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Coupling of creatine kinase to glycolytic enzymes at the sarcomeric I-band of skeletal muscle: a biochemical study *in situ*

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Abstract

The specific interaction of muscle type creatine-kinase (MM-CK) with the myofibrillar M-line was demonstrated by exchanging endogenous MM-CK with an excess of fluorescently labeled MM-CK *in situ*, using chemically skinned skeletal muscle fibers and confocal microscopy. No binding of labeled MM-CK was noticed at the I-band of skinned fibers, where the enzyme is additionally located *in vivo*, as shown earlier by immunofluorescence staining of cryosections of intact muscle. However, when rhodamine-labeled MM-CK was diffused into skinned fibers that had been preincubated with phosphofructokinase (PFK), a glycolytic enzyme known to bind to actin, a striking *in vivo*-like interaction of Rh-MM-CK with the I-band was found, presumably mediated by binding of Rh-MM-CK to the glycolytic enzyme. Aldolase, another actin-binding glycolytic enzyme was also able to bind Rh-MM-CK to the I-band, but formation of the complex occurred preferably at long sarcomere length (>3.0 μm). Neither pyruvate kinase, although known for its binding to actin, nor phosphoglycerate kinase (PGK), not directly interacting with the I-band itself, did mediate I-band targeting of MM-CK. Anchoring of MM-CK to the I-band via PFK, but not so via aldolase, was strongly pH-dependent and occurred below pH 7.0. Labeling performed at different sarcomere length indicated that the PFK/MM-CK complex bound to thin filaments of the I-band, but not within the actomyosin overlap zones. The physiological consequences of the structural interaction of MM-CK with PFK at the I-band is discussed with respect to functional coupling of MM-CK to glycolysis, metabolic regulation and channeling in multi-enzyme complexes. The *in situ* binding assay with skinned skeletal muscle fibers described here represents a useful method for further studies of specific protein–protein interactions in a structurally intact contractile system under various precisely controlled conditions.

Introduction

Creatine kinase (CK; EC 2.7.3.2), like many glycolytic enzymes, belongs to the group of 'ambiquitous' enzymes (Wilson, 1978) whose intracellular distribution seems partitioned between strictly soluble and particulate forms that may vary in relative proportion with the metabolic state of the cell. These enzymes, including prominent examples as hexokinase (HK) and phosphofructokinase (PFK), may reversibly interact with cellular organelles, like mitochondria (Lamsen *et al*., 1990) and cytoskeletal structures, like microfilaments (for reviews see Masters, 1981; Ovadi, 1995), respectively. Some cytosolic muscle type creatine-kinase (MM-CK) was shown to specifically interact with the sarcomeric M-line (Turner *et al*., 1973) in an isoenzyme-specific manner (Wallimann *et al*., 1983; Stolz and Wallimann, 1998). The amount of M-line bound MM-CK was sufficient to regenerate *in vitro* all the ATP needed for maximal contraction of myofibrils (Wallimann *et al*., 1984). Functional coupling between CK and cardiac contraction has been demonstrated (Ventura-Clapier *et al*., 1994) and recently a connection between the myosin ATPase cycle and the CK reaction was also shown to facilitate cardiac actomyosin sliding in an *in vitro* assay (Sata *et al*., 1996), thus corroborating earlier results concerning the functional coupling between co-immobilized MM-CK and myosin (Arrio-Dupont *et al*., 1992). In addition to representing a potent intramyofibrillar ATP regenerator, the M-line bound enzyme was also shown to represent a structural element of the M-line (for review see Wallimann and Eppenberger, 1985). Some years later, one could show by immunofluorescent localization on muscle cryosections, that in addition of being fairly tightly bound to the M-line, some MM-CK was also bound, albeit more loosely, to the sarcomeric I-band from where it could be washed out, however with relative ease (Wegmann *et al*., 1992).

To investigate further the interaction of MM-CK with these sarcomeric regions, an assay was established with

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chemically permeabilized muscle fibers, whereby the binding of fluorescently labeled MM-CK could be monitored on-line *in situ* under a variety of well controlled experimental conditions using confocal microscopy (Kraft *et al*., 1995a; Stolz *et al*., 1998). Compared to *in vitro* studies this fiber diffusion assay has the advantage that interactions of the proteins with the cytoskeleton or with contractile proteins can be studied in a structurally intact system under conditions that are primarily physiological or that can be varied and well controlled. Using this method, the specific exchange of labeled MM-CK with endogenous CK at the M-line has been demonstrated earlier (Kraft *et al*., 1995b,c). In addition, using CK mutants, as well as chimerical MM/BB-CK constructs, a region in MM-CK which was responsible for attachment of this enzyme to the M-line could be defined precisely (Stolz and Wallimann, 1998; Hornemann *et al*., 2000). However, in this assay with permeabilized muscle fibers, we did not observe the specific interaction of MM-CK with the I-band as was clearly seen by immunofluorescence staining of frozen sections of chemically fixed intact muscle (Wegmann *et al*., 1992). Apparently, in permeabilized muscle fibers some 'factors' were missing which mediated MM-CK-binding to the I-band of muscle fibers *in vivo*.

