


# Localization and function of M-line-bound creatinekinase

## M-band model and creatine-phosphate shuttle

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**Author(s):**

Wallimann, Theo 

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LOCALIZATION AND FUNCTION OF M-LINE-BOUND CREATINEKINASE.

*Struktur* M-BAND MODEL AND CREATINE-PHOSPHATE SHUTTLE.

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Habilitationsschrift

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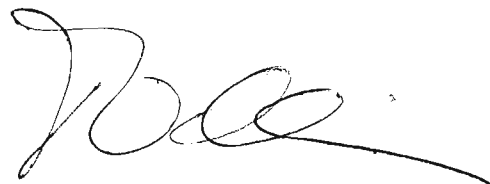
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Eidgenössische Technische Hochschule Zürich

von Theo Wallimann, Institut für Zellbiologie ETH

im November 1983



Dr. Theo Wallimann  
Schürmattstr. 23  
8962 Bergdietikon, AG  
Tel. 044-740 70 47

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## 1. STRUCTURE OF THE M-BAND

### 1.1 M-band structure

The electron-opaque M-line or M-band which transverses the center of the A-band is one of the striking features of cross-striated muscle myofibrils seen in the electron microscope (Fig. 1-5). It appears to be the only myofibrillar structure that directly connects thick filaments to each other. After "in situ" fixation of skeletal muscle, dehydration and standard embedding for electron microscopy, the M-band structure appears as a complex structure being made up of several transverse elements connecting the thick filaments through the bare zone region to give rise to the typical hexagonal thick filament lattice (Franzini-Armstrong and Porter, 1964; Knappeis and Carlsen, 1968; Pepe, 1971; see also Fig. 1). High resolution electron microscopy in combination with image analysis of ultrathin transverse sections of muscle fiber bundles has revealed a hexagonal lattice of thick filaments interconnected by primary m-bridge structures (nomenclature according to Sjöström and Squire, 1977a,b) (Fig. 1, M4) that are 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; Luther et al., 1981; Fig. 1). Recently, ultrathin frozen sections of myofibers cut longitudinally have revealed an even more detailed substructure of the M-band (Sjöström and Squire 1977a,b; Strehler et al., 1983). Besides the three or five main transverse structures

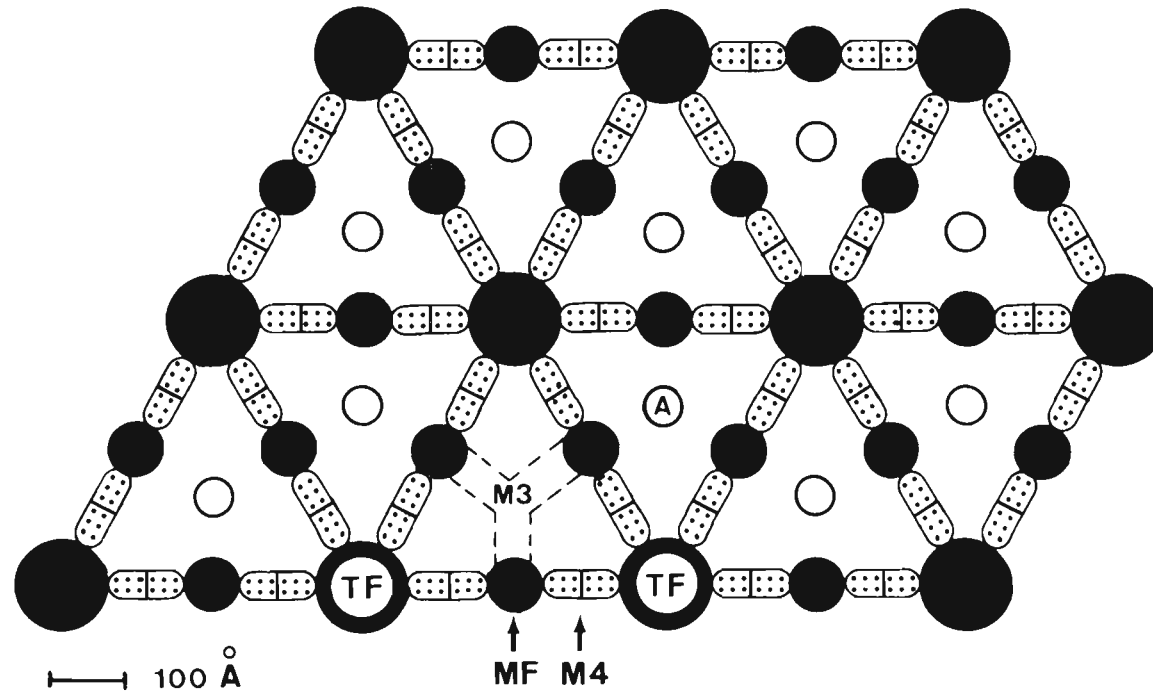
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Figure 1

Model of the transverse structure of the M-band.

Model updated from earlier models by Knappeis and Carlsen (1968), Wallimann et al. (1975), 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) showing the hexagonal lattice of the myosin containing thick filaments (TF) that are interconnected by the primary m-bridge structures (M4, representing one half-m-bridge). The typical circular or <sup>∩</sup>modular 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-bridge (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 are thought to be made up of one MM-CK dimer (Wallimann et al., 1975b; 1983a). The projections of the actin-containing thin filaments at the trigonal points between the thick filaments are indicated by open circles (A).



(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, like the M3 and M3' m-bridges that are probably related to the secondary m-bridges seen in transverse sections, (Fig. 1 and 2, M3). These additional minor substructures 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 M-1 bridges are thought to provide a secondary role in this respect (Luther et al., 1981). <sup>Insert</sup> ~~As~~ <sup>As</sup> a simplified, updated model of the M-band showing the most prominent transverse elements is presented in Fig. 2. <sup>Insert</sup> ~~the~~


The existence of transverse connections between the thick filaments at the M1, the M4 and M4', and the M6 and M6' level (Fig. 2) that, depending on the type of muscle, give rise to three or five main cross-striations in longitudinal sections 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. Though 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 not even in ultrathin longitudinal cryosections which are technically superior to conventional methods. <sup>\*</sup> Therefore, unless proven otherwise, the existence of m-filaments should be seriously questioned (Fig. 2 indicated by a question mark).

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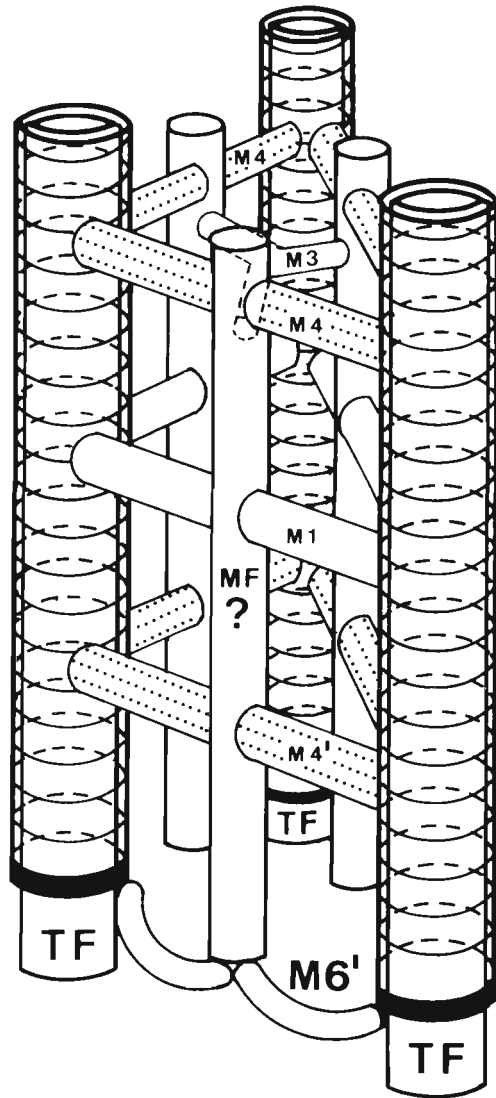
Figure 2

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), Strehler et al. (1983) of a longitudinal section through the M-band of skeletal muscle showing 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). 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 prominent of the transverse elements, the M-1 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 of yet unidentified component(s) within the M-1 bridges (Wallimann et al., 1975; 1983a; Strehler et al., 1983).<sup>\* Therefore</sup> The existence of m-filaments (MF) thought to be running parallel to the thick filaments (TF) along a distance of some 75 nm (Knappeis and Carlsen, 1968) is questionable (?).<sup>\*</sup> The possible location for

*Although in cross-section  
However, the nodular enlargements or circular thickenings are often seen at the m-bridge levels (Fig 1)  
The meeting points of NF and N4, N1 or N4 are consistently observed, the question is it remains unclear  
whether these thickenings at the different levels are longitudinally connected by the postulated N-filament.  
That why a question mark is put at "m-filament" (MF) position in Fig 2.*

myomesin, the M-band protein of 185'000 M<sub>r</sub> (Grove et al., 1984) is the "ensheathment" around the thick filaments  and for the M-protein of 165'000 M<sub>r</sub> (Masaki and Takaiti, 1974) is the "ensheathment" and possibly the M6 and M6' bridges as well (Dr. Lars-Eric Thornell, personal communication).



