumulated during phase C was regenerated rapidly; 2 min later, a linear steady-state reaction was observed, again reflecting the actin-activated Mg^{2+}-ATPase activity, as measured by CP hydrolysis in the presence of excess CK. Thus, >95% inhibition of M-line-bound CK activity by IAA or DNFB (see Table 1) abolished the ATP regeneration potential of myofibrils without interfering significantly with the ATPase activity or calcium sensitivity. (The slopes during phase F in Fig. 7A,B were identical; see Table 1).

2.5. ATP Regeneration Potential after Extraction of M-Line-Bound CK by Low-Ionic-Strength Buffer

pH stat tracings very similar to those shown in Fig. 7b were obtained with myofibrils after treatment with low-ionic-strength buffer (5 mM Tris/HCl, pH 7.8), which is known to extract the M-line-bound CK (Turner *et al.*, 1973). Removal of bound CK was monitored by direct measurement of CK activity (Table 1) and by indirect immunofluorescence staining (not shown). The amount of CK still remaining at the M band after extraction by low-ionic-strength buffer depended on the duration of the treatment. Approximately 92% and 96% of bound CK was extracted by treatments of 20 and 40 min, respectively. The endogenous CK remaining bound at the M band was not sufficient to keep up with ATP hydrolysis, even though ATPase and calcium sensitivity were both lowered slightly by prolonged low-ionic-strength extraction (Table 1), presumably as a result of extraction of actin, regulatory proteins, and some myosin as the myofibrils swelled. Thus, specific extraction of M-line-bound CK, like inhibition of bound CK by IAA or DNFB, abolished the ATP regeneration potential of these myofibrils.

2.6. Effect of Anti-M-CK Antibodies on the ATP Regeneration Potential

Excess of monospecific anti-M-CK IgG had a strong inhibitory effect on the myofibrillar bound CK. Creatine kinase activity in the presence of excess anti-M-CK IgG was lowered to about 20% of the control value obtained with preimmune IgG, as measured by direct pH stat assay (Table 2). However, CK remained associated with the M band, as shown by indirect immunofluorescence staining (Wallimann *et al.*, 1984). Inhibition of endogenous M-line-bound CK by anti-M-CK IgG, like inactivation by IAA and DNFB or extraction of CK by low-ionic-strength buffer, also resulted in a loss of ATP regeneration potential without significantly affecting the actin-activated Mg^{2+}-ATPase activity (Table 2). After addition of excess exogenous CK, a linear steady-state activity similar to that of control IgG-treated myofibrils was measured. That is, pH-stat tracings similar to those shown in Fig. 7B were obtained with anti-M-CK IgG-treated myofibrils (not shown). In contrast to the results with intact antibody, an excess of monovalent anti-M-CK Fab fragment not only abolished most of the CK activity (Table 2), but, as expected,

Table 2. Effect of Anti-M-CK Antibodies on the ATP Regeneration Potential of M-Line-Bound CK[a–e]

Myofibrils treated with:		Actin-activated Mg^{2+}-ATPase activity measured by the combinded CK/ATPase assay (E) in the presence of Ca^{2+} (μmoles CP/min, mg myofs.)		Amount of M-line-bound active CK (F)	
		Without exogenous CK	With exogenous CK	μmoles CP / min, mg myofs.	%
Control IgG	(A)	0.35	0.35	0.79	100
Control Fab	(B)	0.37	0.37	0.76	96
Anti-M-CK IgG	(C)	not measurable	0.34	0.15	19
Anti-M-CK Fab	(D)	not measurable	0.36	0.06	7.6

[a]From Wallimann *et al.* (1984).
[b]The actin-activated Mg^{2+}-ATPase activity of myofibrils treated with an excess of control IgG (A), control Fab (B), anti-M-CK IgG (C), and anti-M-CK Fab (D) measured by the combined CK/ATPase pH-stat assay in the presence of ATP, CP, and Ca^{2+} before and after addition of exogenous CK, expressed as μmoles of CP hydrolyzed per min and mg of myofibrils (E) (Wallimann *et al.*, 1984).
[c]CK activity of myofibrils measured by direct pH-stat assay expressed in μmoles of CP hydrolyzed per min and mg of myofibrils (F).
[d]CK content of control IgG-treated myofibrils was taken as 100%.
[e]Means were averaged from three experiments and standard deviations were less than 12%.

extracted the M-line-bound CK as shown by pH stat measurements (Table 2) and indirect immunofluorescence (Wallimann *et al.*, 1984) as well. Treatment of pectoralis myofibrils with excess anti-M-CK Fab followed by washing to remove MM-CK–Fab complexes also resulted in a loss of endogenous ATP regeneration potential of myofibrils (not shown). The CK still remaining bound to the M band after such a treatment, i.e., approximately 8% of the CK bound originally to the M band (Table 2), was not sufficient to keep up with the rate of ATP hydrolysis that was shown to be unimpaired after addition of excess exogenous CK (Wallimann *et al.*, 1984). Incubation with control IgG or Fab did not interfere with M-line-bound CK activity and had no effect on the ATP regeneration potential (Table 2).

2.7. *Comparison of the ATP Regeneration Potentials of Different Muscle Types*

Depending on muscle type, washed myofibrils prepared under identical conditions contained different amounts of myofibrillar CK. Pectoralis major (PM) and posterior latissimus dorsi (PLD) from chicken, both fast-twitch muscles, showed a higher actin-activated Mg^{2+}-ATPase activity than did the slow-tonic anterior latissimus dorsi (ALD) or chicken heart muscle; they also contained more myofibrillar CK (Table 3). The ATP regeneration potentials of the fast- and slow-twitch skeletal myofibrils were sufficient to keep up with the

Table 3. Comparison of the ATP Regeneration Potential by Myofibrillar Creatine Kinase of Different Striated Muscle Types[a–d]

	CK activity μmoles CP / min, mg myofs.	Actin-activated Mg^{2+}-ATPase ($+Ca^{2+}$) μmoles ATP / min, mg myofs.	ATP-regeneration potential	
Pectoralis major	0.78	0.39	Sufficient	(+)
Anterior latissimus dorsi	0.23	0.19	Just sufficient	(±)
Posterior latissimus dorsi	0.61	0.31	Sufficient	(+)
Heart[d] (chicken)	0.02	0.08	Insufficient	(−)

[a]From Wallimann *et al.* (1984).
[b]Creatine kinase activities of myofibrils, prepared under the same conditions as described for pectoralis major, from the muscles indicated, expressed in μmoles of CP hydrolyzed per min and mg of myofibrils, as measured by the direct pH-stat assay (A) (Walliman *et al.*, 1984).
[c]Actin-activated Mg^{2+}-ATPase activities determined by direct pH-stat measurements of ATP hydrolyzed in the presence of Ca^{2+} expressed in μmoles of ATP hydrolyzed per min and mg of myofibrils.
[d]Myofibrils from adult mammalian hearts (swine and bovine) show (as for chicken skeletal muscle) a sufficient ATP regeneration potential to keep up with the *in vitro* myofibrillar ATPase.

ATPase, whereas chicken heart myofibrils, which are known to lack a clear electron-opaque M-band structure, and M-line-bound CK (Wallimann *et al.*, 1977b), did not have sufficient CK to bring about intramyofibrillar ATP regeneration. However, myofibrils from adult bovine and swine hearts, both of which contain CK bound at the M band and which display a clearly defined electron-opaque M-band structure, were fully competent to regenerate sufficient ATP to keep the actin-activated Mg^{2+}-ATPase of these muscles running at maximal *in vitro* speed (Wallimann *et al.*, 1984).