Since MM-CK was shown to be functionally coupled to glycolysis by removing glycolytically generated ATP into the large phosphocreatine reservoir (Van Waarde *et al*., 1990; for review see Wallimann *et al*., 1992; Wallimann and Hemmer, 1994), and since the glycolytic enzymes are all co-localized at the sarcomeric I-band, with some of them interacting directly with F-actin and thin filaments (Sigel and Pette, 1969; Arnold *et al*., 1971; Clarke and Masters, 1975; Walsh *et al*., 1980; Bronstein and Knull, 1981; for review see Ovadi, 1995; Ovadi and Orosz, 1997), a possible association of MM-CK with some of the glycolytic enzymes seemed likely. Thus, we used the same *in situ* diffusion assay to co-diffuse glycolytic enzymes together with fluorescently labeled MM-CK into permeabilized skeletal muscle fibers and then followed the binding of the latter to myofibrillar substructures directly by confocal microscopy.

Surprisingly, the two glycolytic enzymes found to anchor MM-CK to the I-band, presumably by forming complexes with MM-CK, were phosphofructokinase (PFK; EC 2.7.1.11) and aldolase (EC 4.1.2.13), but not, as we expected, those glycolytics which produce ATP, e.g., phosphoglycerate kinase (PGK; EC 2.7.3) or pyruvate kinase (EC 2.7.1.40). The physiological implication of the structural coupling of MM-CK to PFK, an ATP-consuming glycolytic enzyme which is highly regulated by several metabolic factors (see Ovadi, 1995; Ovadi and Orosz, 1997), is discussed. Finally, some regulatory aspects concerning the dynamic function of glycolytic multi-enzyme complexes, including MM-CK, as well as their reversible association with the thin filament at the I-band, will be considered. A preliminary account of this work has been presented earlier (Kraft *et al*., 1996).

Materials and methods

Enzymes, antibodies and incubation solutions

Creatine kinase (MM-CK) as well as the glycolytic enzymes aldolase, phosphofructokinase (fructose-6-phosphate kinase; PFK), pyruvate kinase and phosphoglycerate kinase, all from rabbit muscle, were obtained from Sigma (Munich, Germany) and from Boehringer (Mannheim, Germany). Fluorescently-labeled secondary antibodies were from Cappel (Dynatech, Zürich, Switzerland). The polyclonal goat antibody against chicken M-line protein preparation containing 165 kDa M-protein as well as 185 kDa myomesin (Eppenberger *et al*., 1981; Grove *et al*., 1984) was generated at the Institute of Cell Biology, ETH Zürich (Switzerland) using standard methods. The polyclonal rabbit anti-chicken MM-CK antibody, also generated at our institute (Wallimann *et al*., 1975), has been described (Stolz *et al*., 1998; Kraft *et al*., 1995b).

Prior to use, the enzymes in ammoniumsulfate solution were dialyzed for at least 12 h at 5°C against the following buffer: 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM dithiotreitol (DTT) and 106 mM potassium-propionate. For subsequent labeling of aldolase with tetramethyl-rhodamine isothiocyanate (TRITC), the protein was dialyzed against phosphate buffered saline (PBS).

All incubations of skeletal muscle fibers occurred in 'relaxing solution' containing 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM MgATP, 5 mM DTT, 20 mM creatine phosphate, 106 mM K-propionate (nominal ionic strength 185 mM), pCa was 8.0. Generally, the pH of this solution was adjusted to pH 7.0, but, as indicated in the results for testing pH-dependent interaction of the enzymes with the sarcomeric proteins, the pH was adjusted in some cases to either pH 6.5 or pH 7.5.

Some of the commercially available glycolytic enzyme preparations were found to contain considerable amounts of proteases. Fibers incubated with these enzymes 'dissolved' over night if no protease inhibitors were added to the solution. To avoid enzymatic degradation of the single muscle fibers in these experiments, a protease inhibitor cocktail was added to the incubation solutions: 10 μM each of leupeptin, antipain, E 64 (trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane) and pepstatin A (all from Sigma), as well as 1 mM of pefabloc (Boehringer Mannheim, Germany) and 1 μg/ml aprotinin (Sigma) were the final concentrations. Addition of protease inhibitors did not alter the binding characteristics of the enzymes studied but allowed us to maintain the structural stability of the fibers during all incubations.