As pointed out earlier (Wallimann et al., 1975a,b; 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 spring-like mechanisms may be envisioned or a dynamic rearrangement of the structure may be postulated. An other 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 towards 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 on the ultrastructural details of the M-band in resting muscle and on the dynamic structural changes that occur during a contraction cycle is needed.

## 1.2 Protein constituents of the M-band

Over the last 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 was 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 (see below) it was probably never completely homogeneous, and its localization 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) which was identified soon afterwards as the muscle isoenzyme of creatine kinase (MM-CK) by Turner et al. (1973) and has subsequently been localized in the M-band by immunohistochemical and immuno-electron microscopic methods (Wallimann et al., 1975a,b; 1978, 1983a). Most recently, a protein previously seen on SDS polyacrylamide gels of myofibrils by Etlinger et al., 1976 and Porzio et al., 1979 and myosin preparations by Offer, 1972, has been identified as a third M-band protein during the course of production of monoclonal antibodies against M-protein. It has been named "Myomesin" and has an  $M_r$  of 185,000 (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 is 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 been 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 conditions (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 where the M-band is (Turner et al., 1973; Wallimann et al., 1977a, 1978; see Fig. 3).

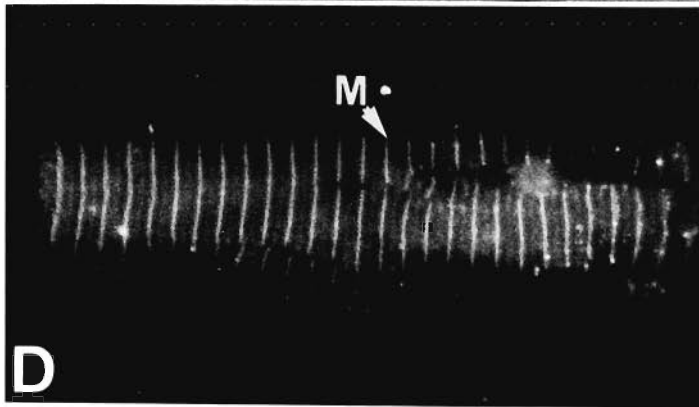
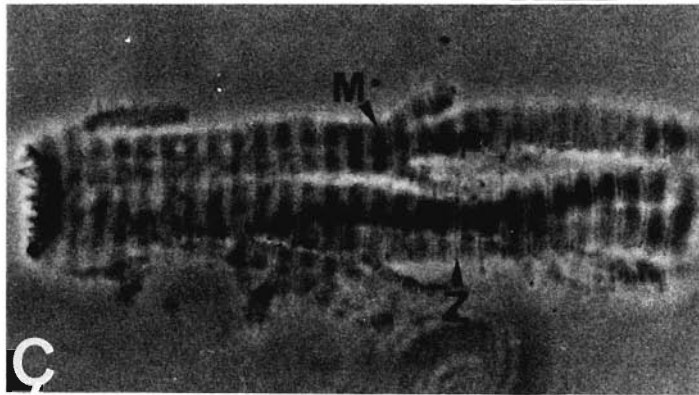
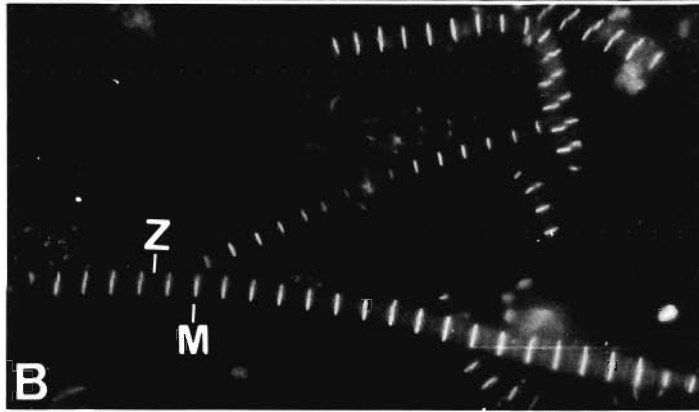
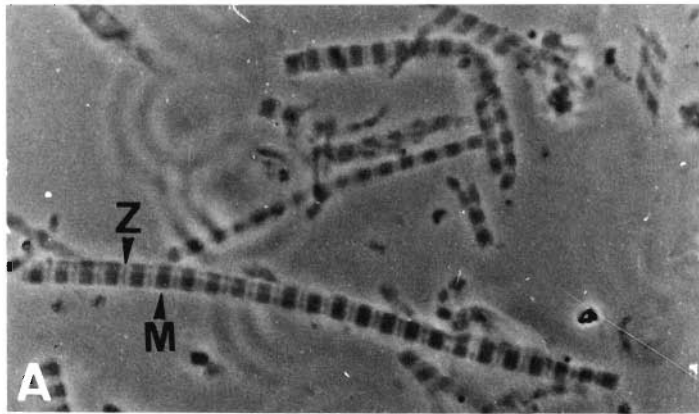
4) The binding of CK to the M-band is isoenzyme specific; that is, only the MM isoenzyme and not the BB or MB forms binds to the M-band (Wallimann et al., 1977a,b). This specificity has also been demonstrated in myogenic cell cultures in myotubes that contain simultaneously all three isoforms of CK (Wallimann et al., 1983b).

5) M-line-bound CK is released by incubation of myofibrils with low ionic strength buffer with a 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).

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 hrs post mortem) kindly provided by the Inst. of Pathology, University of Zürich (head, Dr. J. Ruettnner) were prepared and stained for immunofluorescence as described earlier (Wallimann et al., 1977a). Magnification 1'200 x





6) Incubation of glycerinated, washed muscle fiber bundles with excess anti-MM-CK IgG leads to heavy labelling 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) On the other hand, 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 opaque M-band (Sommer and Johnson, 1969) nor MM-CK are found in chicken heart muscle (Eppenberger et al., 1964; Wallimann et al., 1977b). In addition, in heart myofibrils of