2.8. ATP Regeneration Potential of M-Line-Bound CK in Vivo

Since 1 g of muscle contains roughly 125 mg of myofibrils (see Section 2.2), and since 55% of the myofibrillar protein is myosin, 470,000 M_r, the *in vitro* ATP regeneration potential of the M-line-bound CK (0.8 μmoles CP per mg of myofibrils per min) amounts to 1.8 μmoles of ATP regenerated per gram of wet muscle per sec at 25°C and pH 7.0. Depending on the muscle type, the maximal power output of skeletal muscle during *in vivo* contractions at 20°C was chemically analyzed to be 1.4 and 3.7 μmoles of CP (ATP) hydrolyzed per gram per sec for rat soleus and extensor digitorum longus, respectively (Kushmerick and Davies, 1969; M. J. Kushmerick, personal communication). Values, after correction for temperature, ranging from 2 to 4 μmoles/g per sec were obtained by [^{32}P]-NMR measurements with contracting frog and total muscles (Gadian *et al.*, 1981; Dawson *et al.*, 1977; Kushmerick *et al.*, 1980; Brown, 1982) and values of maximal power output of 1.5–3 μmoles/g per sec were reported for human muscle (McGilvery, 1975; Howald *et al.*, 1978). The M-line-bound CK alone can regenerate enough ATP *in vitro* to support a rate of ATP hydrolysis of 1.8 μmoles/g per sec, and therefore is able to keep up with an ATP turnover rate of the myofibrillar

actin-activated Mg^{2+}-ATPase of more than 6 ATP per second per myosin head under *in vitro* conditions of 25°C, pH 7.0. This rate would correspond to 50 > 100%, depending on muscle type, of ATP turnover measured *in vivo,* indicating that M-line-bound CK alone can maintain a steady, locally high concentration of ATP *in vivo.* Thus, intramyofibrillar regeneration by M-line-bound CK could account for most, or even the entire regeneration, of ATP required for contraction. Under *in vitro* conditions, the presence of excess ATP (4 mM) and CP (10 mM) during the pH stat assay led after a certain time to supercontraction of the myofibrils with corresponding irreversible loss of structure. The myofibrillar ATPase activity as measured by pH stat via CP hydrolysis was linear during supercontraction, and the M-line-bound CK remained associated with supercontracted myofibrils. It is conceivable that, under *in vivo* conditions, the ATP regeneration potential of the M-line-bound CK might be even higher, since compartmentalization and structural integrity of the muscle fiber bundles are conserved, and the 10–20% inhibitory effect on CK activity by 4 mM ATP and Ca^{2+} observed *in vitro* (Wallimann *et al.,* 1984) is alleviated. To test this possibility, single-turnover studies with myofibrils (Sleep, 1981) and tension measurements with chemically skinned fibers (Goldman *et al.,* 1982) should be attempted to avoid supercontraction and to extend the results on ATPase activity and ATP regeneration potential to tension measurements performed in the absence of exogenously added CK. Recent experiments seem to indicate that chemically skinned fibers, generally believed to be free of soluble constituents, readily develop tension upon addition of a limiting amount of ATP and excess CP without the addition of any CK (Ferenczi *et al.,* 1984). This would also indicate that the endogenous CK (presumably bound to the M band) is also sufficient to maintain the ATP needed for continuous tension development (Savabi *et al.,* 1983).

It should be mentioned here that M-line-bound CK does not appear to be an absolute prerequisite for muscle function *per se,* since chicken heart muscle and some slow tonic muscles seem to function adequately within their physiological constraints without a clearly recognizable, electron-opaque M-band structure or M-line-bound CK (Wallimann *et al.,* 1977b), even though the small amount of BB-CK bound at the Z band of chicken heart myofibrils is not sufficient for ATP regeneration (Wallimann *et al.,* 1984). The absence of an M-band structure and M-line-bound CK in chicken heart is an exceptional case that may be related to special hitherto unknown physiological properties of this muscle, since adult mammalian hearts all contain a well-developed M-band structure as well as M-line-bound CK (Wallimann *et al.,* 1977b; Carlsson *et al.,* 1982) sufficient for intramyofibrillar ATP regeneration (Wallimann *et al.,* 1984). As shown recently, differences in the fine structure of the M band, as determined by electron microscopic study with ultrathin frozen sections, turn out to be among the most reliable criteria for discriminating between different muscle fiber types that exhibit distinctly different contractile properties (Thornell, 1980; Sjöström *et al.,* 1982; Thornell and Carlsson, 1984). Therefore, the M band, long thought of as an entity of little significance for muscle contraction, may turn out to exert significant influence on the physio-

logical characteristics of a given muscle fiber type, on the basis of its structural and functional properties.

2.9. Summary of Results

After 10 wash cycles (50 v/w) and incubation with 1% Triton X-100, 0.8 EU of CK activity remains bound per 1 mg chicken pectoralis myofibrils freed of soluble CK, mitochondria, and sarcoplasmic reticulum by differential centrifugation. This activity represents 5% of the total CK present in muscle. The bound CK is specifically located at the M band and contributes to the electron density of this sarcomeric structure (Wallimann *et al.*, 1978, 1983a). Measurement of the combined actin-activated Mg^{2+}-ATPase and CK reactions of such myofibrils in a pH stat assay showed that the M-line-bound CK is active. The amount of M-line-bound CK activity was sufficient to rephosphorylate the ATP hydrolyzed *in vitro* by the actin-activated Mg^{2+}-ATPase of myofibrils, the maximal specific activity of the ATPase being 0.4 ± 0.05 μmoles P_i per min per mg of myofibrils when measured under optimal conditions. The amount of bound CK in pectoralis major (PM) is sufficient to support an ATP turnover rate of 6 ATP per sec per myosin head, corresponding to 50–100% (depending on the muscle type) of the turnover rate *in vivo*. The amount of M-line-bound CK, and concomitantly the ATP regeneration potential, seems to depend on the muscle type: highest in fast muscles (PLD and PM), lower in slow muscles (ALD and mammalian heart), and least in chicken heart. Inhibition of myofibrillar CK activity by DNFB, IAA, and anti-M-CK IgG, or specific extraction of M-line-bound CK by either low ionic strength or incubation with excess of monovalent anti-M-CK Fab abolished the ATP regeneration potential of myofibrils without affecting ATPase activity. Inhibition of myokinase, mitochondrial ADP/ATP-translocase, and respiration did not affect either the ATP regeneration potential or the actin-activated Mg^{2+}-ATPase of the myofibrils, thereby ruling out a significant contribution by adenylate kinase or mitochondria to the observed *in vitro* ATP regeneration.

The M-line-bound CK seems to have the potential for the intramyofibrillar regeneration of most or all of the ATP hydrolyzed by the myofibrillar ATPase during muscle contraction. This finding holds true for all muscles with a well-developed M-band structure and M-line-bound CK. Thus, the intracellular compartmentalization and isoenzyme-specific localization of CK are physiologically significant.