The final concentration of the fluorescently labeled proteins, as well as that of unlabeled proteins, in the incubation medium was adjusted to about 15–20 μM for MM-CK and pyruvate kinase and 30–40 μM for aldolase, PGK and PFK. It should be noted that PFK, as

described by others (Parmeggiani *et al*., 1966), tended to precipitate rather easily under various conditions tested, especially during the labeling procedure, so that its final free concentration turned out to be usually below 30 μM.

Fluorescent labeling

Rhodamine-labeled MM-CK (Rh-MMCK) and rhodamine-labeled pyruvate kinase (Rh-pyruvate kinase) as well as fluorescein-labeled phosphofructokinase (fluorescein-PFK) were obtained by modifying the proteins with rhodamine-X-iodoacetamide (Rh-IAA; Molecular Probes, Inc., USA) or with fluorescein-X-iodoacetamide (Molecular Probes, Inc., USA), respectively, as described in Kraft *et al*. (1995b). Rhodamine labeled MM-CK modified with Rh-IAA at stoichiometric ratios is still enzymatically active. Fluorescently-labeled aldolase (Rh-aldolase) and phosphoglycerate kinase (Rh-PGK) were obtained by modifying the proteins with tetramethyl-rhodamine isothiocyanate (TRITC Isomer R; Sigma, Munich, Germany) according to standard methods (The and Feltkamp, 1970a,b; Goding, 1976). Fluorescent labeling of PFK, however, was rather difficult and caused some precipitation of the enzyme. After the labeling, the excess rhodamine-X iodoacetamide or the excess fluorescein-X-iodoacetamide, respectively, were removed by gelfiltration (Sephadex G-25, Amersham Pharmacia Biotech, Freiburg, Germany) using an elution buffer containing 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT and 34 mM K-propionate.

Preparation and mounting of single skinned skeletal muscle fibers

Preparation of small bundles from rabbit psoas muscle and chemical permeabilization of the membranes ('skinning'), as well as isolation of single muscle fibers from these bundles were carried out according to a method described earlier (Brenner, 1983; Yu and Brenner, 1989; Kraft *et al*., 1995a,b). Directly after dissection, approximately 3×3 mm wide and 6–8 cm long bundles of rabbit psoas muscle were incubated for 30 min at 5°C in skinning solution containing 0.5% Triton X-100, 5 mM KH_2PO_4, 3 mM magnesium acetate, 5 mM EGTA, 1 mM ATP (sodium salt), 50 mM creatine phosphate, 5 mM NaN_3, 10 mM glutathione, 2 mM dithiothreitol, including the same cocktail of protease inhibitors as added to the incubation solution (see above). Subsequently, the muscle was transferred to skinning solution without triton. For isolation of single fibers, segments of about 1–1.5 cm were cut from the psoas muscle bundles. The single fibers were usually kept in 'skinning solution' for at least 4–5 h before incubation with any of the enzymes. The single fibers could also be stored for up to about 4 days in skinning solution with protease inhibitors, DTT and glutathione to prevent degradation during storage. Skinned fibers prepared according to this method have been characterized extensively under various conditions with respect to their intact contractile function and structural stability in mechanical measurements of active force generation and other parameters (Brenner, 1983; Yu and Brenner, 1989; Kraft *et al*., 1995a).

For confocal microscopy, the single muscle fibers were mounted in a flat, home-made flow-through chamber (approximately 250 μm deep) on a microscopic slide or on a large cover slip, depending on the type of microscope used (Kraft *et al*., 1995b; Stolz *et al*., 1998). The chamber was closed with a cover slip so that the fibers were completely surrounded by solution. For changing solution, e.g., when adding the fluorescently labeled proteins or relaxing solution without additional proteins, the new solution was sucked through the chamber. During all incubations, new solution was added repeatedly to prevent concentration gradients. In several experimental runs, the molecules were diffused into the mounted muscle fibers without covering the chamber. The fibers were kept in a drop of solution (100 μl) which could be exchanged and stirred during the incubation. After the last solution exchange the chamber was closed with a cover slip.

Preparation, mounting and incubation of the fibers occurred on ice at 1–5°C avoiding higher temperatures for prolonged periods of time thus keeping the fibers structurally and functionally stable also during long term incubations (up to a few days).