## Figure 4

### Decoration of the M-band by anti-creatine kinase IgG

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

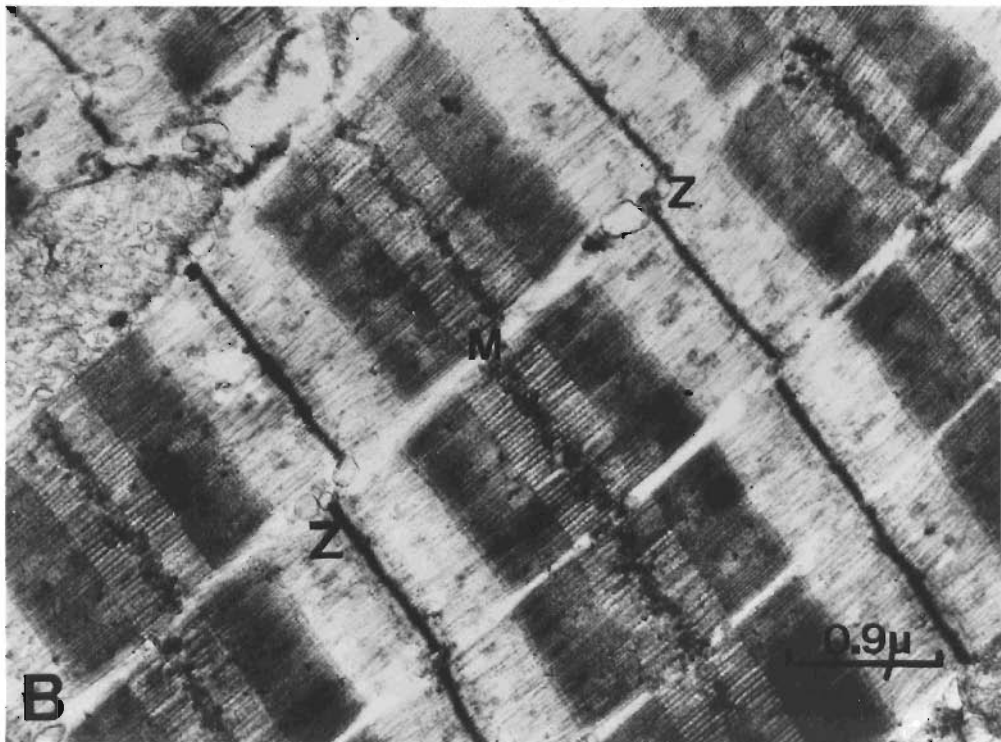
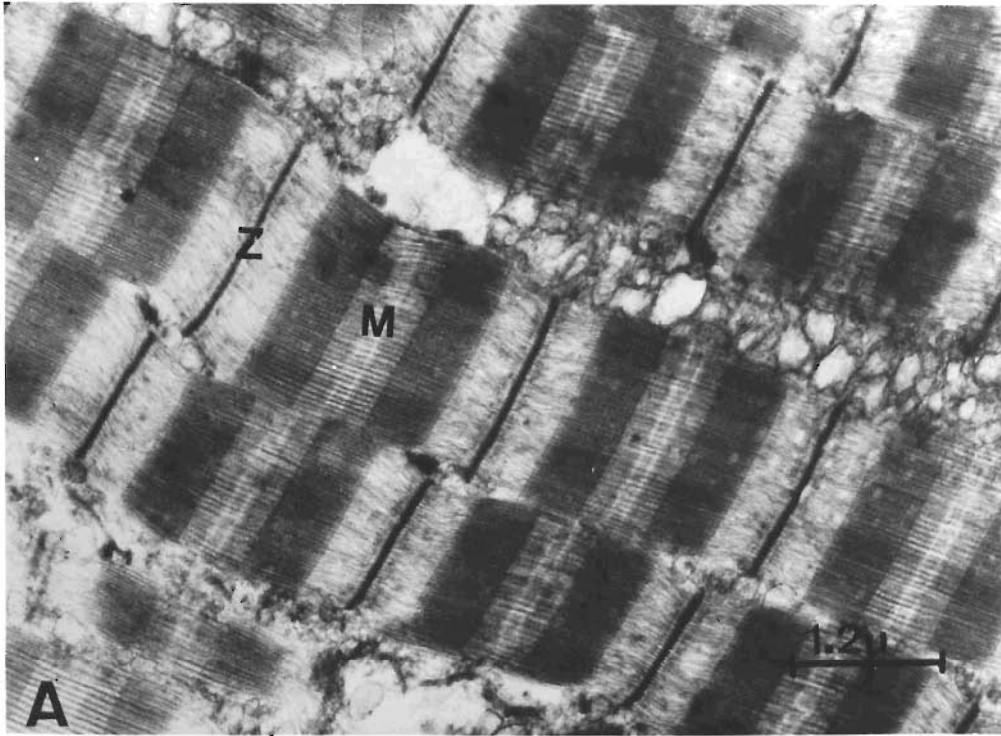
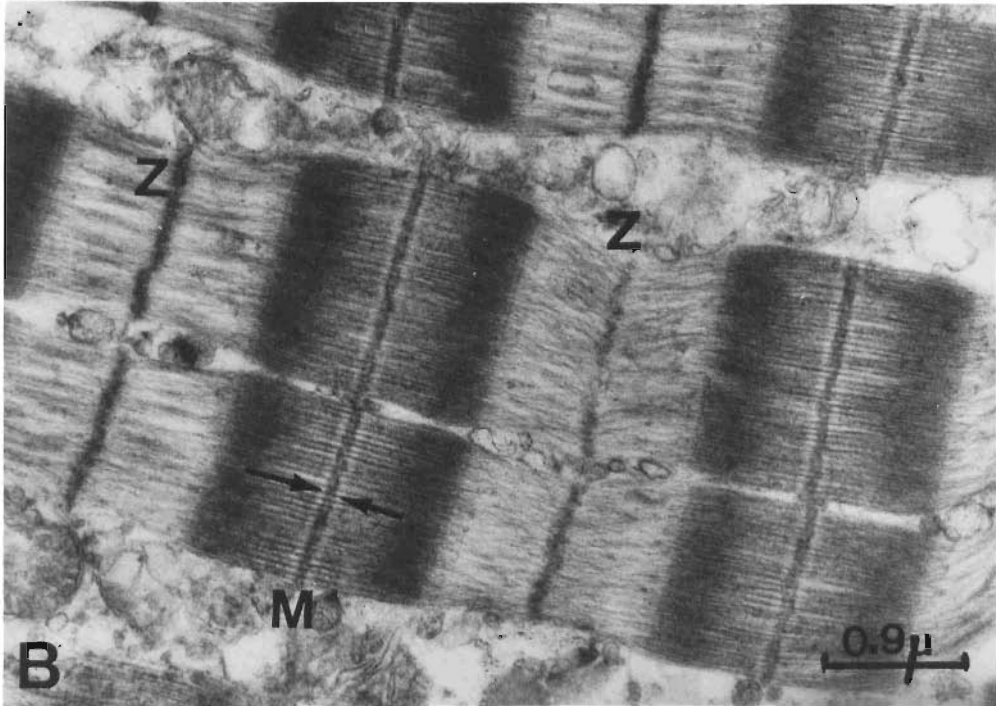
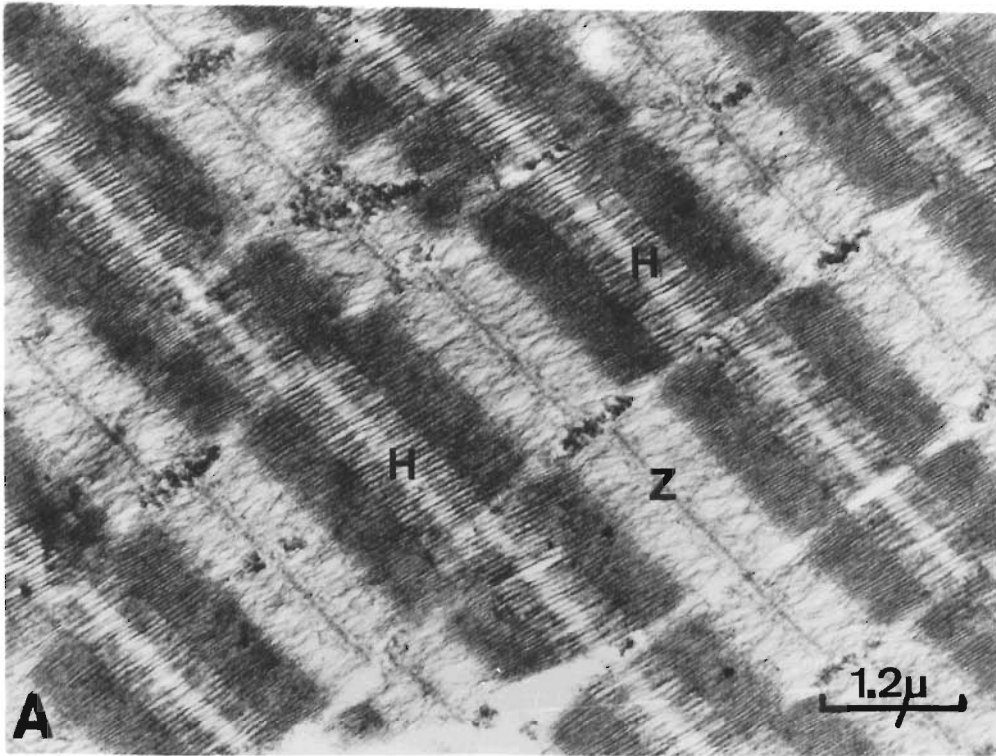


Figure 5

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 (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). Fiberbundle after incubation with a higher concentration (3 mg/ml) of monovalent anti-M-CK Fab (A, note removal of electron-dense material from the M-band by excess of monovalent antibody (Wallimann et al., 1978).

