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# *In situ* compartmentation of creatine kinase in intact sarcomeric muscle: the acto–myosin overlap zone as a molecular sieve

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**Summary**

Creatine kinase isoenzymes (CK = ATP: creatine N-phosphoryl transferase, EC 2.7.3.2) were localized *in situ* in cryosections of intact sarcomeric muscle by immunocytochemical staining. Similar to cardiac muscle, spermatozoa and photoreceptor cells, mitochondrial-type CK (Mi-CK) localization in skeletal muscle was also restricted to mitochondria. Besides the well-documented localization of muscle-type (M-CK) at the M-line and at the sarcoplasmic reticulum, surprisingly, most of the sarcoplasmic M-CK was also highly compartmentalized and was mainly confined to the I-band. The localization of M-CK at the I-band coincided with that of adenylate kinase and aldolase. In intact muscle, the diffusion equilibrium decisively favours occupancy by all three enzymes of the I-band, with the acto–myosin overlap region of the A-band acting as a molecular sieve, excluding to a large extent all three enzymes from the acto–myosin overlap region. This indicates that in intact muscle, this region of the A-band may be less accessible *in vivo* to soluble, sarcoplasmic enzymes than thought before. If muscle were permeabilized by chemical skinning before fixation, I-band CK, as well as aldolase and adenylate kinase, were solubilized and disappeared from the myofibrils, but the fraction of M-CK which was specifically associated with the M-line remained bound to the myofibrils. Implications of these findings are discussed with respect to the functional coupling of I-band–CK with glycolysis, to the formation of large multienzyme complexes of glycolytic enzymes with CK and to the supply of energy for muscle contraction in general.

## Introduction

Creatine kinase catalysing the reaction $PCr^{2-} + MgADP^{-} + H^{+} \leftrightarrow Cr + MgATP^{2-}$ (PCr and Cr standing for phosphocreatine and creatine, respectively) is an enzyme involved in the energy metabolism of cells with high and fluctuating energy demand, e.g. skeletal and cardiac muscle, brain, photoreceptor cells, electric organ and spermatozoa (for review see Wallimann *et al.*, 1989; Wallimann & Eppenberger, 1990).

In adult mammalian skeletal and cardiac muscle, two main CK isoenzymes, muscle specific 'cytosolic' M-CK and mitochondrial Mi-CK, are expressed at relatively high levels (for review see Wallimann & Eppenberger, 1985). Most of the M-CK is soluble as it can be washed out from muscle by buffers of physiological ionic strength but a fraction (5–10%) of the same dimeric M-CK enzyme is bound specifically to myofibrils at the M-line (Turner *et al.*, 1973; Wallimann *et al.*, 1977) where it works as an efficient intramyofibrillar ATP-regenerator (Wallimann *et al.*, 1984). Based on immunoelectron microscopy data, it has been proposed that the M-line-bound M-CK plays also a structural role by forming the so called M-4 and M-4′ m-bridges interlinking the thick filaments in register (Strehler *et al.*, 1983; Wallimann *et al.*, 1983; Wallimann & Eppenberger, 1985).

In addition, some M-CK is specifically bound to the sarcoplasmic reticulum (SR) where it is supporting ATP-dependent $Ca^{2+}$-pumping by the SR-$Ca^{2+}$ ATPase and also regulating local ATP to ADP ratios (Rossi *et al.*, 1990). Furthermore, some CK is associated with the sarcolemma membrane where it is believed to form a functional microcompartment with the $Na^{+}/K^{+}$-ATPase (Sharov *et al.*, 1977; for review see Saks *et al.*, 1978).

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Depending on the muscle fibre type, a significant proportion of the total CK activity (approximately 5–25%) is represented by the mitochondrial Mi-CK isoenzyme. Mi-CK, in contrast to the cytosolic CK isoenzymes, forms octamers which have been characterized in detail both biochemically and structurally (Schlegel *et al.*, 1988a,b, 1990; Wyss *et al.*, 1990; Schnyder *et al.*, 1991a,b). Mi-CK was found to be associated with the inner mitochondrial membrane (Scholte, 1973), to be enriched at the inner/outer mitochondrial membrane contacts (Adams *et al.*, 1989; Biermans *et al.*, 1990; Kottke *et al.*, 1991) and to be functionally coupled to oxidative phosphorylation (Jacobus & Lehninger, 1973; Saks *et al.*, 1980; Erickson-Viitanen *et al.*, 1982; Tombes & Shapiro, 1985). The enzyme is involved in transphosphorylation of matrix-generated ATP to yield PCr, as well as in 'energy channelling' of PCr out of the mitochondria (Schlegel *et al.*, 1990; Kottke *et al.*, 1991; Wallimann *et al.*, 1992).

Here, we show by immunofluorescence and immunogold labelling that Mi-CK in skeletal muscle, as in cardiac muscle (Schlegel *et al.*, 1988a), spermatozoa (Wallimann *et al.*, 1986a) and photoreceptor cells of the retina (Wegmann *et al.*, 1991), is also restricted to mitochondria.

Whereas the compartmentation and function of CK at the sites of energy consumption (myofibrils, SR, sarcolemma etc.) or of energy production (mitochondria) have been studied in some detail, little is known about the localization and the specific function of the bulk of CK, i.e. soluble sarcoplasmic CK. Based on results obtained by immunoperoxidase staining for CK activity, Otsu and colleagues (1989) claimed that in dog heart most creatine kinase M is distributed in the A-band. Here, we have applied *in situ* immunohistochemical techniques on thin frozen sections of intact sarcomeric muscle of chicken and rat prepared according to the methods introduced by Tokuyasu (1973). In order to directly localize M-CK protein itself, we used isoenzyme-specific anti-M-CK antibodies followed by FITC-tagged second antibody, thus avoiding the possible artefacts caused by diffusion of peroxidase stain products.

Our results indicate that in addition to the well-known association of M-CK with the myofibrillar M-line (for review see Wallimann & Eppenberger, 1985) and the sarcoplasmic reticulum (Rossi *et al.*, 1990), most of the cytoplasmic M-CK is also compartmentalized in muscle being located mainly in the I-band region. This holds true also for the glycolytic enzymes which are accumulated at the I-band as well (Dölken *et al.*, 1975). Interestingly, *in situ* M-CK and other sarcoplasmic enzymes, including the significantly smaller adenylate kinase, seem to be mostly excluded from the acto–myosin overlap region of the A-band. Part of this work has been presented in abstract form (Wegmann *et al.*, 1987).

## Materials and methods

### In situ *fixation of skeletal muscle tissue*

Thin (2–3 mm) muscle fibre bundles were excised from the white pectoralis major portion of a freshly killed adult chicken, tied to plastic rods and fixed for 2 h on ice in solution A (100 mM KCl, 1 mM EGTA, 0.5 mM EDTA, 1 mM beta-mercaptoethanol, 0.1 mM phenylmethane-sulfonyl-fluoride (PMSF) at pH 7.0) containing 4% paraformaldehyde (PFA) and 0.2% glutaraldehyde (GA), then washed on ice in solution A, cut into 2 × 2 mm pieces and subsequently infiltrated overnight with 2.3 M sucrose (Tokuyasu, 1973). Small pieces (1 × 1 mm) of fixed and sucrose-infiltrated muscle were placed on cryomicrotome sample holders and frozen by rapid immersion in an excess volume of liquid nitrogen-cooled Freon 22. In one experiment 2 mM Mg ATP was added to the solution A during fixation.

### *Permeabilization of skeletal muscle fibres before fixation*

Muscle fibre bundles were prepared as above, but incubated for three consecutive 3-h periods at −20° C in solution A containing 50% glycerol. In between the permeabilization treatments the fibres were washed in solution A at 4° C to remove soluble material. After the last wash the fibres were fixed, infiltrated with sucrose and frozen as described above. Alternatively, permeabilization was also achieved by incubation with solution A containing 0.1% of saponin, but longer times were needed to wash out completely the soluble enzymes.

### In situ *fixation of cardiac muscle*

Trabecular fibre bundles from adult rat heart were prefixed by holding them with forceps at resting length or slightly stretched for 2–5 min at 37° C in solution A containing 8% PFA and 0.2% GA. After postfixation for 2 h in the same solution on ice, the fibres were washed in solution A, infiltrated in sucrose and frozen as described above.

### *Cryosectioning*

Cryosections of 0.2–0.8 μm thickness were cut at −75 to −95° C with an Ultracut E microtome fitted with a Cryokit FC4 (Reichert–Jung, Vienna). Sections were picked up from the knife with a drop of 2.3 M sucrose (containing 0.1% GA to minimize leakage of soluble proteins) and mounted onto glass coverslips.