Confocal microscopy and image recording

The laser confocal system consisted of a Zeiss Axiophot or an Axiovert fluorescence microscope, respectively, and a Biorad MRC-600 confocal scanner unit. The system was equipped with an argon/krypton mixed gas laser. The images were recorded with a Zeiss Plan Apochromat oil immersion objective 40×/1.0 with a working distance of 0.31 mm.

To reduce background fluorescence and most importantly to remove any unspecifically accumulated proteins, unbound molecules were generally washed out for 10–30 min prior to final image recording using 'relaxing solution' without any added proteins. The images shown in this paper represent typical binding patterns for the respective proteins obtained under the specified conditions. We confirmed this by repeating all labeling experiments at least four times except for labeling of the fibers with fluorescein-PFK alone (repeated three times).

A possible problem with double fluorescence technique is the possibility that emitted light from fluorescein (e.g., FITC-labeled myomesin antibody) is also detected by the photomultiplier for rhodamine fluorescence. This is due to the fact that the rhodamine emission filter does not completely cut off the fluorescein emission. The problem can occur specifically when fluorescein-labeling is very strong. For example, a strong fluorescein signal at the M-line could be mistaken as weak labeling by a rhodamine-conjugated protein, while

it actually represents bleed-through of fluorescein into the rhodamine channel. Therefore, in some cases where rhodamine-labeling was rather weak, we recorded images from fibers with fluorescein- and rhodamine-labels in the double fluorescence mode and in a parallel experiment from fibers with rhodamine-fluorescence only. This allowed us to allocate the sarcomere regions correctly and to ensure that the rhodamine labeling was not 'contaminated' by the fluorescein-label. One such example is represented by the fluorescein-PFK incubation shown in Figure 4b.

Experimental protocol

For all incubations in the present study, 'unfixed' skinned skeletal muscle fibers from rabbit psoas muscle were used. The diffusions occurred under relaxing conditions, i.e., in the absence of calcium while the fibers were kept at 1–5°C in a humid chamber in the dark. Observation of the labeled fibers in the confocal microscope took place at room temperature. No reagents were added to the solutions for reduction of photobleaching.

In most cases, prior to addition of the unlabeled or fluorescently labeled molecules, the fibers were incubated for more than 2 h in a solution containing an antibody against the M-line protein myomesin (anti-myomesin-Ab, 1:100), serving as an internal M-line marker. Subsequently, this antibody was labeled with a secondary, FITC-labeled antibody or a CY-5-labeled antibody for at least 2 h. The specific binding of the anti-myomesin antibody to the M-line of the sarcomeres allowed us to precisely identify the M-line in the images of the fibers by double fluorescence technique. In experiments where a combination of an unlabeled enzyme and Rh-MM-CK was used, the fibers were first incubated with the unlabeled enzyme for at least 3 h and subsequently Rh-MM-CK was added to the enzyme solution. In general, all fibers were incubated for at least 4 h (or over night) in Rh-MM-CK, plus the respective unlabeled enzyme, before the first images were recorded.

After relatively short incubation times only the outer layers of the fibers were equilibrated with the proteins while there was still a diffusion gradient towards the center of the fiber. This was particularly the case for the incubation with anti-myomesin antibody and the secondary, FITC-labeled antibody as well as for aldolase plus Rh-MM-CK or PFK plus Rh-MM-CK incubation (see e.g., Figures 2b, 5 and 6). The same slow equilibration was observed previously for several other molecules and seems to be due to rather complex diffusion and binding properties particularly of larger molecules to specific binding sites of the highly structured sarcomeric muscle (Kraft *et al*., 1995b).

Specific binding, as it can be observed by confocal microscopy, was characterized as follows (see also Kraft *et al*., 1995): (i) Specific distribution of the fluorescently labeled molecules and therefore much higher intensity in a particular compartment of each sarcomere, e.g., along the M-line, (ii) always only very slow loss of the bound molecules upon washing with solution containing neither labeled nor unlabeled molecules and (iii) often relatively slow equilibration of the labeled molecules over the whole cross-section of the muscle fiber.