H, H-zone; M, M-band; Z, Z-band.



some mammals such as rat, incorporation of CK into the M-band and the concomitant appearance of an electron-dense M-band structure are delayed 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 (Dr.. Lisbeth 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, it is concluded that CK is 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 half the length of an m-bridge as indicated in Fig. 1 and Fig. 2. This interpretation is supported by the fact that the amount of CK activity and hence the number of MM-CK molecules which are extracted from the M-band correspond to the number of half-m-bridges of both M4 and M4' substriations (one CK molecule or one half-m-bridge per some 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 (13x4 nm) (Knappeis and Carlsen, 1968; Luther and Squire, 1978; Wallimann et al., 1975b, 1977, 1983a).

#### 1.4 Interactions between M-band proteins

Studies of the interactions between M-protein, probably containing as an impurity a slightly degraded form of myomesin as well (Grove et al., 1984), MM-CK and myosin as well as 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 not been able to confirm these findings or have found only weak interactions between the above proteins in vitro (Woodhead and Lowey, 1983). From the detailed study by the latter authors, one can infer only weak interactions of both MM-CK and M-protein with myosin and no interaction of 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, like myomesin and others, do exist and may play important structural or possibly enzymatic roles. 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  $M_r$  M-band proteins (Martin 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 (Martin Bähler, personal communication). More recently experiments with reversible phorbol ester treatment of myogenic cell cultures also indicate that the M-band and thus some of the M-band proteins are responsible for the structural integrity of A-segments (Doetschman and Eppenberger, 1984). Even though studies by electron microscopy 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 M-protein and myomesin both are bound more tightly at the M-band than 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", e.g., 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, 1982; Wang and Ramirez-Mitchell, 1983). Such intramyofibrillar connections at the M-band level (Street, 1983), besides the well documented Z to Z-band connections (Brecker 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. Such a harness would render to the muscle fiber its elasticity, prevent breakage and permit lateral transmission of tension (Street, 1983), with the M-band as well as the Z-band representing the major myofibrillar anchorage structures (Street, 1983).

### 1.6 Conclusion

Besides the two proteins of high  $M_r$ , 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 immuno-labelling studies of ultrathin frozen sections of muscle with monoclonal antibodies against the two proteins (Dr. Lars-Eric Thornell, personal communication).

However, more information is needed for the unambiguous assignment of the individual M-band components to the structural elements of the M-band. Since the numbers of distinctly different structural M-band elements found by electron microscopy is larger than the number of described M-band proteins it is most 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 with 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 the high  $M_r$  ones. So far the only M-band protein with a known function is MM-CK. Since the M-line-bound MM-CK most likely serves 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) (E.C. 2.7.3.2.) to yield ATP as the actual source of energy for contraction. CK is involved in maintaining proper intracellular ATP/ADP ratios and CP pool sizes and is therefore a key enzyme in muscle energetics (for review see Carlson and Wilkie, 1974).

CK ( $M_r$  80,000) is a dimeric enzyme known to exist as isoproteins. Three isoenzymes formed by combination of either two homologous (MM-(muscle) and BB-(brain)CK or heterologous (MB-CK)) subunits 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, which is 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 so

that 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). As a consequence, in fully differentiated chicken skeletal muscle the predominant form of CK is the MM type which is present at high levels (approx. 5 mg of CK per gram wet weight of muscle) while MB or BB-CK are undetectable (Eppenberger et al., 1964; Wallimann et al., 1977a,b). For a long time these CK isoenzymes were considered to be strictly cytoplasmic and thus soluble; rapid ATP synthesis (by glycolytic enzymes) and ATP regeneration (CK-catalysed 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 also specifically bound at strategically important locations (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 (CK-MiMi), is bound to the outer side of the inner mitochondrial membrane (Jacobs et al., 1964; Scholte et al., 1973a,b; 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 which allows

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, 1982b; 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  $\text{Ca}^{2+}$ -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  $(\text{Na}^{+}+\text{K}^{+})$ -ATPase has been proposed (Grosse et al. 1980).

Furthermore, as mentioned above 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 et al., 1975a,b, 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 thus is a myofibrillar structural protein (Wallimann et al., 1975b, 1983a, see above). 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, 1975a; Saks et al., 1976a; Bessman and Geiger, 1981). Even though an association of CK with myosin has been suggested earlier (Yagi and Mase, 1962; Botts and Stone, 1968), there was no direct experimental evidence that the

myofibrillar M-line-bound CK had an enzymatic function. Recent results (see below, Wallimann et al., 1982, 1984) provide experimental evidence for the physiological significance for myofibrillar CK by demonstrating that the bound CK is enzymatically active and that it acts as a potent intramyofibrillar ATP regenerating system. 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 hydrolysed 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 be discussed and put within the context of the CP-shuttle mentioned before.

## 2.2 Content of total and M-line-bound CK in skeletal muscle

The predominantly white, fast-twitch glycolytic pectoralis major from chicken contains approximately 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

approximately 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 per mg of myofibrillar protein. Since the protein content of chicken pectoral muscle is 18 % of its wet weight (T. Wallimann, unpublished) and 69% of the total muscle protein represents myofibrillar protein (Huxley, 1972), one gram 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 approximately 4.5% of the total CK present in chicken pectoralis muscle (2200 EU/gram wet weight). This relative value of M-line-bound CK which was 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 approximately 1 CK per 40 myosins (for details of assumptions, e.g., number of myosin molecules per thick filament etc. 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 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 (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 allows a continuous non-destructive survey of the combined CK/ATPase reactions and an accurate determination of steady-state rates (Fig. 7a,b). The assay has been optimized for both the CK and the myofibrillar ATPase activities (conditions given in Fig 7a,b; for details see Wallimann et al., 1984). Provided that CK (endogenous M-line-bound or exogenously added) is present in excess, the ADP produced by the actin-activated  $Mg^{2+}$  ATPase is rephosphorylated by CK via CP which results 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 due to recharging of some of the ADP present during phase C (Fig. 7a, phase E). Upon



## 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 hydrolysed is 0.33 ( $bH^+ - aH^+ = 0.33$ ) This way 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 phosphoryl-creatine;  $H^+$  are protons; a and b are molar fractions of protons liberated per hydrolysed ATP and protons consumed per hydrolysed CP, respectively; X is the initial number of CP molecules present (for details see Wallimann et al., 1984).

Figure 6

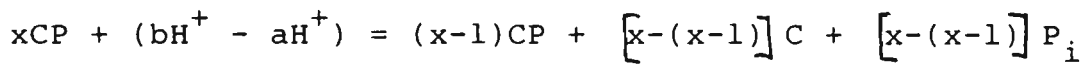
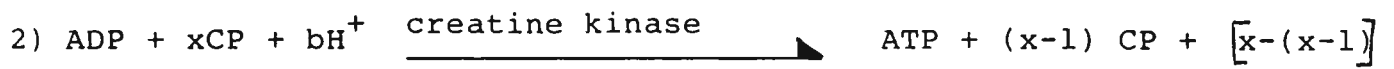
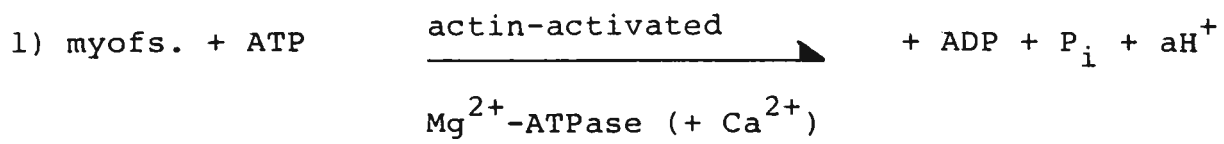


Figure 7a

ATP regeneration potential of CK bound at the M-band of washed pectoralis myofibrils

Copy of original pH-stat tracing (upper figure), a) 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 hydrolysed. The myofibrillar ATPase is supported first by the endogenous, M-line-bound CK only (C) and then by endogenous plus excess of exogenously, added CK (F).