3. Incorporation of M-Line-Bound CK into the CP Shuttle

The results presented in Section 2 provide evidence for a functional role of M-line-bound CK as a potent intramyofibrillar ATP regenerator. This finding is incorporated into a schematic model of CK function and energy flux in muscle in Fig. 8. This updated version of a model proposed earlier by Wallimann (1975) depicts the CP shuttle in muscle, a term coined by Bessman and Geiger in 1981. The various localizations of CK isoenzymes, mitochondri-

al CK-MiMi (No. 2) (Jacobs *et al.*, 1964; Scholte, 1973; Scholte *et al.*, 1973; Jacobus and Lehninger, 1973), sarcoplasmic MM-CK (No. 3), M-line-bound MM-CK (No. 4) (Turner *et al.*, 1973; Wallimann *et al.*, 1977a, 1978, 1983a), and CK bound to sarcoplasmic reticulum or plasma membrane (No. 7) (Baskin and Deamer, 1970; Khan *et al.*, 1971; Sharov *et al.*, 1977; Saks *et al.*, 1977) are depicted as numbers in black squares and white circles in Fig. 8. The model shows microcompartmentalization and functional coupling of CK isoenzymes with energy-producing (oxidative phosphorylation, ATP/ADP-translocase (No. 1), and glycolysis) and energy-consuming (muscle contraction (No. 5), Ca^{2+} pumps and Na^+/K^+-pumps (No. 8)) processes (Bessman and Fonyo, 1966; Jacobus and Lehninger, 1973; Saks *et al.*, 1975, 1976a,b, 1978, 1980; Seraydarian and Abbott, 1976; Yang *et al.*, 1977; Booth and Clark, 1978; Newsholme *et al.*, 1978; Iyengar and Iyengar, 1980; Moreadith and Jacobus, 1982; Erickson-Viitanen *et al.*, 1982a,b; Bessman and Geiger, 1981; Schlösser *et al.*, 1982; Wallimann *et al.*, 1983a, 1984; Paul, 1983). The individual compartments are connected by the CP shuttle and buffered by excess soluble CK (3) and a large CP pool (Naegle, 1970). The directions of energy flux within working muscle that lead to the CP shuttle are indicated by arrowheads.

The model takes into account the large size of the CP pool(s) relative to the ATP pool(s) in resting muscle (Fitch *et al.*, 1968; Fitch, 1977; Burt *et al.*, 1976; Dawson *et al.*, 1977; Gadian *et al.*, 1981), as well as the changes in pH (Burt *et al.*, 1976; Dawson *et al.*, 1977; Hochachka and Mommsen, 1983) and concentrations of energy-rich phosphates that occur during prolonged contractions, metabolic blocking, ischemia, anoxia, or during recovery (Infante and Davies, 1965; Seraydarian *et al.*, 1968, 1969; Dhalla *et al.*, 1972; McGilvery and Murray, 1974; Saks *et al.*, 1978; Ventura-Clapier and Vassort, 1980). The model also incorporates the existence of compartmentalized, protected ATP pools (Gudbjarnason *et al.*, 1970; Nunally and Hollis, 1979) as well as the idea of privileged access of substrates to the different microcompartments (Perry, 1954; Saks *et al.*, 1978; Bessman *et al.*, 1980). The CP shuttle represents an intricate network of energy-utilizing and -producing activities that are mediated by the communication of two different CK isoenzymes—MiMi-CK and MM-CK—which are strategically located at these sites and are able to respond quickly to changes in the local environment and to communicate via large buffer pools of soluble CK and CP. Small compartmentalized pools of "metabolically active" adenosine nucleotide phosphates are turned over at relatively high rates (Gudbjarnason *et al.*, 1970; Nunally and Hollis, 1979) at sites of energy production (Fig. 8, mitochondrial and glycolytic ATP/ADP) and sites of energy consumption (Fig. 8, myofibrillar ATP/ADP) and are connected via the CK/(CP/C) system (Fig. 8: 2,3,4,7, and C/CP). CP is considered not only as a metabolically inert storage and buffering form, but also as a transport form of energy, thus eliminating the need of shuttling the adenosine nucleotide phosphates present in muscle in relatively small concentrations (Bessman and Geiger, 1981), the benefit of which is to render the cell metabolic system capable of being highly responsive to small changes in energy (ATP/ADP) potentials.

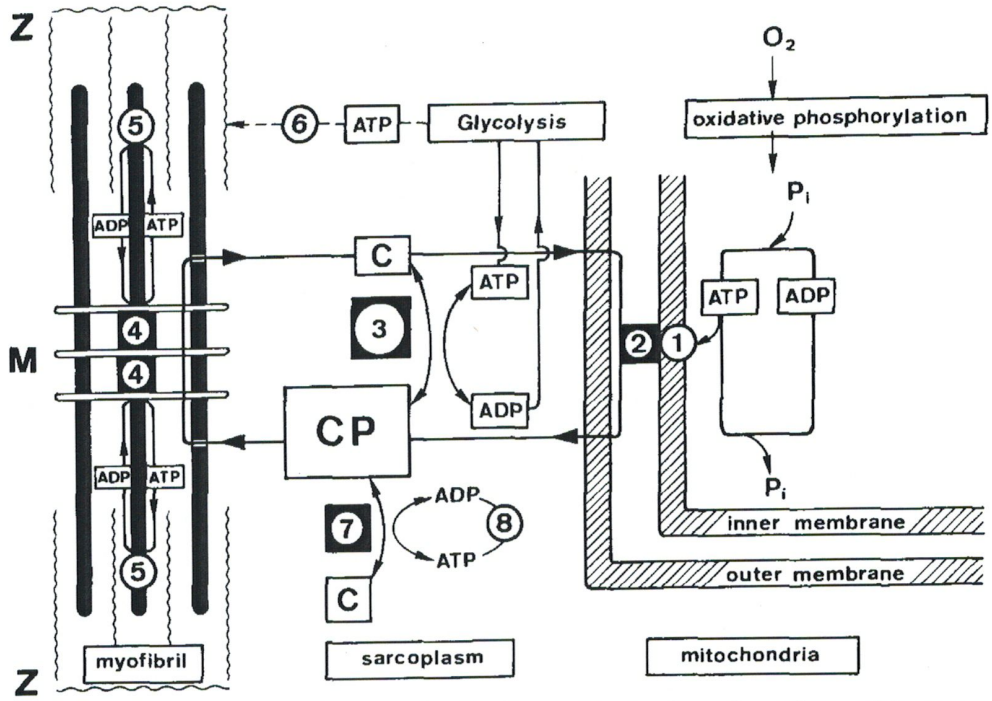

Figure 8. Creatine kinase function and the CP shuttle. Updated version of a model proposed earlier (Wallimann, 1975) of CK action within muscle that is compatible with the CP shuttle proposed independently by Bessman and Geiger in 1981. The various locations of CK isoenzymes in muscle (Nos. 2, 3, 4, and 7) and the microcompartmentalization CK with the mitochondrial ATP/ADP translocase (1), the myofibrillar ATPase (5) and the membrane-associated ATPases (8) are depicted in conjunction with a simplified model of energy flux in a mixed, glyocolytic, and oxidative skeletal muscle or in heart muscle. Phosphorylcreatine (CP) is depicted as an inert pool as well as a transport form of energy. The model shows cyclic hydrolysis and rephosphorylation of small adenosine nucleotide pools connected to the large CP pool(s) *via* creatine kinase present within the cellular compartments at strategically important locations. The flux of energy within working muscle from sites of ATP-generaton (oxidative phosphorylation and glycolysis) to sites of ATP utilization with a high-energy demand is mediated by a CP shuttle. The model also illustrates privileged access of substrates to the various functionally coupled microcompartments and emphasizes the existence of distinct, separated adenosine nucleotide–phosphate pools. (From Nunally and Hollis, 1979.) ATP/ADP translocase system (No. 1); mitochondrial CK isoenzyme (MiMi-CK) (No. 2); sarcoplasmic, soluble MM-CK isoenzyme (No. 3); M-line-bound MM-CK (No. 4); myofibrillar, actin-activated Mg^{2+}-ATPase (No. 5); glycolytic ATP directly available for muscle contraction (No. 6) if such a pathway existed; minor amounts of CK located at different subcellular compartments with high-energy requirements (No. 7), e.g., at sarcoplasmic reticulum or plasma membrane where CK is thought to be kinetically coupled to the Ca^{2+}-ATPase (Ca^{2+} pump) and the (Na^+/K^+) ATPase (Na^+/K^+ pump), respectively (No. 8). See text for details of this model. M, M band; Z, Z line.