### *Indirect immunofluorescence staining*

To wash out sucrose, coverslips with sections were placed on droplets of 0.1 M glycine in phosphate-buffered saline (PBS: 0.15 M NaCl, 7.5 mM phosphate at pH 7.4). Subsequently, after blocking unspecific binding sites with PBG (0.2% gelatine, 0.5% bovine serum albumin (BSA) and 0.1 M glycine in PBS, pH 7.4) for 30 min at room temperature, the sections were incubated for 1 h at room temperature with different primary antibodies: (1) rabbit anti-chicken M-CK and Mi-CK antisera, IgG and affinity-purified IgG, characterized extensively in this laboratory (Wallimann *et al.*, 1977, 1986a,b; Schlegel *et al.*, 1988a,b; Wegmann *et al.*, 1991), as well as monoclonal anti-chicken M-CK antibodies (gift from Dr L. Cerny, this laboratory); (2) sheep anti-human M-CK (Merck, Darmstadt, Germany); (3) rabbit anti-chicken adenylate kinase antibody (characterized by immunoblotting and immunoprecipitation; R. Schneider,

Masters Thesis, Institute of Cell Biology, ETH–Zurich); (4) rabbit anti-chicken aldolase antiserum (Lebherz, 1975; kind gift from Dr Lebherz, UCSD, San Diego, California); (5) rabbit anti-chicken C-protein, characterized extensively (Bähler *et al.*, 1985a,b). Antibodies were diluted appropriately (usually between 1:100 to 1:250) with PBS containing 10% of normal goat serum. Affinity purified anti- M-CK and anti-Mi-CK IgGs were diluted to 1–5 mg $ml^{-1}$. Preimmune sera or IgG were used at the same concentrations as controls.

After three consecutive washes with PBG for 10 min each, sections were incubated for 1 h at room temperature with FITC-conjugated goat anti-rabbit IgG (Cappel, Dynatech, Zürich), diluted 1:100 in PBG. The sections were washed extensively with PBS and mounted in a polyvinyl alcohol-based embedding medium (Lennette, 1978) with 1 mg $ml^{-1}$ of para-phenylene-diamine added as bleaching protectant (Valnes & Brandtzaeg, 1985). A Zeiss standard model 18 microscope equipped with a Planapo 63× oil immersion objective and a type III RS (fluoresceine/rhodamine) filter was used.

*Densitometry*

Photonegatives of cryosections stained by immunofluorescence were scanned by a computer-assisted Model 620 Video densitometer (Bio-Rad, Glattbrugg, Switzerland). Raw data obtained after scanning along the long axis of immunostained fibres were directly plotted without curve fitting etc. A scan along the outside of the fibre was taken as the background fluorescence baseline. Care was taken that signal darkness of negatives was well within the linear range of the film. A ratio of the fluorescence intensities at the M-line over the I-band was determined semiquantitatively by measuring the peak areas of a total of 120 M- and I-band fluorescence intensities from 25 scans.

*Immunoelectron microscopy*

For electron microscopy, ultrathin (40–70 nm thick) cryosections of chicken skeletal muscle were cut at −95° C according to Tokuyasu (1973) as described above for light microscopy, but the sections were picked up with a droplet of 2.3 M sucrose in PBS containing 2% PFA and 0.2% GA and mounted on collodion-coated 200–400 mesh EM copper grids. Sucrose was washed away and traces of GA were quenched by 0.2 M glycine in PBS at pH 7.4. Immunolabelling was performed with rabbit anti-chicken mitochondrial CK (anti-Mi-CK) antiserum (diluted 1:200 in PBG) or with affinity-purified anti-Mi-CK IgG (diluted with PBG to 1–5 μg $ml^{-1}$). Antiserum and antibody had been characterized extensively before (Schlegel *et al.*, 1988a,b; Wegmann *et al.*, 1991). As second antibody, a goat anti-rabbit IgG conjugated to 5 nm colloidal gold particles (Janssen Pharmaceutica, Beerse, Belgium), diluted 1:100 in PBG, was used. Incubation times were 2 h at room temperature for each antibody, with extensive washing before and after incubation with the second antibody. Immunostained sections were postfixed with 0.2% GA to prevent removal of immunogold. Subsequently, after contrasting for 10 min with 4% neutral and for 2 min with 2% acidic uranyl acetate, the sections were supported on the grid by 1.3% methylcellulose and, after drying, were looked at in a Jeol 100C electron microscope at 100 kV and photographed on Agfa (Scientia) 6.5 × 9 cm plates.

## Results

*Association of M-CK with the M-line and confinement of cytosolic M-CK to the I-band*

Indirect immunofluorescence staining of semithin cryosections (according to Tokuyasu, 1973) of chemically-fixed chicken pectoralis muscle fibre bundles with anti-M-CK antibodies, followed by FITC-conjugated secondary antibody, resulted in strong staining of the I-band, as well as the M-line, whereas the acto–myosin overlap region on both sides of the A-band was stained either only very weakly or not at all (Fig. 1a and b). This is shown in more detail at higher magnification with chicken skeletal muscle in Fig. 2, and with rat cardiac muscle in Fig. 3. As a control, unspecific staining with preimmune antibody followed by FITC-conjugated second antibody was negligible (Fig. 1e and f). A similar staining pattern was also obtained if intact muscle fibre bundles were fixed in solution A containing 2 mM MgATP, supposedly relaxing the acto–myosin cross-bridges. The correct assignment of the staining to sarcomeric substructures is important here because there is some controversy about CK localization in striated muscle in the literature (Otsu *et al.*, 1989; see below). The immunofluorescence staining pattern we observed can easily be assigned to the respective sarcomeric substructures owing to its clarity and brightness as well as to the widths of the stained bands (see also tracing of immunofluorescence intensity in Fig. 2C). The same conclusion is also achieved by superposition of phase contrast and immunofluorescence pictures (not shown), and was corroborated by double immunofluorescence staining. In addition to an anti-M-CK antibody, a specific anti-M-line-protein antibody was used (not shown) which was demonstrated earlier to label exclusively the M-line or the myosin head-free bare zone of isolated thick filaments (Bähler *et al.*, 1985c). These results unambiguously prove that the narrow fluorescent stripe assigned to M-line CK (coinciding with anti-M-line protein staining) is indeed the M-line, whereas the wide, more intensely fluorescent zones correspond to the I-bands (see Figs 2 and 3) and not to the A-bands as claimed by Otsu and colleagues (1989).

For Figures 1a and b, 2 and 3, muscle fibres were fixed *in situ* before freezing and cryosectioning. However, if muscle fibre bundles were first permeabilized by chemical skinning before fixation at low temperatures, e.g. by incubation in relaxing solution plus glycerol or 0.1% saponin, and then treated as above, the I-band fluorescence was completely lost, but the M-line staining remained equally strong as before (Fig. 1c and d). A similar, but generally weaker staining pattern was also observed with monoclonal anti-M-CK antibodies (not shown). This indicates that, in contrast to the fraction of M-CK which remains firmly bound to the M-line, the bulk of soluble sarcoplasmic M-CK is only loosely associated *in situ* within the I-band from where it can be