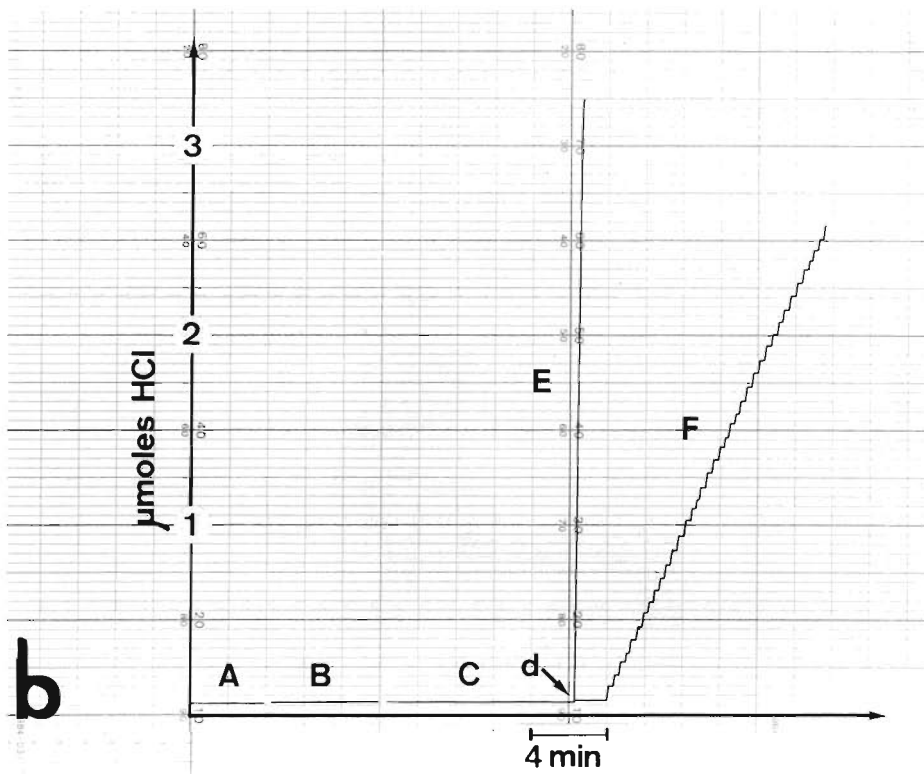
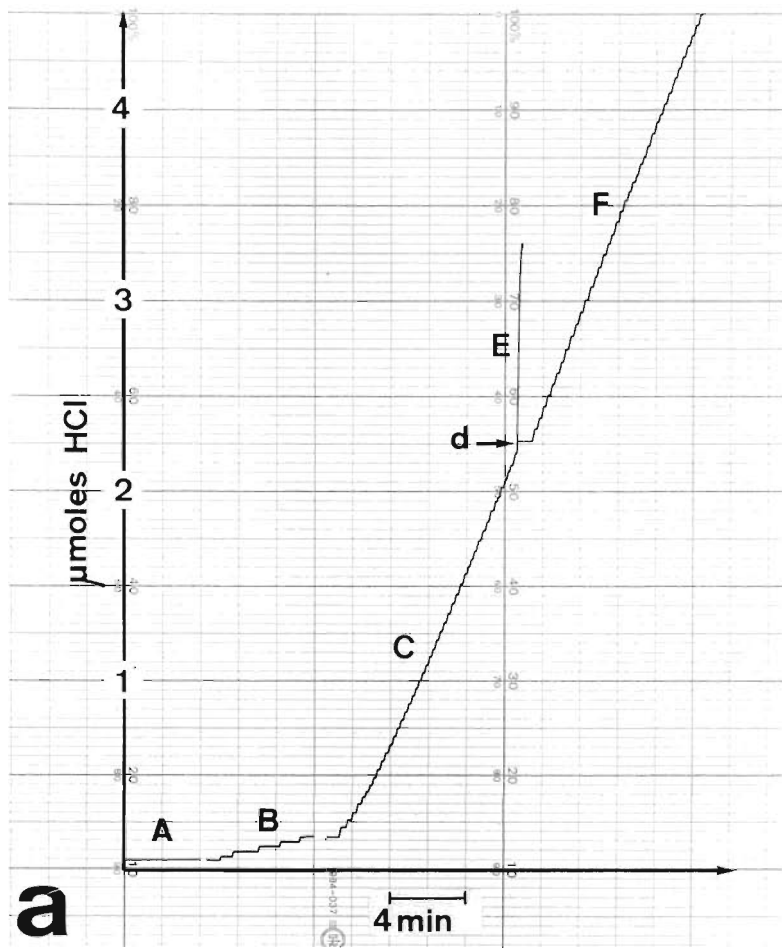
Blank reaction of assay mix (75 mM KCl, 10 mM  $MgCl_2$ , 0.1 mM EGTA, 4 mM ATP and 10 mM CP at pH 7.0) (A). Addition of 2 mg of washed myofibrils giving rise to some myofibrillar ATPase activity in the absence of  $Ca^{2+}$  (B). Steady-state rate after the addition of 0.2 mM  $Ca^{2+}$  of the actin-activated  $Mg^{2+}$ -ATPase activity supported only by the endogenous, M-line-bound CK (C). Addition of an excess (20 EU) of purified MM-CK (d). Fast recharging reaction (E) by the excess of exogenous CK of ADP that was present at a certain steady-state level during the previous reaction phase (C) leading to a new, lower steady-state level of free ADP during the next phase (F). New steady-state rate of the actin-activated  $Mg^{2+}$ -ATPase supported by the endogenous plus the excess of exogenous CK (F). Note the identical slopes in C and F. Under above conditions the net consumption of HCl per mole of CP hydrolysed at pH 7.0 and 25°C was 0.33 (see Fig. 6).

Fig. 7b

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 hydrolysed. The myofibrillar ATPase is supported first by endogenous, M-line-bound CK only (C) and then by endogenous plus excess of exogenously, added CK (F). Blank reaction of assay mix (A). Addition of 2 mg of washed myofibrils that had been treated with  $50\mu m$  of DNFB to specifically inactivate M-line-bound CK (B). Addition of  $0.2\text{ mM }Ca^{2+}$  (at the beginning of phase C) and subsequent addition of an excess (20 EU) of purified MM-CK (d). Recharging of accumulated ADP (E). New steady-state rate of the actin-activated  $Mg^{2+}$ -ATPase activity driven by the endogenous plus excess of exogenous CK (F).

Note the complete loss of the ATP regeneration potential after inactivation by DNFB of the M-line-bound CK (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 I) 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 II).



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) which 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-line-bound CK was sufficient to regenerate all ATP hydrolysed 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 that were optimized for both the actin-activated  $Mg^{2+}$ -ATPase and CK reactions. ATP-regeneration potential and actin-activated  $Mg^{2+}$ -ATPase activity, i.e., the steady-state rates shown in phase C and phase F in Figure 7a, were not significantly altered after pre-incubation of the myofibrils with 1 mM  $p^{1}p^{5}$ -diadenosine-5'-pentaphosphate (Ap-5-A), 200  $\mu$ M atractyloside (AT) or 50  $\mu$ M 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<sup>o</sup> with washing solution containing 1% Triton X-100. 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 less than 5% of 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 between pH 6.6 and 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  $\mu$ M dinitrofluorobenzene (DNFB); Infante and Davis, 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  $\text{Ca}^{2+}$  (Fig. 7b, phase C). Thus, although the endogenous myofibrillar CK was still bound at the M-line as demonstrated by indirect immunofluorescence (not shown), it was inactivated and therefore not able to support regeneration of the ATP hydrolysed by the myofibrillar ATPase (Table I). Due to continuous hydrolysis of ATP by the myofibrillar ATPase, which

was not affected significantly by IAA and DNFB (Table I), a continuous drop in pH was observed during phase C (Figure 7b). After addition of excess exogenous CK (Fig. 7b, at point d, where the pH had dropped to 6.7), the ADP which had accumulated during phase C was regenerated rapidly and two minutes later a linear steady-state reaction was observed reflecting again 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 (Table I) abolished the ATP regeneration potential of myofibrils without significantly interfering with the ATPase activity or calcium sensitivity (slopes during phase F in both Fig. 7a and Fig. 7b were identical, see also Table I).

#### 2.5 ATP regeneration potential after extraction of M-line-bound CK by low ionic strength buffer

Very similar pH-stat tracings as 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 I) and 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 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 I

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.