In order to appreciate the proposed model of energy flux in muscle (CP shuttle), some facts about the pool sizes of energy-rich phosphates and their compartmentalization, as well as the changes in levels of these metabolites and changes in intracellular pH that take place during contraction, will be discussed in the following sections.

3.1. Energy-Rich Phosphates in Resting Muscle

In resting muscle, the value for the *in vivo* intracellular concentrations of CP (25–35 mM), C (5–10 mM), ATP (4–5 mM), ADP (very low, not determined exactly), P_i (1 mM) and free Mg^{2+} (4 mM) as determined by nondestructive [^{31}P]-NMR methods (Burt *et al.*, 1976; Dawson *et al.*, 1977; Gadian *et al.*, 1981; Cohen and Burt, 1977) are, except for a somewhat higher value for CP content, in good agreement with the values obtained by chemical determinations (Fitch and Shields, 1966; Fitch *et al.*, 1974). Thus, in resting muscle most of the total phosphate recorded by NMR is PC and the ratio of CP to ATP is on the order of 5–6 : 1 (Burt *et al.*, 1976). Less than 10% of the ATP is associated with myosin (Perry, 1952; Barany and Barany, 1972), but some 60% of the total ADP (<1 mM) is bound to the F-actin filaments (Seraydarian *et al.*, 1962; McGilvery and Murray, 1974). Direct phosphorylation of actin-bound ADP does not seem to take place (Moos, 1964; West *et al.*, 1967).

3.2. Changes in Levels of Energy-Rich Phosphates during Muscle Contraction in Vivo

The prime source of energy for muscle contraction is the hydrolysis of ATP by the myofibrillar actin-activated Mg^{2+}-ATPase (for review, see Taylor, 1972). However, in living muscle contraction is not accompanied by measurable changes in intracellular ATP but rather by hydrolysis of CP. The net breakdown of CP into C and P_i, while ATP levels remain constant, was unequivocally demonstrated by blocking the glycolytic pathway, but not the CK activity, with small concentrations of IAA (Carlson and Siger, 1960). It was possible to show the breakdown of ATP during contraction only if the CK system had been completely blocked by DNFB (Cain and Davies, 1962). Upon inhibition of CK activity with DNFB, only a small fraction of the total intracellular ATP can be used for muscle contraction and, even though the ATP levels are still around 70–80% that of normal values, the muscle fails to respond to further stimulation (Infante and Davies, 1965).

These earlier results, which already pointed to the crucial function of the CK/CP system in muscle-energy metabolism and to the possibility of compartmentalization within the high-energy P_i system, were fully confirmed by modern, noninvasive [^{31}P]-NMR techniques where a net breakdown of CP during single tetanic contractions could be recorded. Again, no changes in intracellular ATP are observed and long-term changes in CP are only pronounced if recovery is impeded by turning off the perfusion pump (Dawson *et al.*, 1977; Neurohr *et al.*, 1983).

A very important step in recognizing the function of the CK/(CP/C) system as a most important metabolic regulatory parameter of muscle energetics was taken by Gudbjarnason *et al.* (1970) and Dhalla *et al.* (1972), who showed that contractility in heart is significantly impaired during fatigue and periods of nonperfusion (ischemia). The loss of contractility paralleled the marked fall of CP levels, with ATP remaining unchanged or still at relatively

high levels. The decrease in peak tension of heart during strong metabolic inhibition was also associated with a decrease in CP but not ATP (Ventura-Clapier and Vassort, 1980; Neurohr *et al.*, 1983). In addition, recovery from severe anoxia of contractile activity followed the subsequent replenishment of CP, while ATP levels remained as low as 20% of the resting value (Naegle, 1970).

Theoretical modeling of CK function in muscle based on simulating the concentration distribution of the various compounds that are involved in energy metabolism as high-energy phosphate is consumed (McGilvery and Murray, 1974) is in accord with older observations in laboratory animals and has been repeatedly confirmed by human biopsies (for review, see Howald *et al.*, 1978). These studies show that an increasing work load is mainly reflected by a decline in CP with little changes in ATP and ADP, in absolute terms, until near-exhaustion. Under prolonged heavy exercise, at maximum energy output that eventually leads to exhaustion, CP levels in humans can be lowered to 30% of original levels with ATP remaining relatively high at 80% of the resting concentration (Karlsson and Saltin, 1970). Under these extreme conditions measurable but still low levels of ADP may activate yet another ATP-regeneration system, i.e., the myokinase reaction ($2ADP \rightarrow ATP + AMP$). The AMP formed by myokinase is removed from primary metabolism by AMP-deaminase, which also is located at the A band (Ashby and Frieden, 1977; Ashby *et al.*, 1979) to yield IMP (Kushmerick and Davies, 1969).

3.3. Effects of Local Changes in Substrate Concentrations and pH Value during Contraction

The first event in the activation of the myofibrillar ATPase by calcium is hydrolysis of ATP, leading to a local accumulation of ADP and H^+, both of which actually serve as substrates for the Lohmann reaction by CK. Availability of ADP plus CP and the lowering of intramyofibrillar pH will activate the M-line-bound CK in the direction of ATP regeneration (pH optimum of the CK reaction is between pH 6.5 and 6.6; Wallimann *et al.*, 1984). On the other hand, the myofibrillar ATPase (pH optimum between pH 7.5–7.8, Wallimann *et al.*, 1984) is slightly inhibited by lowering of the pH. It is during this first phase that M-line-bound CK would act as a rapidly responding intramyofibrillar ATP-regenerator. Upon functional coupling of the myofibrillar CK with the myofibrillar ATPase, a slight alkalinization, measurable *in vivo* (Dawson *et al.*, 1977), is to be expected as CP is continuously being hydrolyzed (see Fig. 6).

During the second phase, after prolonged stimulation when CP reserves are slowly depleted, the rate of glycolysis as a secondary backup system increases. Transphosphorylation by sarcoplasmic CK of glycolytically derived ATP as well as direct hydrolysis of glycolytic ATP (if the latter pathway exists *in vivo*) then leads to a net production of protons (Hochachka and Mommsen, 1983) and to a concomitant lowering of the intracellular pH that can deviate (depending on muscle type) more than 0.5 pH units from the normal pH

values in resting muscle, pH 6.8–7.2 (Roos and Boron, 1981), as shown by direct NMR measurements (Dawson *et al.*, 1977).

During a third phase, in oxidative and mixed muscles, protons are absorbed by mitochondria as a consequence of oxidative phosphorylation of ADP (Jacobus and Lehninger, 1973; Saks *et al.*, 1978), counterbalancing a further sharp pH drop during prolonged exercise. The changes in local pH and the increased availability of creatine and mitochondrial matrix generated ATP then lead to increased mitochondrial CK activity. Functional coupling via ATP/ADP translocase of mitochondrial CK action with oxidative phosphorylation leads to a net production of CP by mitochondria that is fed into the CP pool and is then transported to the sites of action in the myofibrillar compartment (Bessman and Fonyo, 1966; Jacobus and Lehninger, 1973; Saks *et al.*, 1980; Moreadith and Jacobus, 1982; Erickson-Viitanen *et al.*, 1982a). Thus, proton-mediated augmentation of glycolysis and oxidative phosphorylation, preceding the usually reported inhibitory effects on glycolysis, may play an important role for overall muscle energetics and recovery (see review by Hochachka and Mommsen, 1983). As far as M-line-bound CK is concerned, local changes in intracellular pH as well as availability of substrates seem to be effective in regulating myofibrillar ATP hydrolysis and regeneration. The presence of bound CK within the myofibrillar compartment might ensure rapid ATP regeneration during early phases of contraction when CP is still in excess. Such a function of M-line-bound CK at the receiving end of the CP shuttle is supported by experimental data and is illustrated in the CP-shuttle model (Fig. 8).