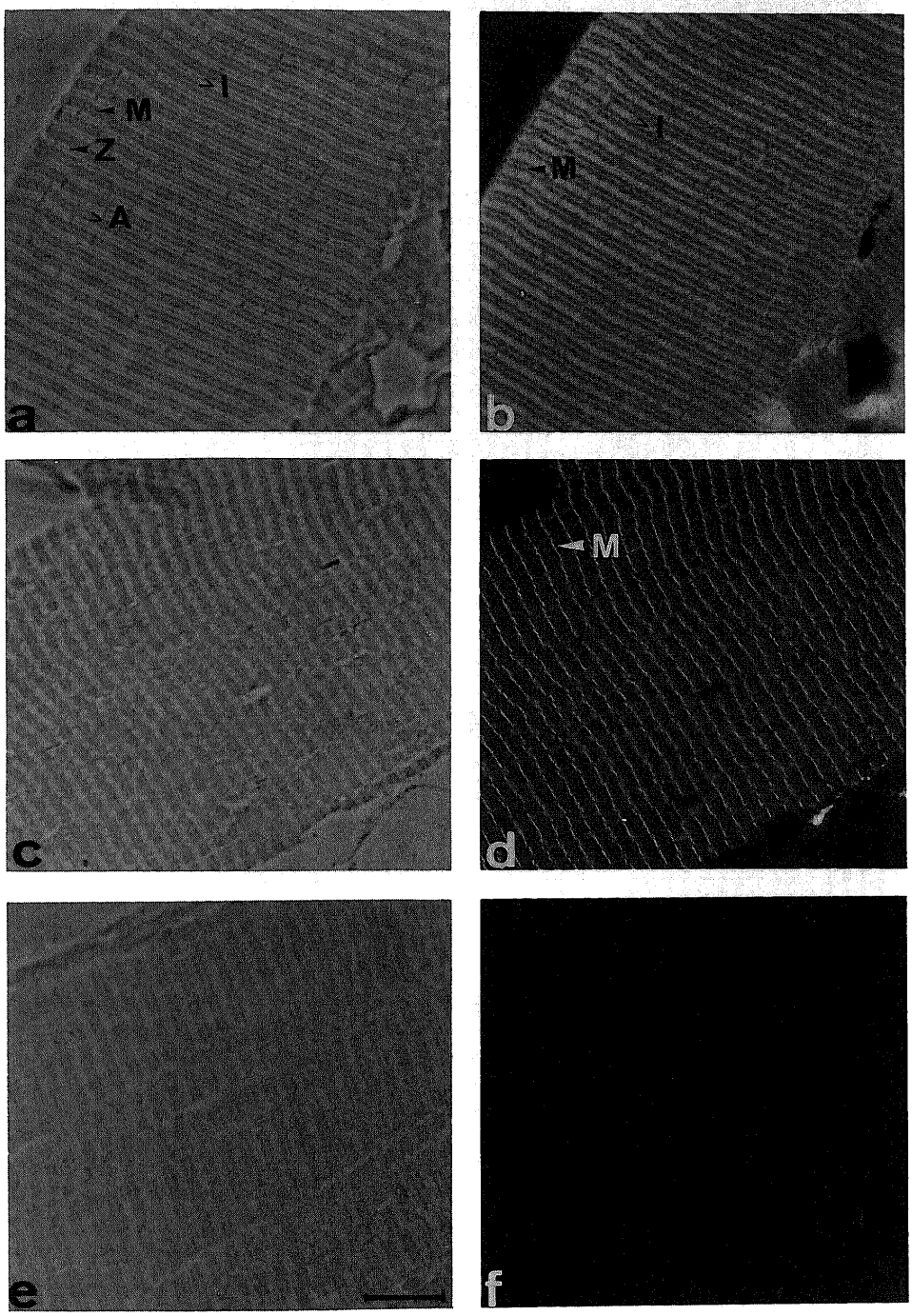


**Fig. 1.** *In situ* immunohistochemical localization of M-CK in skeletal muscle. (a and b) Semithin (0.3–0.5 μm) cryosections of chicken pectoralis muscle, prepared according to Tokuyasu (1973) and stained with specific rabbit anti-chicken M-CK antiserum (diluted 1:200), followed by FITC-conjugated fluorescent second antibody (diluted 1:150). Note the sharply delineated staining of the M-lines (M), broad staining of the I-bands (I), often resolving I-band halves owing to sparing of Z-lines (Z), and weakness of immunostaining form the acto–myosin overlap zone of the A-bands (A) in the fluorescence picture (b); (c and d) same, but with muscle permeabilized and washed before fixation to remove soluble CK. Note that M-line-bound CK (M) remains firmly bound to myofibrils whereas the soluble cytosolic M-CK is washed out quantitatively from the I-band (d); (e and f) same as shown in (a) and (b), but staining with control serum followed by FITC-conjugated second antibody (diluted 1:150) (f); corresponding phase contrast picture (e). Note absence of unspecific staining in the control; (a, c and e) corresponding phase contrast pictures. Bar = 10 μm.

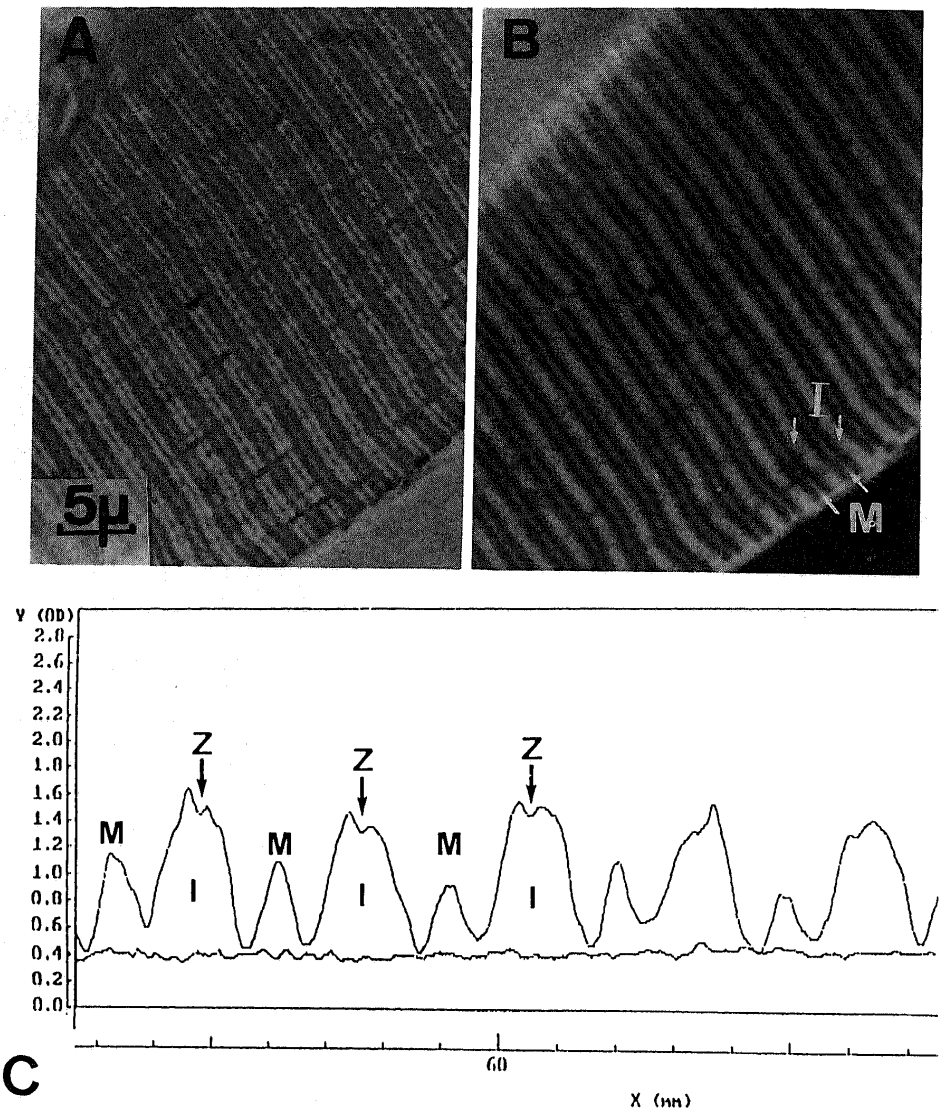


**Fig. 2.** *In situ* indirect immunofluorescence staining of skeletal muscle by anti-M-CK antibodies. Higher magnification of semithin (0.2 µm) cryosection of chicken pectoralis muscle stained by affinity-purified rabbit anti-chicken M-CK IgG at a concentration of 2 µg $ml^{-1}$, followed by 1:150 diluted goat anti-rabbit IgG conjugated with FITC (B); corresponding phase contrast picture (A). Note strong staining of the M- and I-bands but very weak staining of even absence of fluorescence from the acto–myosin overlap zones of the A-band. Note also the separation of I-band halves by unstained Z-disks in (B) = 5 µm. (C) Representative scan of immunofluorescence intensity by densitometer tracing of the photonegative from the muscle fibre stained for M-CK shown in (B) along the fibre axis (upper tracing) and just along the periphery outside of the fibre (lower background tracing). Note strong M- and I-band fluorescence signal with some indication of sparing the Z-line (Z) indicated by the division of the fluorescence signal in two I-band halves (C), best seen by observing picture (B) perpendicularly to the fibre long axis. Bar = 5 µm.

removed by permeabilization of the sarcolemma and repeated washing at physiological salt conditions (Fig. 1c and d), whereas the resistance to extraction of M-line-bound CK is indicative of a rather strong association of M-CK at the M-line (for review see Wallimann & Eppenberger, 1985).