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 (A); myofibrils after treatment with 10 mM IAA at 4°C, pH 7.0 for 12 hrs (B); myofibrils after treatment with 50 µM DNFB at 4°C, pH 7.0 for 3 hrs (C); myofibrils after M-band extraction by incubation with low ionic strength buffer (5 mM Tris, pH 7.8) for 15 min (D) and 45 min (E).

Myofibrillar actin-activated  $Mg^{2+}$ -ATPase activity obtained by direct pH-stat measurements of ATP hydrolysed in the absence and presence of  $Ca^{2+}$  is expressed in µmoles of ATP hydrolysed per min and mg of myofibrils (F) (Wallimann and Szent-Györgyi 1981; Wallimann et al., 1984); calcium sensitivity of myofibrils  $\left(1 - \frac{\text{ATPase in EGTA}}{\text{ATPase in } Ca^{2+}}\right) \times 100$  is expressed in percent (G) (Wallimann and Szent-Györgyi, 1981). 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 hydrolysed per min and mg of myofibrils (H) (Wallimann et al. 1984); CK activity of myofibrils measured by direct pH-stat assay expressed in µmoles

of CP hydrolysed per min and mg myofibrils (I); the CK content of untreated myofibrils was taken as 100 %. Means were taken from four experiments. Standard deviations were less than 10 %.

Table 1

	actin-activated Mg <sup>2+</sup> -ATPase activity measured <u>directly</u> (F)		calcium sensitivity (G)	actin-activated Mg <sup>2+</sup> -ATPase activity, measured by the combined CK/ATPase assay (H) in the presence of Ca <sup>2+</sup>		amount of M-line-bound active CK (I)	
	μmoles ATP/min, mg myofs.		%	μmoles CP / min, mg myofs.		μmoles CP /min, mg myofs. %	
	EGTA	Ca <sup>2+</sup>		without exogenous CK	with exogenous CK		
untreated myofibrils (A)	0.022	0.37	94	0.37	0.37	0.8	100
myofibrils after 10 mM IAA (B)	0.039	0.33	88	none	0.33	0.001	0.13
myofibrils after 50 μm DNFB (C)	--	--	--	none	0.31	0.02	2.5
myofibrils after 15 min. of 5 mM Tris pH 7.8 (D)	0.038	0.33	88	none	0.33	0.06	7.5
myofibrils after 45 min. of 5 mM Tris pH 7.8 (E)	0.040	0.29	86	none	0.29	0.03	3.8

(Table I), 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. CK 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 II). 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 II). 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 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 II), but, as expected, also extracted the M-line-bound CK as shown by pH-stat measurements (Table II) and indirect immunofluorescence (Wallimann et al., 1984). Treatment of pectoralis myofibrils with excess anti-M-CK Fab followed by

## Table II

### Effect of anti-M-CK antibodies on the ATP regeneration potential of M-line-bound CK.

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 is expressed as  $\mu$ moles of CP hydrolysed per min and mg of myofibrils (E) (Wallimann et al. 1984). CK activity of myofibrils measured by direct pH stat-assay is expressed in  $\mu$ moles of CP hydrolysed per min and mg of myofibrils (F). The CK content of control IgG-treated myofibrils was taken as 100 %. Means were averaged from 3 experiments. Standard deviations were less than 12 %.

Table II

myofibrils treated with:	actin-activated Mg <sup>2+</sup> -ATPase activity measured by the combined CK/ATPase assay (E) in the presence of Ca <sup>2+</sup>		amount of M-line-bound active CK (F)	
	$\mu$ moles CP / min, mg myofs. without exogenous CK	$\mu$ moles CP / min, mg myofs. with exogenous CK	$\mu$ moles 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)	none	0.34	0.15	19
anti-M-CK Fab (D)	none	0.36	0.06	7.6

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 (approximately 8% of the CK bound originally to the M-band, Table II) were 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 II).

## 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 the slow-tonic anterior latissimus dorsi (ALD) or chicken heart muscle, and they also contained more myofibrillar CK (Table III). The ATP regeneration potentials of the fast and slow skeletal myofibrils were sufficient to keep up with the 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 for intra-myofibrillar ATP regeneration. However, myofibrils from adult bovine and swine hearts both of which contain CK bound at the M-band and display a clearly defined electron opaque M-band structure, were fully competent to

Table III

Comparison of the ATP regeneration potential by myofibrillar creatine kinase of different striated muscle types.

Creatine kinase activities of myofibrils, prepared under the same conditions as described for pectoralis major, from the muscles indicated are expressed in  $\mu$ moles of CP hydrolysed per min and mg of myofibrils as measured by the direct pH-stat assay (A) (Wallimann et al. 1984). Actin-activated  $Mg^{2+}$ -ATPase activities determined by direct pH-stat measurements of ATP hydrolysed in the presence of  $Ca^{2+}$  is expressed in  $\mu$ moles of ATP hydrolysed per min and mg of myofibrils.



Table III

	CK activity	actin-activated Mg <sup>2+</sup> -ATPase (+Ca <sup>2+</sup> )	ATP-regeneration potential	
	$\frac{\mu\text{moles CP}}{\text{min, mg myofs}}$	$\frac{\mu\text{moles ATP}}{\text{min, mg myofs}}$		
Pectoralis major	0.78	0.39	sufficient	(+)
Anterior latissimus dorsi	0.23	0.19	just sufficient	( <u>+</u> )
Posterior latissimus dorsi	0.61	0.31	sufficient	(+)
Heart* (chicken)	0.02	.08	not sufficient	(-)

\*

Foot note: myofibrils from adult mammalian hearts (swine and bovine) show, like chicken skeletal muscle, a sufficient ATP regeneration potential to keep up with the in vitro myofibrillar ATPase.

regenerate sufficient ATP to keep the actin-activated  $Mg^{2+}$ -ATPase of these muscles running at their maximal in vitro speed (Wallimann et al., 1984).

## 2.8 ATP regeneration potential of M-line-bound CK in vivo

Since one gram of muscle contains roughly 125 mg of myofibrils (see section 2.2) and since 55% of the myofibrillar protein is myosin with a  $M_r$  of 470,000, the in vitro ATP regeneration potential of the M-line-bound CK (0.8  $\mu$ moles CP per mg myofs per min) amounts to 1.8  $\mu$ moles of ATP regenerated per gram of wet muscle per sec at 25<sup>o</sup> and pH 7.0. Depending on the muscle type, the maximal power output of skeletal muscle during in vivo contractions at 20<sup>o</sup> was measured by chemical analysis to be 1.4 and 3.7  $\mu$ moles of CP (ATP) hydrolysed per gram per sec for rat soleus and extensor digitorum longus, respectively (Kushmerick and Davies, 1969; Kushmerick and Crow, in preparation). Values, after correction for temperature, ranging from 2 - 4  $\mu$ moles per gram per sec were obtained by <sup>32</sup>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  $\mu$ moles per gram 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  $\mu$ moles per gram per sec and hence 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 at 25<sup>o</sup> C and 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 of or even the entire regeneration of ATP required for contraction. Under the in vitro conditions the presence of excess ATP (4 mM) and CP (10 mM) during the pH-stat assay lead 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 may even be 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  $\text{Ca}^{2+}$  which is 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 that are performed in the absence of exogenously added CK. Recent experiments seem to indicate that chemically skinned fibers, that are 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., 1983). 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 seem 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) that is sufficient for intramyofibrillar ATP regeneration (Wallimann et al., 1984). As shown recently, differences of M-band fine structure as seen in the electron microscope with ultrathin frozen sections turn out to be one of the most reliable criteria to discriminate between different muscle fiber types exhibiting distinctly different contractile properties (Thornell, 1980; Sjöström et al., 1982). Therefore, the M-band, long thought of as something of little significance for muscle contraction, may turn out, in its structural and functional properties, to influence significantly the physiological characteristics of a given muscle fiber type.