3.4. Compartmentalization in Muscle

As early as 1961, Lee and Visscher concluded from perfusion studies using ^{14}C-labeled creatine that there may be more than one compartment of CP, C, and adenosine nucleotide phosphates in the heart. Later on, the early cessation of contractility in ischemic heart led Gudbjarnason *et al.* (1970) to suggest the existence of functionally compartmentalized pools of ATP and CP. These investigators postulated, as did Naegle in 1970, that transfer of energy-rich phosphates from the site of synthesis to the sites of utilization is of critical importance in the maintenance of muscle contraction. The fact that contractility is severely affected as a consequence of falling CP levels, even though intracellular ATP remains unchanged (Cain and Davies, 1962; Infante and Davies, 1965), would also indicate that only a small portion of the intracellular ATP presumably derived from glycolysis is directly available for muscle contraction (Fig. 8, No. 6), as concluded by Seraydarian *et al.* (1968, 1969), Naegle (1970), McGilvery and Murray (1974), Saks *et al.* (1978). Recently, segregation of enzymes and substrates involved in energy metabolism and compartmentalization of metabolic pathways, i.e., glycolysis and oxidative phosphorylation, was suggested by Paul (1983). Evidence from [^{31}P]-NMR saturation transfer experiments of energy-rich phosphate kinetics in normal and ischemic perfused hearts also point to a compartmentalized system of CK

action (Brown *et al.*, 1978). Direct evidence, also by NMR techniques, for the existence of "protected" ATP pools which are not easily reached by Mn^{2+} and thus differ in their ability to interact with the CK system, was presented by Nunally and Hollis in 1979; this work suggested the existence of ATP compartments at the myofibrillar and mitochondrial side of the CP shuttle.

The apparent paradox that ATP—the prime source of energy for muscle contraction—is restored by rephosphorylation from CP, while at the same time the maintainance of CP levels must be accomplished by ATP, can be explained by incorporating the concept of compartmentalization in muscle (Nunally and Hollis, 1979) into the CP-shuttle model. Since CK catalyzes both reactions, the forward and backward events would have to be separated in space in order to guarantee an effective functioning (Nunally and Hollis, 1979).

3.5. Kinetic Properties of Myofibrillar CK and Privileged Access of CP

Myofibrillar CK is similar or identical to the soluble MM-CK isoenzyme (Turner *et al.*, 1973). It is evident from the K_m values for CP and C (K_m values of MM-CK for CP and C are 1.7 mM and 16 mM, respectively (Saks *et al.*, 1976a), that for the isolated MM-CK isoenzyme the reverse reaction (formation of ATP, Lohmann reaction) is kinetically preferred. The high affinity of MM-CK for Mg-ADP (K_m = 0.08 mM) causes the latter to be effectively trapped and rephosphorylated into ATP by CK as long as CP is present at concentrations of ~3–4 mM or higher. The possibility that ATP generated by CK within the myofibrillar compartment may be more accessible for hydrolysis than is extramyofibrillar ATP, is supported by other evidence. As early as 1954, Perry showed that in the presence of CK and CP, 3 μm of ADP caused a shortening of glycerinated myofibrils, whereas a similar degree of shortening could not be observed unless >60 μm ATP was added directly to the myofibrils. These results were confirmed in our laboratory with freshly isolated, washed myofibrils without addition of exogenous CK (T. Schlösser and T. Wallimann, unpublished data). The observation by Maughan *et al.* (1978) that a CK/CP backup system did improve the mechanical performance of mechanically disrupted cardiac cells even in the presence of high concentrations of ATP, points into the same direction. Recently, Bessman and co-workers (1980) have shown that by adding [γ^{32}P]-ATP, unlabeled ADP and CP to isolated myofibrils the label in the inorganic P_i formed was greatly diluted, indicating that ATP formed by CP via CK can reach the actin-activated Mg^{2+}-ATPase active site of myosin more readily than labeled ATP from the medium. Glycerinated muscle fiber bundles containing native CK, in the presence of small amounts of ADP and physiological concentration of CP, produced faster, stronger contractions and faster, more complete relaxation than did equimolar higher concentrations of ATP (Savabi *et al.*, 1983). These data fit well with our direct measurements of the ATP regeneration potential of M-line-bound CK and support a tight, functional coupling of CK and myosin ATPase. The data indicate that ATP generated within the myofibrillar "compartment" by CK and CP may be more easily available for the myofibrillar

ATPase. Because of its molecular size, charge, and ability to bind to a large number of proteins, ATP, within the highly organized fibrillar structure of muscle, may be even less mobile than its twofold smaller-than-CP *in vitro* diffusion coefficient would indicate (Naegle, 1968).

For these reasons, the availability (concentration × diffusibility) of CP (which is a smaller, less charged molecule that binds only to CK) for the myofibrillar "compartment" can be estimated to be >20 times higher than that of ATP.

3.6. Why CK at the M Band?

The question arises as to why a relatively small amount of the total CK is located specifically at the M band. It is found at the central bare zone of the thick filament, at a distance of 0.1–0.8 μm (depending on the state of contraction) from the sites (actomyosin overlap zones to either side), where ATP is hydrolyzed. One of the reasons may simply be that there is no room left for CK to be located at the A band, without interfering with the structurally and topologically intricate cyclic attachment and detachment of myosin cross-bridges for a number of myosin-associated proteins, e.g., C protein, H protein, and X protein (Starr and Offer, 1971; Ashby *et al.*, 1979; Starr and Offer, 1983) are already occupying specific sites on the thick filament. If CK cannot be directly attached to at the sites of ATP hydrolysis, a location between the A-band halves seems the next best alternative. Given the evidence by Eisenberg *et al.* (1979) for highly anisotropic diffusion, even for 100 M_r compounds within muscle-fiber bundles, diffusion of sarcoplasma perpendicular to the myofilament axes is slower than along the filaments; the presence of CK within the M-band disk may be advantageous for intramyofibrillar ATP regeneration, especially at the very center of the myofibril.

It should be mentioned that it is still unresolved as to whether the interfilament space in muscle, especially in the actomyosin overlap region, is indiscriminately accessible to all sarcoplasmic constituents. It is perhaps indicative that metabolites, glycolytic enzymes, and soluble CK all seem to be preferentially concentrated in the I-band region (Arnold and Pette, 1970), even though some of them, such as CK, do not bind to thin filaments (Bronstein and Knull, 1981). To answer this question, the influence of structurally bound water around thick and thin filaments as well as the *in vivo* fluid dynamics would also have to be considered. Unfortunately, little information is available on these factors.

During contraction, the two thin filament lattices of a sarcomere are being pulled by the thick filaments toward the center of the sarcomere. Thus, despite the fact that during this process the overall volume of muscle does not change, it is conceivable that the pistonlike interdigitation of sliding thick- and thin-filament lattices (Huxley, 1973) is also moving sarcoplasma, enriched in ADP, H^+, and P_i, toward the M band, where immediate ATP regeneration by the M-line-bound CK would take place. In addition, the power-stroke movement of individual myosin heads may generate a "sarcoplasmic streaming." Due to the fact that in striated muscle myosin molecules are organized into

bipolar thick filaments, which pull on the thin filaments the direction of the sarcoplasmic streaming would be toward the M line, opposite to the cytoplasmic streaming observed by Sheetz and Spudich (1983), who showed that myosin-coated fluorescence beads can "walk" along organized actin cables in the direction of the Z line. In this context a localization of CK in the central bare zone region of the thick filament, at the M band, between the two A-band halves would seem to be physiologically advantageous for rapid intramyofibrillar ATP regeneration. Results (from our laboratory) from immunofluorescence of unwashed *in situ* fixed and cryosectioned whole muscle tissue show that CK is mainly located in the I band (sparing the Z line) and in the M band, but little or none is found within both the A-band halves (G. Wegmann, personal communication).