Our *in situ* immunolocalization results of CK in muscle are conflicting with those reported by Otsu and colleagues (1989) who claimed that most of the CK is distributed in the A-band. One explanation for this discrepancy may be that these authors applied peroxidase-base immunohistochemistry which has the disadvantage that the reaction product (oxidized diaminobenzidine) is diffusible. It is conceivable that similar to histochemistry based on enzyme activity staining involving a phenazine methosulphate-tetrazolium salt capture reaction (Raap *et al.*, 1983), the stain product of peroxidase may also be prone to diffusion. By subsequent washing after staining some of the more easily accessible stain may be removed whereas some stain product may get trapped at membranes (Biermans *et al.*, 1990) and also at those cellular sites with a high density of cytoskeletal or myofibrillar filaments, e.g. at the acto–myosin overlap region. The immunofluorescence method used in our experiments, however, monitors directly the binding of fluorescent antibody to

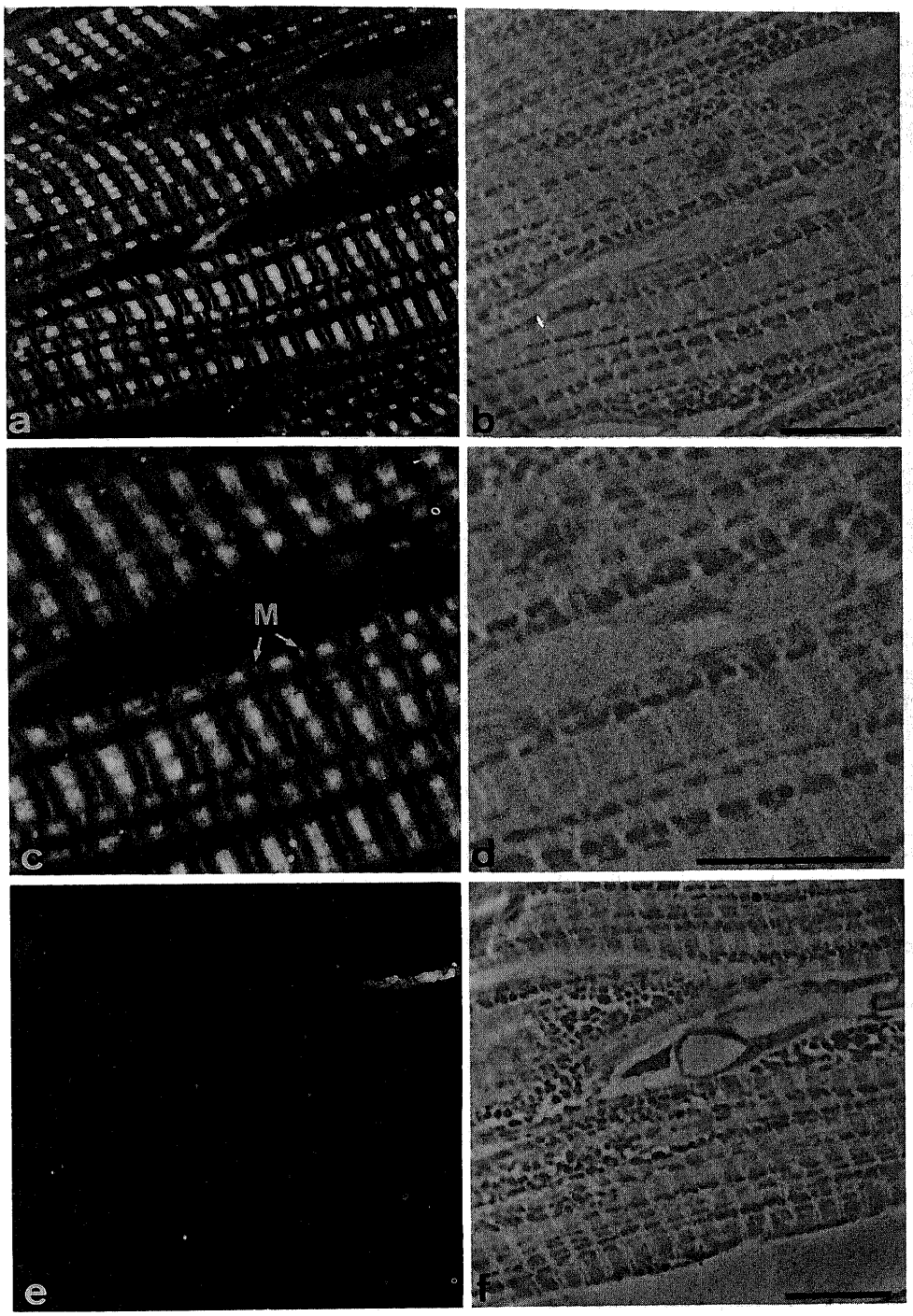


**Fig. 3.** *In situ* immunohistochemical localization of M-CK in cardiac muscle. Indirect immunofluorescence staining of semithin (0.5 μm) frozen sections (according to Tokuyasu, 1973) of adult rat heart with sheep anti-human M-CK IgG at 1:200 dilution, followed by FITC-conjugated second antibody at 1:100 dilution, shown here at low (a) and higher magnification (c) with the parallel phase contrast pictures (b and d), and the corresponding control with non-immune IgG (e and f). Note similar staining of M-lines (M) and I-bands as seen with chicken skeletal muscle shown in Figs 1 and 2. Bar = 10 μm.

subcellular structures and therefore seems to be more reliable.

In an approach to determine the relative quantities of the M-CK bound to the M-line and that associated with the I-band, photonegatives of structurally well-preserved muscle fibres, stained by indirect immunofluorescence with anti-M-CK antibody, were scanned by densitometry and the areas of M- and I-band fluorescence peaks determined semiquantitatively by planimetry (Fig. 2C). The results indicate that approximately 20 ± 5% of the *myofibrillar* M-CK is localized at the M-line. Considering the facts that, in addition to the I-band CK, a certain quantity of sarcoplasmic CK is also intramyofibrillar and that in skeletal muscle some 5–10% of CK is of

mitochondrial origin, the above relative value is in line with earlier measurements using isolated, washed myofibrils where approximately 5–10% of the *total* CK was found to be bound to the M-line of sarcomeric muscle (see Wallimann & Eppenberger, 1985).

The very low immunofluorescence staining by anti-M-CK of the acto–myosin overlap zone on both sides of the A-band, as well of the weaker staining of the Z-disk in comparison with the I-band can be seen if the fluorescence picture is viewed perpendicular to the long axis of the muscle fibre (Fig. 2B). The same is also indicated by the densitometry tracings which show fluorescence peaks at the M- (M) and I-band (I) with little or no fluorescence staining at the acto–myosin overlap region. Owing to the limited resolution of the method caused by stray-light fluorescence from the intensively stained I-band halves, the separation of the two I-band halves by an unstained Z-disk is only indicated by a small dimple in the I-band peak (Z) (Fig. 2C), but can be seen better by eye in Fig. 2B.

Basically the same immunolocalization pattern was observed after staining of intact rat cardiac muscle fibres with anti-M-CK antibody (Fig. 3), where the A-band halves on both sides of the M-line also remained mostly unstained, indicating a much lower concentration or even absence of M-CK from the acto–myosin overlap zones (Fig. 3a and c). The accessibility of antibodies after cryosectioning to this sarcomeric region of the A-band halves did not pose a problem because these very same sarcomer regions were stained perfectly well with anti-C-protein (Bähler *et al.*, 1985b) (see Fig. 4f), as well as with anti-86 kDa protein antibodies (Bähler *et al.*, 1985a) (not shown).

Interestingly, the immunofluorescence staining patterns obtained with anti-adenylate kinase (Fig. 4b) and anti-aldolase antibodies (Fig. 4d) on chemically fixed and subsequently cryosectioned muscle, showing staining of the I-band halves as well as of the H-zone or M-line region, were quite similar to those obtained with anti-M-CK antibody (compare Fig. 1b and Fig 4b and d). However, in contrast to the anti-M-CK staining where approximately 20% of the fluorescence intensity was at the M-line, the relative staining intensity by anti-adenylate kinase and anti-aldolase antibodies of the M-line region versus the I-band region was only 8–10% at the H-zone versus the I-bands, as measured by densitometry. Most importantly, the immunofluorescence staining with the latter two antibodies was abolished from both the M- and I-band regions if muscle was permeabilized and washed before chemical fixation (not shown), whereas after the same treatment the M-line-bound M-CK still remained firmly bound to the myofibrils (Fig. 1d). This indicates that in contrast to M-CK the two soluble enzymes aldolase and adenylate kinase are not associated with the M-line, but rather occupy some of the void volume of the H-zone. Furthermore, in contrast to M-CK, both enzymes were washed out more readily from the H-zone than from the I-band (not shown). These results indicate only a loose association of the two latter enzymes at the I-band, possibly with thin filaments (Masters *et al.*, 1987) and/or glycolytic enzymes present in this sarcomeric region (Dölken *et al.*, 1975; Pette, 1975).

Taken together, our results show that in intact sarcomeric muscle the larger empty space within the thin filament lattice region of the I-band halves as well as the H-zone are readily accessible to soluble sarcoplasmic enzymes but, surprisingly, much less so the empty space within the acto–myosin overlap zone. As in skinned muscle *in vitro*, after removing most of the sarcoplasmic components, the latter region seems to be rather accessible for infusion of proteins, e.g. myosin S1, antibodies and peptides, it has been widely assumed that this is the case also *in vivo* in the intact muscle as used here. However, as our results point out this may not exactly be the case.