## 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 remain bound per 1 mg chicken pectoralis myofibrils which have been freed of soluble CK, mitochondria and SR 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). By measuring the combined actin-activated  $Mg^{2+}$ -ATPase and CK reactions of such myofibrils in a pH-stat assay it was shown that the M-line-bound CK was active. The amount of M-line-bound CK activity was sufficient to rephosphorylate the ATP hydrolysed in vitro by the actin-activated  $Mg^{2+}$ -ATPase of myofibrils, the maximal specific activity of the ATPase being  $0.4 \pm 0.05$   $\mu$ moles  $P_i$  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 being 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 the ATP regeneration potential or the

actin-activated  $Mg^{2+}$ -ATPase of the myofibrils, thus 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 hydrolysed 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 above provide evidence for a functional role of M-line-bound CK as a potent intramyofibrillar ATP-regenerator. This finding is here incorporated into a schematic model of CK function and energy flux in muscle. Figure 8 shows an updated version of a model proposed earlier (Wallimann, 1975a) depicting the "CP-shuttle" in muscle, a term coined by Bessman and Geiger in 1981. The various localizations of CK isoenzymes, mitochondrial 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 black-squared, white-circled numbers. 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, 1982b; Bessman and Geiger, 1981; Schlösser et al., 1982; Wallimann et al., 1983, 1984; Paul, 1983). The individual "compartments" are connected by the CP-shuttle and buffered by

## Figure 8

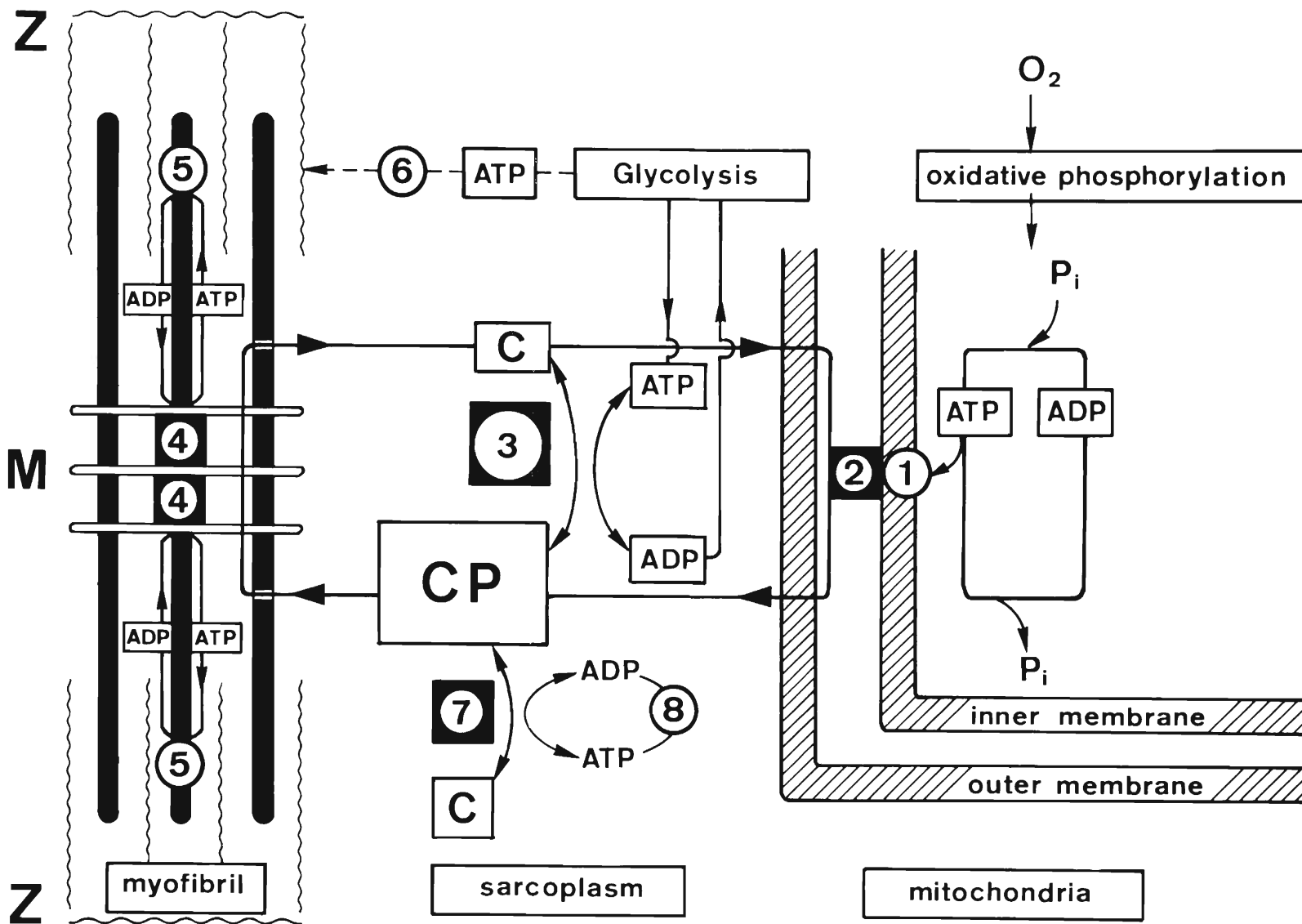
### Creatine kinase function and the CP-shuttle

Updated version of a model proposed earlier (Wallimann, 1975a) 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 (2,3,4,7) 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, glycolytic and oxidative skeletal muscle or in heart muscle. Phosphoryl-creatine (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 that are 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-generation (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 (Nunally and Hollis, 1979).

ATP/ADP translocase system (No.1); mitochondrial CK isoenzyme (CK-MiMi) (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  $\text{Ca}^{2+}$ -ATPase ( $\text{Ca}^{2+}$ -pump) and the  $\text{Na}^+/\text{K}^+$ -ATPase ( $\text{Na}^+/\text{K}^+$ -pump), respectively (No.8). Details of this model are given in the text.



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; 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, 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, CK-MiMi and MM-CK, which are strategically located at these sites, able to quickly respond 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 of adenosine nucleotide phosphates that are present in muscle in relatively small concentrations (Bessman and Geiger, 1981) the benefit of which is to make the cell metabolic system capable of being highly responsive to small changes in energy (ATP/ADP) potentials.

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 chapters.

### 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 non-destructive  $^{31}P$ -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 et al., 1974). Thus in resting muscle most of the total phosphate recorded by NMR is PC and the ratio of CP:ATP is in 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., 1972; 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 Davis, 1962). Upon inhibition of CK activity with DNFB only a small fraction of the total intracellular ATP can be utilized for muscle contraction and, even though the ATP levels are still around 70-80% of the 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, non-invasive  $^{31}P$ -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 non-perfusion (ischemia). The loss of contractility paralleled the marked fall of CP levels, with ATP remaining unchanged or still at relatively high level. The decrease in peak tension of heart during strong metabolic inhibition was also associated with a decrease in CP but not ATP (Ventura-Clapier, 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 (Naegele, 1970).

Theoretical modelling 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 man 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 ( $2 \text{ ADP} \longrightarrow \text{ATP} + \text{AMP}$ ). 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  $\text{H}^+$ , both actually serving 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 alkalization, measurable in vivo (Dawson et al., 1977), is to be expected as CP is continuously being hydrolysed (see Fig. 6).

During the second phase, after prolonged stimulation when CP reserves are slowly depleted, the rate of glycolysis as a secondary back-up system increases. Transphosphorylation by sarcoplasmic CK of glycolytically derived ATP as well as direct

hydrolysis of glycolytic ATP (if latter pathway exists in vivo) then leads to a net production of protons (Hochachka and Mommsen, 1983) and 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 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 seems to be effective in regulating myofibrillar ATP hydrolysis and regeneration. The presence of bound CK within the "myofibrillar compartment" might assure 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  $^{14}\text{C}$ -labelled 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. The authors 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, 1963), 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  $^{31}\text{P}$ -NMR saturation transfer experiments of energy-rich phosphate kinetics in normal and ischemic perfused hearts point also to a compartmentalized system of CK action (Brown et al., 1978). Direct evidence, also by NMR

techniques, for the existence of "protected" ATP pools that are not easily reached by  $Mn^{2+}$  and differ in their ability to interact with the CK system was presented by Nunally and Hollis in 1979 and, as a consequence, the existence of ATP compartments at the myofibrillar and mitochondrial side of the CP-shuttle was postulated.