3.7. Soluble Sarcoplasmic CK

If transphosphorylation by mitochondrial CK is able to keep up with the production of matrix-generated ATP in mitochondria (Saks *et al.*, 1978), and likewise, M-line-bound CK is sufficient, provided there is an excess of CP to regenerate the ATP used for muscle contraction, what, then, is the function of the bulk of CK that is soluble?

Creatine is synthesized in a stepwise manner in liver, kidney, and pancreas, but not in muscle, and is carried by the blood (at concentrations of <0.1 mM) to the muscle tissues where it is concentrated, mostly as CP to ~30 mM (for review, see Fitch, 1977). Two mechanisms—a saturable entry process and an active transport system in combination with intracellular trapping—have been proposed as involved in maintaining these high concentrations of CP and C in muscle (Fitch and Shields, 1966; Fitch *et al.*, 1968). The extent of the involvement of soluble CK in intracellular trapping of C or CP is not known. Since the K_m of MM-CK for CP (1.5 mM) is 10-fold lower than the K_m for C (Saks *et al.*, 1976a), the 5–7 g of CK per kg of wet muscle could theoretically trap by direct binding only 100 μmoles of CP/kg, whereas the *in vivo* concentration of CP is ~25 mM. Thus the vast amounts of soluble CK in muscle cannot be considered a sink for CP.

We propose that some if not all of the sarcoplasmic CK is functionally coupled to glycolysis and is playing an important role during anaerobic recovery by replenishing the depleting CP pool(s) through transphosphorylation of glycolytically generated ATP. Evidence that the glycolytic system may be under the direct control of the CK/CP/C system has been reported and CP has been proposed to act as an inhibitor of several glycolytic enzymes, but only the inhibition of glyceraldehyde 3-phosphate dehydrogenase by CP occurs at levels consistent with the *in vivo* situation in resting muscle (Oguchi *et al.*, 1973). The reported inhibitions of phosphofructokinase and pyruvate kinase by CP (Uyeda and Racker, 1965; Kemp, 1973) have been shown to be an artifact of an impurity in CP preparations (Fitch *et al.*, 1979). In this context, it would be interesting to know whether creatine exerts a positive control effect on glycolysis.

Although evidence for direct coupling of the CK system to glycolysis is

sparse, the sarcoplasmic CK seems to be functionally coupled to glycolysis, in light of several observations.

1. The amount of soluble CK and the glycolytic potential of muscle are correlated such that glycolytic muscles (e.g., chicken pectoralis major) contain, in absolute terms, approximately four times higher levels of soluble CK and a much higher ratio of sarcoplasmic versus mitochondrial CK when compared with oxidative muscles (Wallimann *et al.*, 1984).
2. The ATP produced by glycolysis upon stimulation of muscle does not accumulate but is immediately and efficiently transphosphorylated to replenish the CP level and maintain a high CP/C ratio.
3. Depletion of CP in a model of defective muscle glycolysis has been directly demonstrated (Brumback *et al.*, 1983).
4. Glycolytic enzymes and soluble CK are in close proximity, contained mostly within the I band of the sarcomere (Arnold and Pette, 1970; Bronstein and Knull, 1981), where the interfilament spacing is larger compared with the actomyosin overlap zone, allowing a concentration of myoplasm at this site, where functionally coupled microcompartments may be formed.

The high concentration of soluble CK would serve to react quickly in a sensitive fashion to any local changes in the energy charge ratios. That is, by replenishing depleted CP, sarcoplasmic CK would efficiently remove excess "metabolically active" ATP formed during anaerobic recovery by glycolysis, but at the same time it would maintain the high ATP/ADP ratio so crucial in directing many metabolic processes (Veech *et al.*, 1979). There is no direct evidence that glycolytically produced ATP is directly used for muscle contraction without prior transphosphorylation by sarcoplasmic CK into CP *in vivo* (Cain and Davies, 1962; McGilvery and Murray, 1974). In contrast to the myofibrillar M-line-bound CK involved in rapid *in situ* ATP regeneration, thereby depleting CP, sarcoplasmic CK would be mainly responsible for replenishing the depleted CP pool(s). In glycolytic muscle, the anaerobic route of recovery via glycolysis and in oxidative muscle the aerobic route via mitochondrial oxidative phosphorylation is preferentially taken. In mixed muscle, the two recovery systems are fine-tuned to act in a concerted fashion to meet the energy requirements of contracting muscle at any point of time (Zammit and Newsholme, 1976). Here, the high buffering capacity of sarcoplasmic CK is thought to play a crucial role in communicating between the two recovery systems and maintain the proper energy charge ratios best suited to both systems.

3.8. Mitochondrial CK

The mitochondrial CK isoenzyme, MiMi-CK, discovered by Jacobs *et al.* in 1964, is present in significant amounts in mitochondria from skeletal muscle, brain, and heart (Jacobus and Lehninger, 1973). The possible importance of this isoenzyme of CK for energetics, especially in the oxidative heart muscle, has attracted considerable attention. MiMi-CK is thought to be located outside

the atractyloside-sensitive ATP/ADP translocase system at the outer surface of the inner mitochondrial membrane (Jacobs *et al.*, 1964; Scholte *et al.*, 1973; Jacobus and Lehninger, 1973; Sharov *et al.*, 1977; Iyengar and Iyengar, 1980; Saks *et al.*, 1980; Moreadith and Jacobus, 1982). In the presence of creatine and trace amounts of ADP to initiate oxidative phosphorylation, mitochondria from muscle, heart, and brain release CP into the extramitochondrial space. It has therefore been proposed that MiMi-CK participates in the production of CP in concert with oxidative phosphorylation (Bessman and Fonyo, 1966; Jacobus and Lehninger, 1973; Saks *et al.*, 1975, 1978; Booth and Clark, 1978). Thus, MiMi-CK would work in the direction of CP synthesis, opposite to the direction of myofibrillar, M-line-bound CK which works in the direction of ATP regeneration (Fig. 8).

Although solubilized MiMi-CK and sarcoplasmic MM-CK isoenzymes differ somewhat in their kinetic properties (Saks *et al.*, 1975, 1976), and although these differences are enhanced upon binding of MiMi-CK to the inner mitochondrial membrane (K_m for ATP of bound MiMi-CK is reduced by factor of 5–7 upon binding), the forward CK reaction—production of CP—is still kinetically less favorable for both isoenzymes (Saks *et al.*, 1978, 1980). Thus, the tempting idea that the different functional roles of the CK isoenzymes at the mitochondrial and myofibrillar ends of the CP shuttle were solely determined by their different kinetic parameters had to be abandoned (Saks *et al.*, 1978). It is even more surprising that in isolated heart mitochondria CK is still able to phosphorylate creatine at high rates, even in the presence of concentrations of CP as high as those found *in vivo* (Saks *et al.*, 1975, 1978), indicating that the inhibition of CK by CP is much weaker in intact mitochondria than with solubilized MiMi-CK (Saks *et al.*, 1980). On the basis of these findings, the concept of compartmentalization of MiMi-CK into a functionally coupled microcompartment together with oxidative phosphorylation and the concept of privileged access of substrates within such compartments has been put forward (Saks *et al.*, 1976, 1978, 1980). Experiments with radioactive phosphate or [γ-^{32}P]-ATP showed privileged access of newly synthesized matrix-generated ATP to MiMi-CK prior to mixing with the total ATP pool (Yang *et al.*, 1977; Erickson-Viitanen *et al.*, 1982). The production of CP via matrix-generated ATP is dependent on oxidative phosphorylation and ATP transport, since no CP is produced by mitochondria that were treated with oligomycin or atractylosides (Jacobus and Lehninger, 1973; Booth and Clark, 1978). In addition, newly formed ADP generated by MiMi-CK is preferentially rechanneled into the mitochondrial matrix and is in such close proximity to the ATP/ADP translocase system that it effectively overcomes the atractyloside inhibition of respiration (Moreadith and Jacobus, 1982). Thus, as a net product of oxidative phosphorylation, a tight functional coupling of and MiMi-CK translocase is assumed to yield CP, which then is fed into the large extramitochondrial CP pool(s) (Fig. 8). The influx of creatine into the mitochondria and the transphosphorylation of matrix-generated ATP transported by ATP/ADP translocase to the functionally coupled MiMi-CK leads to a consequent maintenance of relatively high local ADP concentration within