*Mitochondrial CK*

Owing to the relatively tight association of Mi-CK with mitochondrial membranes (Rojo *et al.*, 1991a,b), this CK isoenzyme could not only be demonstrated in skeletal muscle at the location of mitochondria by immunofluorescence (Fig. 5), but also directly within mitochondria by immunogold methods (Fig. 6). Whereas immunofluorescence staining of chicken pectoralis muscle with specific anti-Mi-CK antibodies showed spotted staining of the rather sparse mitochondria present in this muscle occurring in parallel lines corresponding to gaps between myofibrils (Fig. 5), immunogold staining of ultrathin cryosections of the same muscle revealed the presence of Mi-CK within mitochondria, presumably along cristae membranes, as well as at peripheral sites within the mitochondria (Fig. 6a and b). These results are consistent with anti-Mi-CK immunogold labelling of adult rat heart tissue and cardiomyocytes (Eppenberger–Eberhardt *et al.*, 1991), as well as of photoreceptor cells of chicken retina (Wegmann *et al.*, 1991). In the latter case, using low-temperature embedding in Lowicryl, the localization of Mi-CK along individual cristae membranes as well as at those peripheral sites where the inner and outer mitochondrial membranes were in close contact, was clearly visible. The results presented here confirm that Mi-CK is also specifically restricted to mitochondria in fast-twitch glycolytic skeletal muscle where the mitochondrial content as well as the relative proportion of Mi-CK versus M-CK are significantly lower than in oxidative muscles (for review see Wallimann *et al.*, 1992).

## Discussion

*Subcellular compartmentation of CK: strongly bound M-CK at the M-line and soluble M-CK mainly restricted to the I-band*

In a variety of tissues, CK isoenzymes are subcellularly compartmentalized in an isoenzyme-specific manner

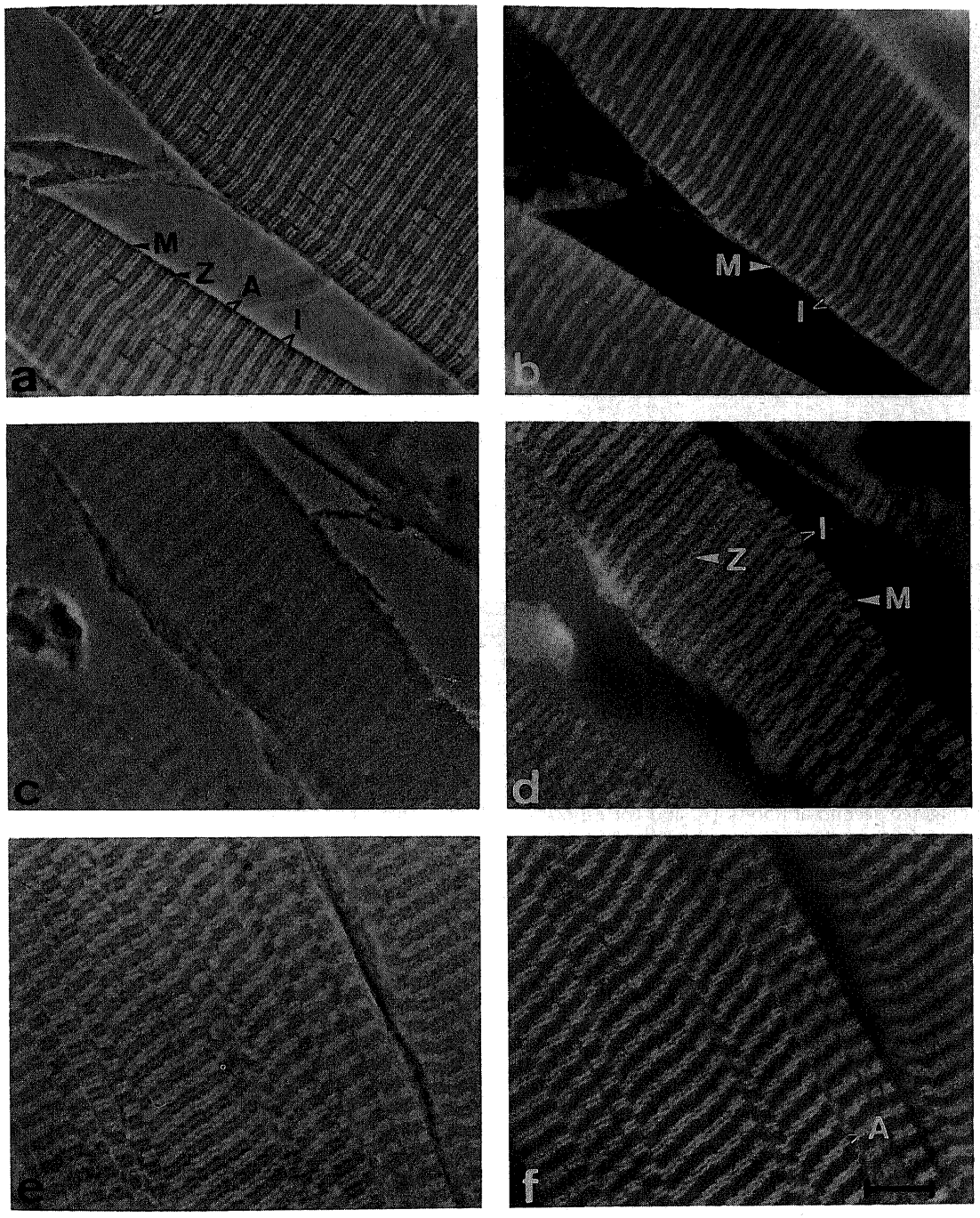


**Fig. 4.** Compartmentation of cytosolic adenylate kinase and aldolase in skeletal muscle *in situ*. *In situ* localization of adenylate kinase (a and b), aldolase (c and d) and C-protein (e and f) by indirect immunofluorescence localization on semithin cryosections (0.3–0.5 μm) of chicken pectoralis muscle showing immunofluorescence (on the right) and corresponding phase contrast pictures (on the left). Notice the similarity in the staining patterns for adenylate kinase (b) and aldolase (d) with M-CK (Fig. 1), with strong staining of the I-band and the M-line region (M), as well as with sparing of the acto–myosin overlap zone of both A-band halves. However, in contrast to M-CK, the immunofluorescence staining disappears completely, also from the M-line region, if the muscle is permeabilized and washed before fixation (not shown here). Notice the strong staining of the acto–myosin overlap zones of the A-band if the same muscle was stained under identical conditions as above, but with anti-C-protein antibody as a control (e and f). A, A-band; Z, Z-line. Bar = 10 μm.

(Eppenberger *et al.*, 1983; Wallimann *et al.*, 1986a,b). Fractions of the 'cytosolic isoenzymes' muscle type M-CK or brain-type B-CK are in part concentrated and specifically localized at sites of energy consumption as well as sites of energy production, whereas Mi-CK is specifically localized in mitochondria. This is corroborated here with *in situ* immunohistochemistry on frozen sections of intact skeletal and cardiac muscle. Whereas the diffusion equilibrium for soluble cytosolic M-CK decisively favours occupancy of the I-band, rather than the A-band, a small

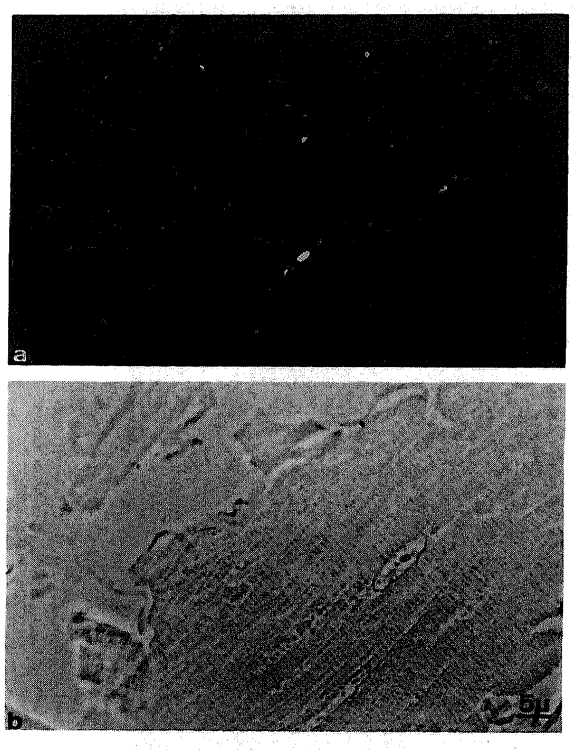

**Fig. 5.** *In situ* immunofluorescence staining for mitochondrial CK in fast skeletal muscle. Semithin (0.4 μm) frozen sections of chicken pectoralis major muscle stained by indirect immunofluorescence for Mi-CK using 1:100 diluted anti-sarcomeric $Mi_b$-CK antiserum (Schlegel *et al.*, 1988a,b), followed by 1:150 diluted FITC-conjugated second antibody (a), and corresponding phase contrast picture (b). Notice absence of immunofluorescence staining on myofibrils but spot-like staining along parallel lines corresponding to the gaps between myofibrils at places where mitochondria are clustered.