By incorporating the concept of compartmentalization in muscle (Nunally and Hollis, 1979) into the CP-shuttle model, 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 maintenance of CP levels must be accomplished by ATP can be explained. Since CK catalyses both these reactions, the forward and backward events would have to be separated in space 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., 1976) that for the isolated MM-CK isoenzyme the reverse reaction (formation of ATP, Lohmann reaction) is kinetically preferred. Due to the high affinity of MM-CK for MgADP ( $K_m = 0.08$  mM) the latter will be effectively trapped and rephosphorylated into ATP by CK as long as CP is present at concentrations of about 3-4 mM or higher. The idea that ATP generated by CK within the myofibrillar compartment may be more accessible for hydrolysis than extramyofibrillar ATP is

supported by other evidence. Already in 1954 Perry showed that in the presence of CK and CP, 3  $\mu$ m of ADP caused a shortening of glycerinated myofibrils, whereas a similar degree of shortening could not be observed unless more than 60  $\mu$ m 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). 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 coworkers (1980) have shown that by adding  $\gamma$ - $^{32}$ P-ATP, unlabelled 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 labelled 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 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. Due to 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 two-fold smaller than CP, in vitro diffusion coefficient would indicate (Naegle, 1968).

For these reasons the availability (concentration x 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; i.e., at the central bare zone of the thick filament, at a distance of 0.1-0.8  $\mu\text{m}$ , depending on the state of contraction, from the sites (acto-myosin overlap zones to either side) where ATP is hydrolysed. 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, X-protein and others (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 inbetween the A-band halves seems the next best alternative. Since diffusion of sarcoplasm perpendicular to the myofilament axes is bound to be 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 here that it is still unresolved as to whether the interfilament space in muscle, especially in the acto-myosin 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, like CK, do not bind to thin filaments (Bronstein et al., 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, there is little information available on these factors.

During contraction the two thin filament lattices of a sarcomere are being pulled by the thick filaments towards 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 piston like interdigitation of sliding thick and thin filament lattices (Huxley, 1973) is also moving sarcoplasm, enriched in ADP,  $H^+$  and  $P_i$ , towards 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 towards 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 towards 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.

### 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 that 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 stepwise in liver, kidney and pancreas, but not in muscle, and is carried by the blood (at concentrations of less than 0.1 mM) to the muscle tissues where it is concentrated, mostly as CP to approximately 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 to be 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 tenfold lower than the  $K_m$  for C (Saks et al., 1976a), the 5-7 grams of CK per kg of wet muscle could theoretically trap by direct binding only 100 umoles of CP/kg, whereas the in vivo concentration of CP is approximately 25 mM. Thus the vast amounts of soluble CK in muscle cannot be considered as 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 inhibition of phosphofructokinase and pyruvate kinase by CP (Uyeda and Racker, 1965; Kemp, 1973) has been shown to be an artifact caused by an impurity in CP preparations (Fitch et al., 1979). In this context it would be very 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, for 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 4 times higher levels of soluble CK and a much higher ratio of sarcoplasmic versus mitochondrial CK when compared to 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 acto-myosin 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, sarcoplasmic CK, by replenishing depleted CP, 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 that is 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 sarcomplasmic CK into CP in vivo (Cain and Davis, 1962; McGilvery and Murray, 1974). In contrast to the myofibrillar M-line-bound CK that is involved in rapid "in situ" ATP regeneration, hence 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 suited best for both systems.



### 3.8 Mitochondrial CK

The mitochondrial CK isoenzyme, CK-MiMi, 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. CK-MiMi is thought to be located outside the atractyloside-sensitive ATP/ADP translocase system at the outer surface of the inner mitochondrial membrane (Jacobus 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 CK-MiMi 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, CK-MiMi 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 CK-MiMi 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 CK-MiMi to the inner mitochondrial membrane ( $K_m$  for ATP of bound CK-MiMi 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 CK-MiMi (Saks et al., 1980). Based on these findings the concept of compartmentalization of CK-MiMi 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). It was shown by experiments with radioactive phosphate or  $\gamma$ - $^{32}$ P-ATP that there is privileged access of newly synthesized matrix-generated ATP to CK-MiMi 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 CK-MiMi is preferentially rechannelled 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, a tight functional coupling of CK-MiMi and translocase is assumed to yield, as a net product of oxidative phosphorylation, 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 that is transported by ATP/ADP translocase to the functionally coupled CK-MiMi 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 1983b), 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 CK-MiMi 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 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 towards 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 site (CK-MiMi) 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 co-ordination 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 neuro-transmission 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 CK-MiMi, CP which then is available for energy requiring reactions. Under these conditions CK-MiMi 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

utilising side (myofibrils,  $\text{Na}^+/\text{K}^+$ -pumps,  $\text{Ca}^{2+}$ -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 the proper CP/P and ATP/ADP ratios that 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 start 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 channelling of energy are given by the structural arrangement and the local environment. Thus, in muscle, the mitochondria and the sites of glycolysis where 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 as 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. Based on the experimental data it

seems that M-line-bound CK, a potent intramyofibrillar ATP regenerating system at the distal end of the CP shuttle, able to support an ATP turnover rate of 6 ATP per sec per myosin head (Wallimann et al., 1984), is of physiological importance to 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 hydrolysed 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 where the maintenance of a critical ATP/ADP ratio is essential (Iyengar et al., 1983), depend by and large on a phosphagen metabolism. Thus, they all contain relatively high concentration of phosphagen and phosphagen kinases, that is, CP and CK in vertebrates. Based on these facts we propose that many or all of the processes in life which critically depend on ATP,

e.g., build-up and maintenance of potentials, propagation and transmission of signals (synaptic membranes), ATP-driven ion-pumps ( $\text{Na}^+/\text{K}^+$ -ATPase), calcium sequestration ( $\text{Ca}^{2+}$ ATPases), contraction and 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), all of which depend on locally high and immediately available energy supply, are coupled with phosphagen kinases (CK in vertebrates) to form efficient, subcellular, functionally coupled microcompartments where small amounts of ATP are locally turned over in a cyclic manner just 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., 1983; Cande, 1983) have recently been inferred to be mediated by CK. Efforts are underway in our laboratory to find comparable "compartments" within excitable cells that are functionally coupled with CK (Barrantes et al., 1983a,b) and to directly localize the enzymes involved.

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## 6. CURRICULUM VITAE

NAME: Theo A. Wallimann

### PERSONAL DATA:

Birth Date: October 13, 1946  
Place of Birth: Luzern, Switzerland  
Citizenship: Swiss; with permanent residence visa for USA  
Marital Status: Married  
Wife's Maiden Name: Beatrice Zueger  
Children: Rebecca, Aug. 12, 1976  
Daniel, June 23, 1979

### EDUCATION:

Public Elementary School Sept.1953 - Sept.1959  
Gymnasium of the Kantonsschule Obwalden in Sarnen,Switzerland Sept.1959 - Sept.1967  
Maturity Certificate  
Swiss Federal Institute of Technology in Zürich (ETH) Sept.1967 - Sept.1971  
Diploma in sciences, major: Biology  
Swiss Federal Institute of Technology in Zürich (ETH) January 1975, Ph.D. in Cell-Biology at the Institute of Cell-Biology ETH with Prof. Hans Eppenberger and Prof. Heinrich Ursprung  
Swiss Federal Institute of Technology in Zürich (ETH) February 1975  
Certification for teaching high school and college Biology with general background in pedagogics and didactics.  
Biol.Dept.,Brandeis University Waltham,Boston,Mass.USA April 1975 - October 1980  
Postdoctoral Fellow with Prof. Andrew G.Szent-Györgyi at Brandeis University

### PRESENT POSITION:

Nov. 1980 - present  
Oberassistent at the Institute for Cell Biology, ETH Zürich, with Prof. Hans Eppenberger

### HONORS:

- A two-year Fellowship from the Swiss Commission for Molecular Biology (Sept. 1972 - Sept. 1974).
- A postdoctoral Fellowship from the Swiss National Science Foundation (March 1975 - December 1976).
- Price and medal of the Swiss Federal Institute of Technology in Zürich Switzerland (February 1975) for Ph.D. Thesis
- A postdoctoral Fellowship from the Muscular Dystrophy Association of America (MDA) (January 76 - October 1980).

MEMBERSHIPS:

- Schweizerische Gesellschaft für Biochemie (SGB)
- Federation of European Chemists (FEBS)
- Biophysical Society of America
- American Society for Cell Biology

PUBLICATIONS:

H. Ursprung, M. Conscience-Egli, D.J. Fox, and Theo Wallimann.  
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