the mitochondrial compartment, thereby ensuring continued respiration of mitochondria upon prolonged stimulation of muscle (Saks *et al.*, 1974). As a consequence, no transport or diffusion of extramitochondrial ADP from sites of energy consumption to the mitochondria would be needed, and mitochondrial function would be efficiently controlled by creatine (Fig. 8). In spite of some controversy about the direct coupling of mitochondrial CK with translocase (Altschuld and Brierley, 1977; Borrebaek, 1980) and the recent suggestion of an additional mechanism for compartmentalization stressing the importance of the outer mitochondrial membrane as a partial diffusion barrier limiting the efflux of matrix-generated ATP (Erickson-Viitanen *et al.*, 1982b), the overwhelming body of evidence clearly indicates that compartmentalization of mitochondrial CK does exist. It also suggests that the enzyme is linked to oxidative phosphorylation in such a manner that it expresses a preference for the source of substrate to finally yield CP as a net product of respiration in both muscle and brain mitochondria (for reviews, see Saks *et al.*, 1978; Bessman and Geiger, 1981).

The importance of MiMi-CK as part of the CP shuttle in energy metabolism and contractility of heart muscle is also stressed by developmental studies, showing that besides the well-known isoenzyme transition from B- to M-CK that takes place during myogenesis (Eppenberger *et al.*, 1964), the mitochondrial CK isoenzyme is not found until about 6 days after birth in the myocardial tissue of mice, rats, and rabbits (Hall and DeLuca, 1975). This is also the time when, during postnatal development, incorporation of MM-CK into the M band of these mammalian heart muscles begins (Carlsson *et al.*, 1982) and a general maturation of the heart muscle toward its full contractile potential takes place (Hopkins *et al.*, 1973; Baldwin *et al.*, 1977). Thus, the coordinate postnatal appearance of the enzymes involved in the CP producing side (MiMi-CK) and in the receiving end of the CP shuttle (M-line-bound MM-CK) emphasizes the need for functional coupling of the two systems in the integrated CP shuttle for optimal muscle function. During brain development, CK also increases markedly at a time when greater coordination of complex nervous activity is becoming apparent, and the similar developmental pattern of CK to that of hexokinase suggests that CK is involved in the overall coordination of energy metabolism and neurotransmission in the fully active adult brain (Booth and Clark, 1978; Norwood *et al.*, 1983).

3.9. Conclusion and Future Prospects

The important aspect of the CP-shuttle model is that according to the acceptor-function theory (Bessman and Fonyo, 1966), oxidative phosphorylation is stimulated by creatine to yield (via transphosphorylation of matrix generated ATP by MiMi-CK) CP, which then is available for energy-requiring reactions. Under these conditions MiMi-CK controls oxidative phosphorylation in mitochondria through the steady-state level of intramitochondrial ADP. As a consequence, both on the energy-producing side (mitochondria, glycolysis) and on the energy-utilizing side (myofibrils, Na^+/K^+ pumps,

Ca^{2+} pumps) of the CP shuttle, small, spacially separated pools of ATP/ADP are being rapidly turned over (Nunally and Hollis, 1979). This turnover is in opposite directions and in a cyclic manner, which is accomplished by each of these sites being a functionally coupled microcompartment with CK isoenzymes. The conversation between the sites is mediated by a CP shuttle and by the proper CP/P and ATP/ADP ratios, which are adjusted by an excess of soluble sarcoplasmic CK (Fig. 8). As stated by Moreadith and Jacobus (1982), microcompartmentalization of CK in the mitochondria and myofibrils may not have to be obligatorily coupled. Each "compartment" may accept its substrates from other sources if the preferred source is not available; e.g., myofibrils readily contract *in vitro* upon direct addition of ATP, and mitochondria may begin respiration *in vitro* with exogenous ADP with no CP or C added in either case. Within the proposed CP-shuttle model we do not imply exclusive access, but would like to stress that preferred pathways do exist also *in vivo* at these microcompartments where, upon stimulation of muscle and during recovery, preferences of substrates and proper channeling of energy are given by the structural arrangement and the local environment. Thus, in muscle, the mitochondria and the sites of glycolysis at which high-energy phosphate synthesis, conversion, and trapping systems are located, represent the origins of the CP shuttle that deliver CP to many subcellular target microcompartments, which may be considered receiving ends of the CP shuttle. One such compartment is represented by the M-line-bound CK, which is functionally coupled to the myofibrillar ATPase of myosin filaments. On the basis of the experimental data, it seems that M-line-bound CK, a potent intramyofibrillar ATP-regenerating system at the distal end of the CP shuttle, which is able to support an ATP turnover rate of 6 ATP per sec per myosin head (Wallimann *et al.*, 1984), is physiologically significant in muscle contraction by contributing to the basic muscle characteristics of speed and frequency of contraction, contractility, and overall muscle performance.

After incorporation of the M-line-bound CK as an ATP-regenerating system at the myofibrillar, receiving end of the CP shuttle, the model illustrates that during normal performance of muscle, the intracellular ATP level remains constant. If some allowance is given for recovery, only small changes in the CP level occur, because the CP transphosphorylated by M-line-bound CK to yield ATP as a direct source for muscle contraction is replenished by the bulk of soluble CK, first via glycolytically generated ATP and then by mitochondrial CK via matrix-generated ATP (Fig. 8). The ATP-regeneration potential of M-line-bound CK has a capacity that may account *in vivo*, in muscles with a well-developed M-band structure and M-line-bound CK, for the intramyofibrillar regeneration of most if not all of the ATP hydrolyzed by the myofibrillar ATPase during muscle contraction.

Excitable tissues (e.g., brain, nerves, muscle, and electric organ) as well as tissues of high-energy demand (e.g., eggs and early embryos), in which the maintenance of a critical ATP/ADP ratio is essential (Iyengar *et al.*, 1983), depend by and large on phosphagen metabolism. Thus, these tissues contain a relatively high concentration of phosphagen and phosphagen kinases, i.e.,

CP and CK in vertebrates. Many or all of the life processes that critically depend on ATP, such as buildup and maintenance of potentials, as well as propagation and transmission of signals (synaptic membranes), ATP-driven ion pumps (Na^+/K^+-ATPase), calcium sequestration (Ca^{2+} ATPases), contraction, motility (myosin ATPase and dynein ATPases), as well as general functions of cell anabolism (Carpenter *et al.*, 1983), cell division, proliferation, and differentiation (Iyengar *et al.*, 1983; Koons *et al.*, 1982), depend on locally high and immediately available energy supply. We propose that these are coupled with phosphagen kinases (CK in vertebrates) to form efficient, subcellular, functionally coupled microcompartments in which small amounts of ATP are turned over locally in a cyclic manner, as shown in Fig. 8. Similar functionally coupled microcompartments, working in the reverse direction, are located at sites of ATP production (mitochondria, glycolysis), where the association with CK warrants rapid removal of "metabolically active" ATP to build up CP as an energy storage and transport form (Fig. 8). Important functions in cell motility (Eckert *et al.*, 1980; Fuseler *et al.*, 1981) and spindle elongation (Koons *et al.*, 1982; Cande, 1983) have recently been inferred to be mediated by CK. Efforts are under way in our laboratory to find comparable compartments within excitable cells that are functionally coupled with CK (Barrantes *et al.*, 1983a,b) and to localize directly the enzymes involved.