but significant portion of M-CK is bound to the M-line (Fig. 7). The binding of M-CK to the M-line can be explained by the association properties of the enzyme with the thick filament backbone in the bare zone region (Bähler *et al.*, 1985c), where M-CK has been shown to constitute a genuine structural element of the M-line, that is the M4 m-bridges (Strehler *et al.*, 1983; Wallimann *et al.*, 1983), and where this very M-line CK also fulfils an enzymatic role as a potent intramyofibrillar ATP-regenerator (Wallimann *et al.*, 1984). In light of the fact that the actomyosin overlap region seems mostly inaccessible to sarcoplasmic enzymes (see below) the localization of M-CK at the M-line, just between the two A-band halves, seems also physiologically advantageous (see Wallimann & Eppenberger, 1985). This is substantiated by the findings that the diffusibility of adenine nucleotides, compared with PCr and Cr, is significantly slower *in vivo* (Yoshizaki *et al.*, 1990), with the diffusion coefficients being higher along the myofilament axis compared with diffusion radially to the filament lattice (Eisenberg *et al.*, 1979). Evidence for intracellular compartmentation of high-energy phosphates and creatine pools (Saks *et al.*, 1984; Miller & Horowitz, 1986; Zahler *et al.*, 1987; Savabi, 1988; Ishida & Paul, 1989; for details see Wallimann *et al.*, 1992), and for directional substrate channelling in muscle (Winegrad *et al.*, 1989; Arrio-Dupont, 1988) is arguing against the concept of free diffusion of molecules within a cell (Meyer *et al.*, 1984), and physiological studies showing the dependence of relaxation and tension development of intact muscle or skinned muscle fibres on myofibrillar-bound M-CK and on the PCr content (Spande & Schottelius, 1970; Ventura-Clapier *et al.*, 1987a,b; Seraydarian, 1980; Bessman & Carpenter, 1985; Veksler *et al.*, 1988; Hoerter *et al.*, 1988, 1991) are supporting our earlier findings concerning the physiological importance of M-line bound CK (Wallimann *et al.*, 1984, 1992).

The *in situ* compartmentation of soluble sarcoplasmic M-CK to the I-band and its 'exclusion' from the A-band acto–myosin overlap region, which is after all quite surprising, needs some explanation. The finding presented here indicates that the diffusion equilibrium for M-CK and the other sarcoplasmic enzymes decisively favours occupancy of the I-band.

The fact that a similar partition was also found for adenylate kinase, a much smaller enzyme with a $M_r$ of 20–25 000, and for the glycolytic enzyme aldolase, indicates that the sarcomeric space, where thick and thin filaments interdigitate by the action of crossbridges, is not readily accessible even to relatively small soluble sarcoplasmic enzymes. One would think that soluble proteins with similar dimensions as myosin crossbridges (with a length of 20–22 nm and with a $M_r$ of approximately 150 000 (Vibert & Cohen, 1988), or smaller molecules like dimeric M-CK (with dimensions of 4 × 10 nm and a $M_r$ of 80 000) or the even smaller adenylate kinase should be able to diffuse relatively unhindered within the acto–myosin lattice. The fact that this seems not to be the case *in vivo* may be physiologically relevant and indicates that this region of the A-band may act *in vivo* as a molecular sieve affecting even small to average-sized proteins. *In vitro*, chemically-skinned muscle fibres or glycerol-treated muscle can also act as molecular sieves, as shown by partial exclusion of polymeric molecules such as PVP, from the overlap region of the A-band (Matsubara *et al.*, 1984), but on the other hand molecules like myosin S1 or antibodies are able to penetrate even into the acto–myosin overlap region as demonstrated by indirect immunolabelling for C-protein (Fig. 4f).

*In vivo*, the average pore size of the intracellular cytoskeletal network, estimated by fluorescence recovery after photobleaching to be only 20–40 nm in the cytoplasma of non-muscle cells (Valnes & Brandtzaeg, 1985), is likely to be reduced significantly in muscle owing to the abundance of highly organized myofilaments and is probably much smaller than anticipated (Goodsell, 1991). Taking the cylindrical radii of the thick and thin filaments as 20 nm and 8 nm, respectively, and an average centre to centre distance between thick filaments of 45 nm, the distance between the surface of thick and thin filaments

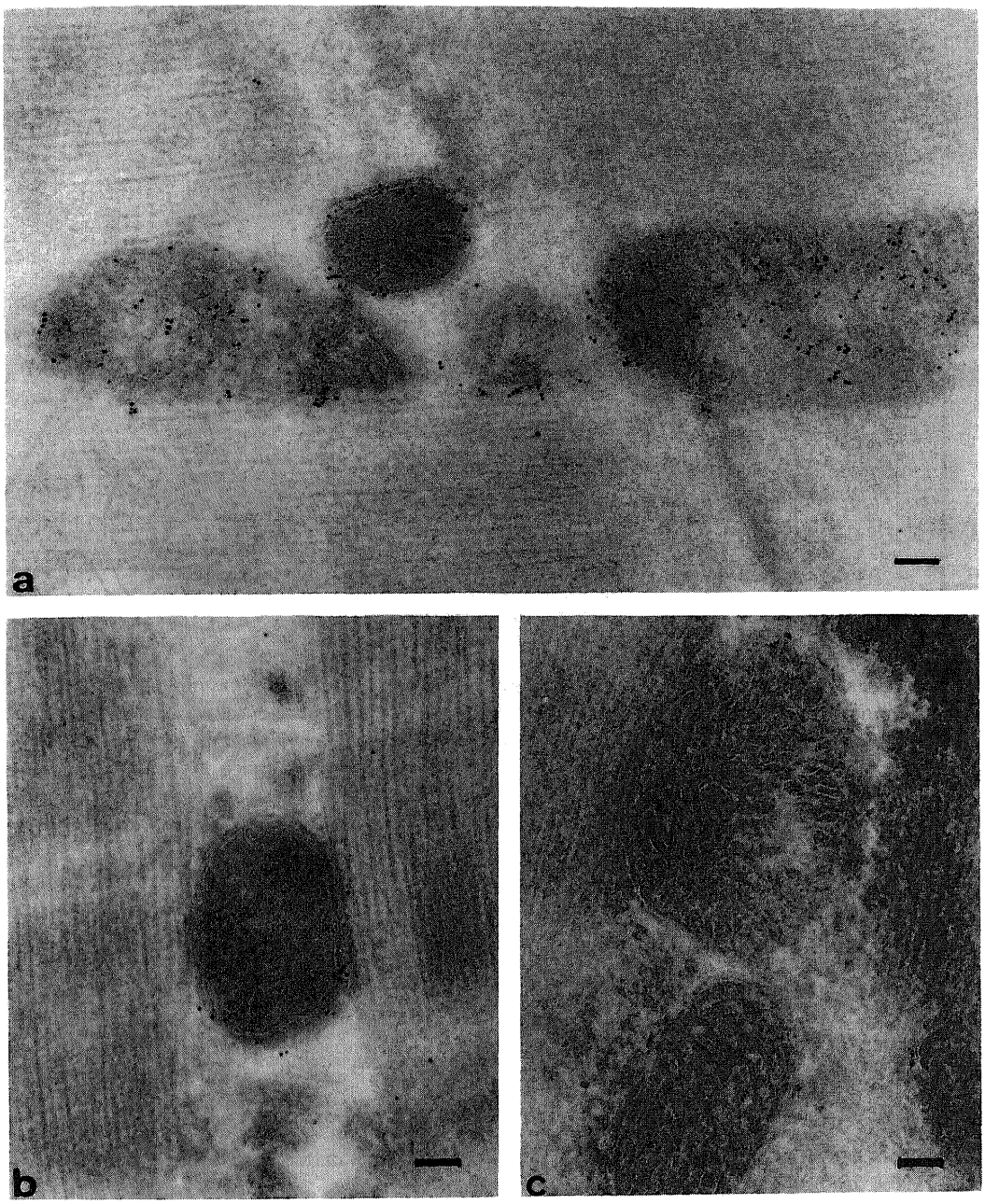


**Fig. 6.** Ultrastructural localization of mitochondrial Mi-CK in fast skeletal muscle. Immunogold localization of Mi-CK on ultrathin cryosections (50–70 nm thick) of chicken pectoralis muscle prepared according to Tokuyasu (1973) using anti-sarcomeric $Mi_b$-CK antibodies (1:100 diluted anti-serum in panel (a); and affinity purified IgG fraction diluted to 1–2 μg $ml^{-1}$ in panel (b)), both followed by 5 nm-gold-conjugated second antibody. Control with preimmune serum plus same concentration of second antibody (c). Notice specific staining of skeletal muscle mitochondria by anti-Mi-CK antibody both within the mitochondria and at the periphery (a and b), as well as absence of colloidal gold in the control. Bar = 0.1 μm.

in a hexagonal lattice would be approximately 11 nm only (Fig. 8). Considering a minimal layer of 3 nm thickness of vicinal water surrounding the myofilaments (Morel, 1985 suggested 3–9 nm) leading to an unstirred layer, and an average Stoke's radius of 5 nm for the M-CK dimer (Morimoto & Harrington, 1972 determined hydrodynamically the dimensions as being 12–14 nm × 5 nm), the freely accessible space to the centre of the M-CK dimer in the acto–myosin overlap zone is only approximately 8% of the total empty space between filaments in this sarcomeric region (see white areas in Fig. 8, modified according to Matsubara *et al.*, 1984). The above number does not take into account the presence of rigor cross-bridges attached to thin filaments which will additionally impose diffusional barriers, and the fact that the transverse filament spacing decreases by about 11% in rigor muscle due to lateral forces exerted by the crossbridges (Matsubara *et al.*, 1984). Thus, the acto–myosin region clearly seems to be able to act as a molecular sieve, the extent of exclusion being a function of the size

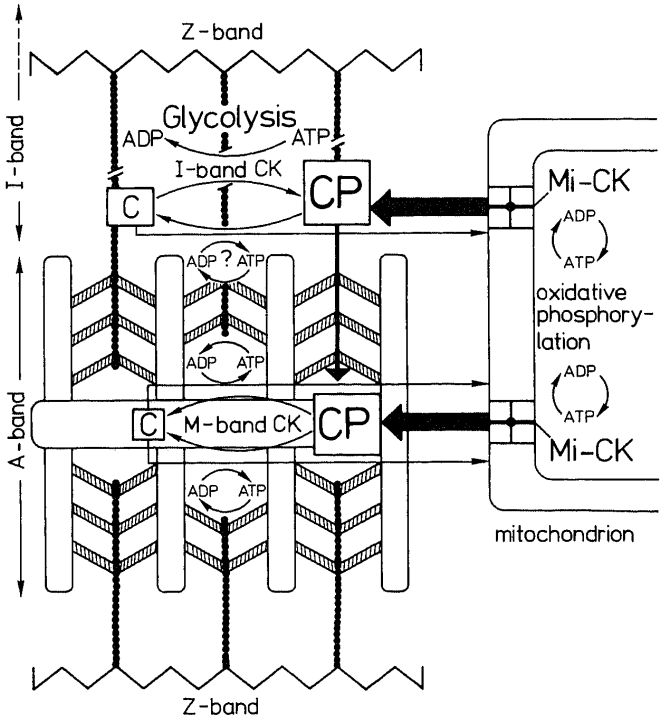


**Fig. 7.** CK–PCr system in sarcomeric muscle. The compartmented localization of CK isoenzymes in muscle at places of energy production (glycolysis and mitochondria) energy consumption (myofibrils) represent the cellular basis for the phospho-creatine circuit model (Wallimann *et al.*, 1989). Visualized here are the specific and rather tight association of M-CK with the M-line (see Wallimann & Eppenberger, 1985), as well as the occupancy by M-CK of the I-band space in conjunction with the glycolytic enzymes, which are also localized there. At the M-line the enzyme is functionally coupled to the myofibrillar actin-activated $Mg^{2+}$-ATPase (Wallimann *et al.*, 1984) which, during contractions, utilizes ATP within the A-band halves on both sides of the M-line. In the I-band, M-CK is functionally coupled to glycolysis and participates in the formation of large but loosly associated multienzyme complexes with glycolytic enzymes (Maughan & Wegner, 1989; Dillon & Clark, 1990). Phospho-creatine (CP), produced by oxidative phosphorylation in the mitochondria via mitochondria CK (Mi-CK) or by glycolysis via loosely bound I-band CK, is available to the M-band CK for efficient regeneration of ATP hydrolyzed by the myofibrillar ATPase during contraction. CP is also available to the SR- and sarcolemma-bound CK (not shown) for the regeneration of ATP utilized by the respective ion pumps located in these membranes (Grosse *et al.*, 1980; Rossi *et al.*, 1990). While I-band CK is functionally coupled to glycolysis, Mi-CK is located along the cristae membranes as well as at contact sites between inner and outer membranes (Kottke *et al.*, 1991), where the octameric enzyme is functionally coupled to oxidative phosphorylation and to the ATP/ADP translocase. The question mark indicates the still open question of whether glycolytic ATP can directly be utilized as such for contraction. We propose, however, that glycolytic ATP, generated by glycolytic enzymes also located in the I-band (Pette, 1975), is transphosphorylated at its site of generation via the I-band CK and that CP and not ATP is diffusing along the myofilament lattice within the sarcomere and is delivered to the A-band haloes via I-band CK. For details of a more generalized PCr circuit model see (Wallimann *et al.*, 1989, 1992 and references therein).

of the macromolecules. Surprisingly, even average-sized enzymes like CK and myokinase seem to be mostly excluded from this sarcomeric region (Fig. 8). Of possible importance in this respect are also the fixed protein charges of thick and thin filaments leading to different Donnan potentials at the A- and I-band, depending on the physiological state of muscle (Bartels & Elliott, 1985). Taken together, all the above factors, molecular sieving, vicinal water and Donnan potentials may all contribute to the described exclusion of sarcoplasmic proteins as well as divalent anions such as $MgATP^{2-}$ and $PCr^{2-}$ from certain sarcomeric regions. This would argue strongly against the concept of the muscle cell as a well-mixed bag of enzymes. The absence of immunofluorescence staining of the A-band halves in intact muscle was certainly not an artefact caused by chemical fixation because relatively thick cryosections of quickly frozen muscle without fixation gave a similar staining, although the structural preservation of the samples suffered and unfixed M-CK had a strong tendency to leak out from the I-band. Also, it is very unlikely that this phenomenon can be explained by masking of epitopes as a result of binding of the proteins to acto–myosin, for one would have to suppose that this would be the case simultaneously for all three proteins, M-CK, aldolase and myokinase, and for all epitopes recognized by the polyclonal antibodies. This seems very unlikely the case for M-CK because the strongly-bound M-CK interlinked in the tight M-line protein-meshwork is recognized perfectly well by the antibody (Wallimann & Eppenberger, 1985).

The staining pattern was independent of small sarcomere length variations unavoidably occurring in frozen