Addendum

The complexity of the M-band structure is revealed by high-resolution electron microscopy of negatively stained ultrathin cryosections of muscle (Fig. 9). Micrographs of chicken pectoralis M-band regions typically show a 5-line striation pattern made up of the five m-bridge arrays: M1, M4, M4′, M6, and M6′ (nomenclature according to Sjöström and Squire, 19771,b) (Fig. 9A). Additional less prevalent striations (M2, M3, M5, Fig. 9A) are also seen. The micrograph of the very thin section shown in Fig. 9A illustrates further that the m-bridges M1, M4, and M6 are qualitatively different from each other. The striations M4 and M4′ are very distinct and sharp, while M1 and M6 and M6′ are broader and more fuzzy. No evidence of m-filaments is present, although the resolution is very good, e.g., note that lines M2 and M3, with a width of ~2 nm, are clearly resolved (Thornell and Carlsson, 1984). The different appearances and the diversity of the M-band structure is obvious when different muscle-fiber types within the same species or muscles from different species are compared (Fig. 9). Three principal types of m-bridge striation patterns are distinguished. For example, a 5-line pattern similar to that seen in Fig. 9A, and B is observed in undifferentiated skeletal muscle fibers of newborn rats, while in 28-day-old rats the M band varies with fiber type, i.e., a 4-line pattern similar to that seen in Fig. 9C is observed in slow-twitch (type-1) fibers of the soleus muscle, while a 3-line pattern similar to that seen in Fig. 9D is observed in the fast-twitch (type-2B) fibers of the extensor digitorum longus, and a 5-line pattern (Fig. 9a and b) is found in the fast-

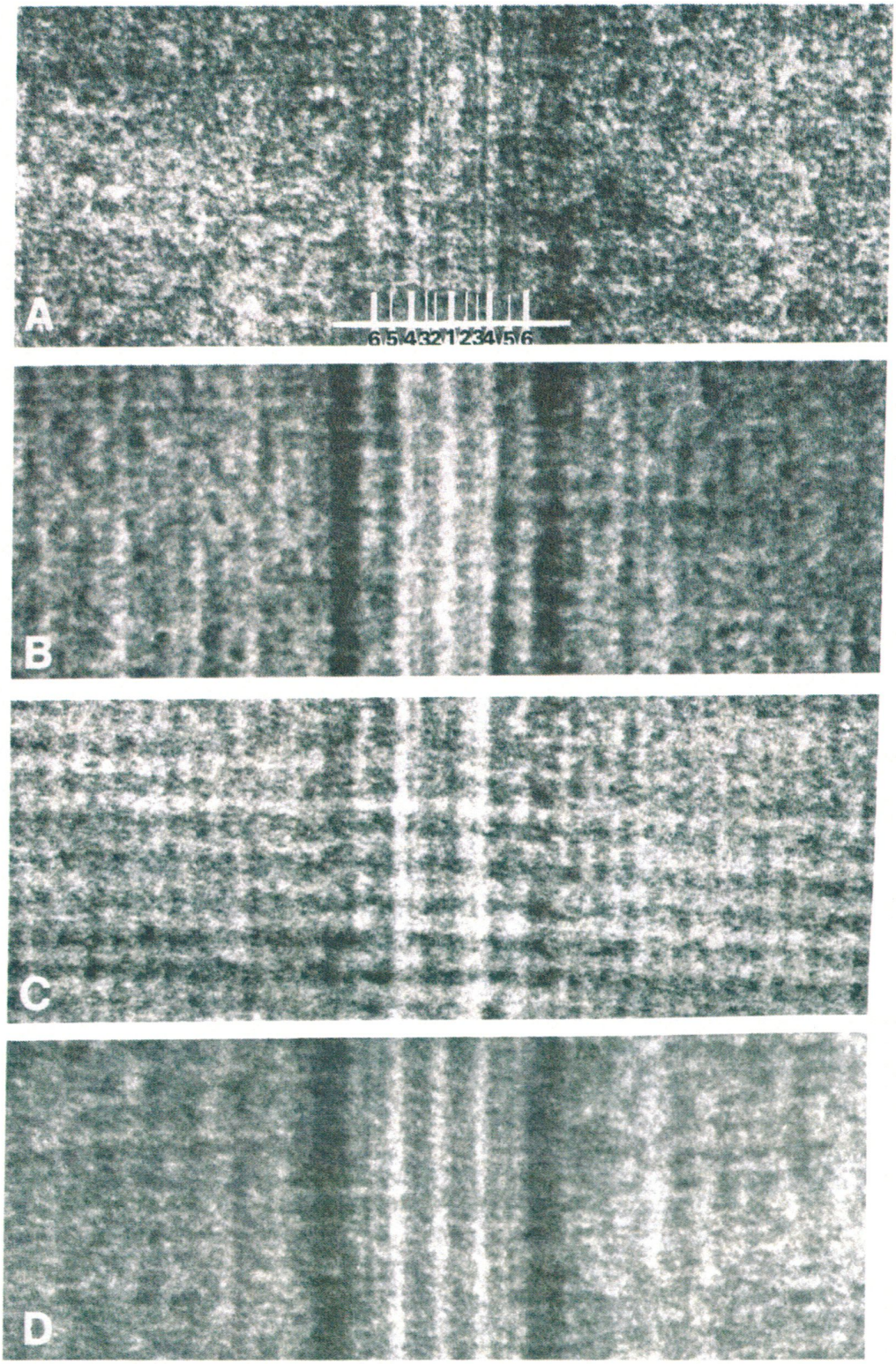

Figure 9. M-band diversity in negatively stained ultrathin cryosections of mature skeletal muscle fibers, as seen by high-resolution electron microscopy at a magnification of ×317,000. The three principal M-band striation patterns (taking into account the most prominent bands only) are shown, e.g., the 5-line pattern (Fig. 9A,B), the 4-line pattern (Fig. 9C) and the 3-line pattern (Fig. 9D). When viewing the micrographs obliquely along the strations their different widths and

twitch (type-2A) fibers of the same muscle (Thornell and Carlsson, 1984). Thus, the M-band structure in rat changes postnatally as the fiber-type specification takes place. No M-band cross-striations are present in the heart of newborn rats, but a distinct 5-line pattern similar to that in Fig. 9B appears with postnatal development at a time when MM-CK is incorporated into the M band (Carlsson *et al.*, 1982; T. Wallimann, unpublished data). These findings support a direct involvement of MM-CK as an integral M-band protein, making up for some of the main m-bridge (M4, M4') structures (Wallimann *et al.*, 1977, 1978, 1983).

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densities are evident. (A) Very thin section from a chicken pectoralis fast-twitch muscle fiber. Nomenclature of striations according to Sjöström and Squire (1977a,b). (B) M band of human tibial muscle (type-1 fiber), a 5-line pattern is apparent. (C) M band of human masseter muscle (type-1 fiber), here the central M1 striation is very weak, giving rise to a 4-line pattern. (D) M band of fish skeletal muscle fibre, here of the 5 lines related to m-bridges only M1 and M4 are strong, leading to a 3-line pattern. Courtesy of S. Karger, Ab, Basel, Switzerland; reprinted from Thornell and Carlsson (1984).

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