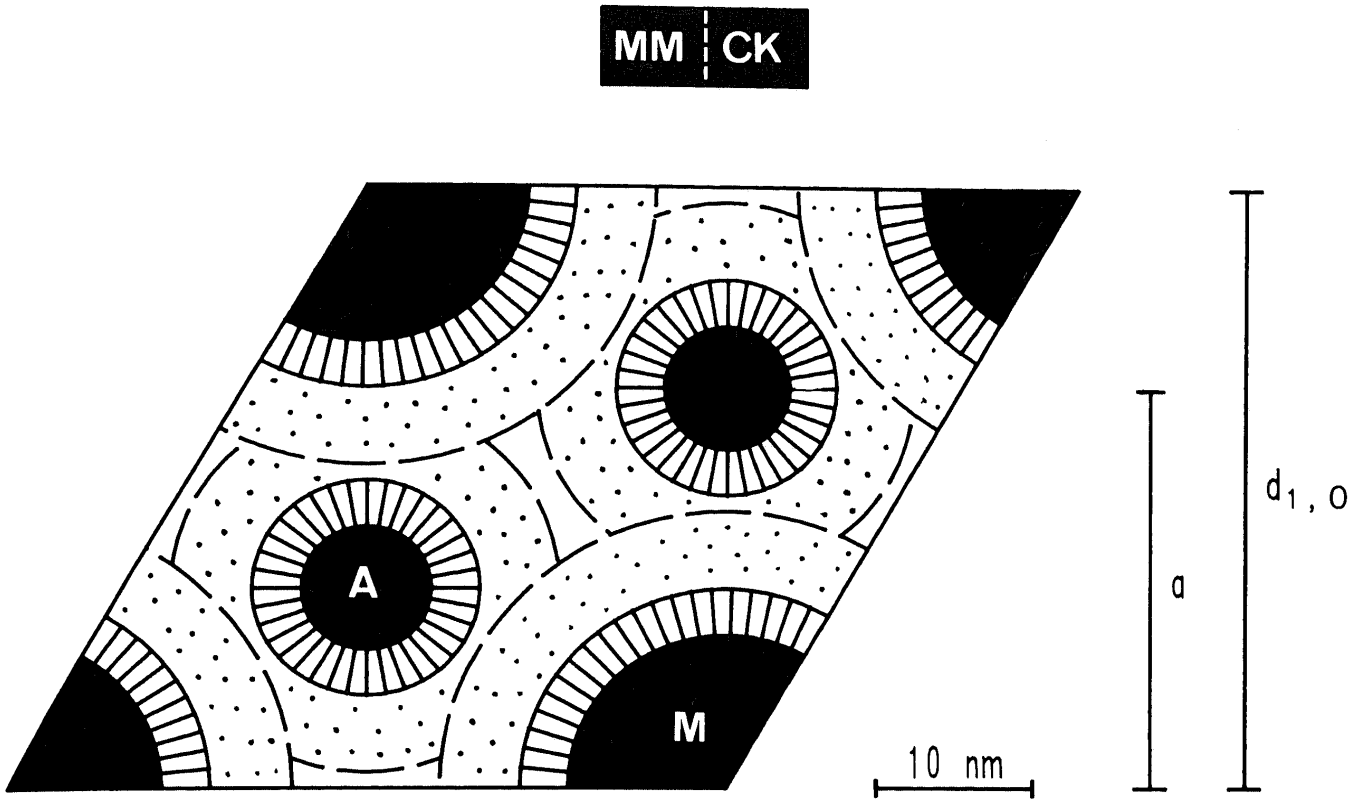


**Fig. 8.** Acto–myosin overlap region of the A-band as a molecular sieve for sarcoplasmic enzymes. Unit cell of the hexagonal filament lattice of a vertebrate muscle in the A-band of the sarcomer displayed according to Matsubara and colleagues (1984), with modifications. (A) thin filaments and (M) thick filaments with cylindrical radii of 4 and 10 nm, respectively, drawn without crossbridges. Regions with radiating spokes represent 3 nm of vicinal water forming an unstirred layer at the periphery of the myofilaments (Morel, 1985, suggested 3–9 nm). Dotted, hatched peripheral regions indicate space unavailable to the centre of the MM-CK dimer with dimensions of 12–14 nm × 5 nm and an average Stoke's radius of approximately 4–5 nm (Morimoto & Harrington, 1972; Wallimann & Eppenberger, 1985). The percentage of free empty space (calculated to be approximately 8%) for M-CK compared with the total space between the myofilaments is represented by the intermittent small white areas. The space occupied by attached crossbridges reaching out from the thick filament backbone towards the thin filaments (not shown here) and the compression of the transverse lattice spacing due to lateral forces exerted by these crossbridges (Matsubara *et al.*, 1984) will further reduce the freely accessible interfilament space for sarcoplasmic proteins (see Discussion). At the I-band, the free interfilament space is significantly larger owing to the complete absence of the thick filament lattice, a fact which favours the diffusion equilibrium towards occupancy of the latter sarcomeric region by sarcoplasmic proteins. $d_{1,0}$, lattice spacing dimensions obtained from X-ray data (Matsubara *et al.*, 1984); a, distance between thick and thin filaments. (Above: MM-CK dimer drawn to scale.)

chicken pectoralis and rat heart muscle (not shown). However, it will be interesting to follow up the *in situ* localization of these enzymes as a function of a wider range of sarcomere lengths by stretching individual muscle fibres in a controlled fashion up to non-overlap length. Whereas the immunolocalizations of M-line-bound and SR-bound M-CK (see electron micrograph on Fig. 5 in Rossi *et al.*, 1990), as well as that of mitochondrial Mi-CK (see below), were quite successfully achieved on an ultrastructural level with immunogold methods on ultrathin cryosections, probably owing to the relatively tight association of CK with these structures, we were unable to get as strong immunogold signals at the I-band on the EM level (Wegmann, 1986) as to be expected from bright immunofluorescence staining of the same sarcomere region in semithin (200–800 nm) cryosections (Figs 1–3). Our interpretation of the rather weak anti-M-CK immunogold labelling at the I-band is that, in contrast to FITC-conjugated antibodies, immunogold only labels the very surface of the tissue sections (Stierhof & Schwarz, 1989) and, as the antigen is solubilized easily from the surface of these very thin (50–70 nm) sections, as generally to be expected of soluble proteins, the immunogold-labelling is expected to be much lower than anticipated from immunofluorescence results at the light microscopic level. This has been consistently confirmed by CK-localization studies in other tissues (Wallimann *et al.*, 1986b). The dissolution of soluble M-CK in muscle took place even after the strongest possible level of chemical fixation had been used, as judged by the preservation of antigen– antibody reactivity with our anti-M-CK antibody that was rather sensitive to fixation. Immunogold labelling of the M-line and I-band of rapidly frozen, cryosubstituted and Lowycryl-embedded muscle specimens was even weaker than with cryosections (Wegmann, 1986).

Based on the immunogold labelling results concerning cytosolic M-CK in muscle (Wegmann, 1986) one cannot, at the moment, rule out completely that on an ultrastructural level there may be some M-CK within the acto–myosin overlap regions after all. However, the immunofluorescence results are of sufficient resolution to show that M-CK in muscle is compartmentalized and that the major part of sarcoplasmic M-CK is confined to the I-band, with much lower amounts of the enzyme being present in the acto–myosin overlap zone.

Besides the partitioning of cytosolic M-CK to the I-band reported here, additional portions of CK have been found to be specifically associated with the sarcoplasmic reticulum (Sharov *et al.*, 1977; Rossi *et al.*, 1990) where CK is functionally coupled to the ATP-dependent $Ca^{2+}$-pump, supporting ATP-driven $Ca^{2+}$-uptake into isolated SR vesicles (Levitsky *et al.*, 1978; Rossi *et al.*, 1990). In addition, some CK has also been found at the sarcolemma membrane (Jockers-Wretou *et al.*, 1977; Sharov *et al.*, 1977) where CK is functionally coupled to the ATP-dependent $Na^+/K^+$-pump (Saks *et al.*, 1977; Grosse *et al.*, 1980; see Wallimann *et al.*, 1989). These *in situ* localizations of CK at membranes are supported by findings that all CK isoenzymes can interact with phospholipid monolayer model membranes (Rojo *et al.*, 1991a).

*Functional coupling of CK with glycogenolysis and glycolysis*

In muscle, the glycolytic enzymes, forming a so-called 'glycolytic complex' (Brooks & Storey, 1988), are located at the I-band (Arnold *et al.*, 1971; Bronstein & Knull, 1981). As shown here, most of the soluble M-CK is also specifically localized at the I-band, and is eluted from skinned muscle fibres together with glycolytic enzymes in the form of large multienzyme complexes (Maughan & Wegner, 1989; Méjean *et al.*, 1989). The amount of soluble M-CK correlates with the glycolytic potential of a muscle (Wallimann & Eppenberger, 1985). This is in accord with immunofluorescence localization of M-CK in different muscle fibre types of the rat (Carlsson *et al.*, 1990). Functional coupling of glycolysis and PCr utilization was also demonstrated by $^{31}$P-NMR (Kuprianov *et al.*, 1980; van Waarde *et al.*, 1990). Thus, the ATP produced by glycolysis upon stimulation of a fast-twitch glycolytic muscle is not accumulated as such but is immediately and efficiently transphosphorylated by CK into PCr to replenish the PCr pool(s) and to maintain a high PCr to Cr ratio in the cell (Fig. 7) (Wallimann *et al.*, 1992).

*Functional coupling of mitochondrial Mi-CK to oxidative phosphorylation*

Interestingly, in cells with high energy demands, cytosolic CK isoenzymes are generally co-expressed with Mi-CK (Jacobs *et al.*, 1964; Jacobus & Lehninger, 1973). Efficient functional coupling between the Mi-CK reaction and oxidative phosphorylation has been established by several research groups using biochemical, kinetic and thermodynamic methods as well as radioisotope analysis. Thus, Mi-CK seems to have privileged access to mitochondrial matrix-generated ATP presented by the adenine nucleotide translocator (ANT) (for reviews and references see Jacobus *et al.*, 1983; Bessman & Carpenter, 1985; Jacobus, 1985; Wallimann *et al.*, 1989; Wallimann & Eppenberger, 1990) (see Fig. 7) (for detailed discussion see Wallimann *et al.*, 1992).

*Main functions of the creatine kinase/phospho–creatine system in muscle*

A model concerning the physiological function of the CK-PCr system in sarcomeric muscle is presented in Fig. 7. The main functions of this system, working as: (i) a 'temporal energy buffer' (Meyer *et al.*, 1984), which is generally accepted and cited in most textbooks, (ii) and as a 'spatial energy buffer' or 'energy transport' system, the so-called CP-shuttle (for reviews see Bessman & Carpenter, 1985; Wallimann *et al.*, 1989, 1992), and (iii) as a regulatory system providing appropriate local ATP to ADP ratios at subcellular sites where CK is functionally coupled to ATP-consuming and ATP-producing processes, are described in detail in a recent review on the PCr circuit (Wallimann *et al.*, 1992).

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## Note added in proof

Recently, Robert and colleagues (1991), based also on results with immunoperoxidases staining, like Otsu and colleagues (1989), suggested that CK, in frog muscle, is localized at the A-band (mainly in the acto-myosin overlap region, see their Fig. 3D). This again points to problems using this indirect technique due to possible diffusion and trapping of stain products to and at places, respectively, where no enzyme is present *in situ* (Raap *et al.*, 1983).

In agreement with our results concerning the molecular sieve effect of the acto-myosin crossbridge zones, recent diffusion experiments of NEM–myosin subfragment 1 (S1) into chemically skinned muscle fibres indicate that it takes 24 h or even longer until S1 saturates the A-band and gets properly orientated inside these skinned fibres (unpublished results in collaboration with Drs T. Kraft and B. Brenner, University of Ulm, Germany).

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