Cryosectioning and immunofluorescent staining

In situ fixation, infiltration with 2.3 M sucrose, cryosectioning and immunofluorescent staining of chicken pectoralis muscle (Figure 1) was carried out according to a previously described method (Wegmann *et al*., 1992). As primary antibody a rabbit-anti-chicken MM-CK antibody was used (Wallimann *et al*., 1983) followed by staining with a fluorescein-conjugated

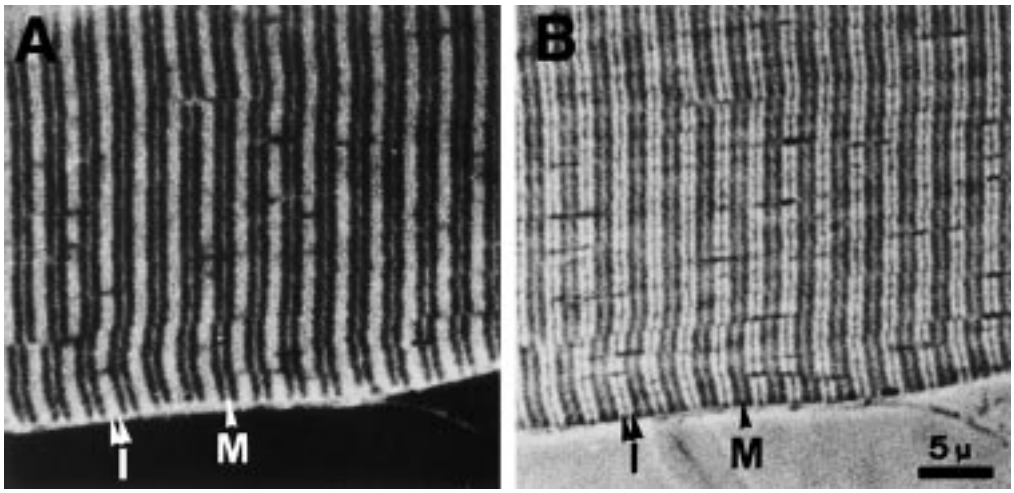

Fig. 1. In-vivo localization of MM-CK in chemically prefixed skeletal muscle. (a) Semi-thin cryosections of chicken pectoralis muscle, stained with a specific MM-CK antiserum, followed by FITC-conjugated fluorescent secondary antibody. Double arrows: I-bands (I) of adjacent sarcomeres, single arrow: M-line (M) of a sarcomere. (b) Corresponding phase contrast image of the same section which allows to identify the different parts of the sarcomere. Note the specific staining of the M-lines as well as of the I-bands with anti-MM-CK antibody in (a).

goat-anti-rabbit IgG secondary antibody. Preparation and staining of mouse gastrocnemius muscle with PFK-antibody (kindly provided by Martin Bähler, Univ. Münster, Germany) occurred according to the same methods.

Results

Sarcomeric binding pattern of MM-CK in native and in chemically skinned skeletal muscle fibers

Myofibrillar creatine kinase (MM-CK) has been shown previously (Wegmann *et al*., 1992) to bind specifically to the M-line, as well as along the I-band of the sarcomeres *in vivo*. This is also demonstrated in Figure 1a which shows a cryo-section of native skeletal muscle labeled with an anti-MM-CK antibody and a secondary, fluorescently labeled antibody. Figure 1b shows the same fiber segment in phase-contrast, where the A- and I-bands of the sarcomeres appear as dark and light bands, respectively. MM-CK staining can be identified as narrow fluorescent band in the M-line and as broader fluorescent band, often sparing the Z-lines, in the I-band of the sarcomeres (Figure 1a).

In order to characterize the specific interactions of MM-CK and other molecules with sarcomeric proteins *in situ*, we used chemically demembranated (skinned) skeletal muscle fibers which allow to study binding of externally added fluorescently labeled molecules in the structurally intact myofibrillar system with confocal microscopy (Kraft *et al*., 1995b). The idea was that the fluorescently labeled exogenous proteins would bind to their binding sites within the sarcomere presumably by filling up empty sites or by replacing or exchanging with the respective endogenous protein. The resulting pattern of high fluorescence within the sarcomere then provides information about the specific binding and sarcomeric distribution of these proteins in skeletal muscle fibers. Another important feature of this fiber assay is that equilibration of the fibers with the fluorescently labeled proteins can be done under many different, well controlled experimental conditions (ionic strength, pH, buffer composition), including physiological conditions, or simulated intracellular pH-shifts.

Surprisingly, if MM-CK is diffused into the chemically skinned skeletal muscle fibers, only the M-line of the sarcomeres becomes fluorescently labeled (Figure 2), which is in contrast to the *in vivo* immunofluorescence study (Figure 1). Figure 2 shows a confocal image of a longitudinal section through a skinned fiber after incubation with Rh-MM-CK for about 3 h (Figure 2a). Prior to Rh-MM-CK incubation, the fiber was labeled with an antibody against the M-line protein myomesin (Figure 2b) so that an unequivocal identification of the sarcomere regions was possible. A comparison of Figure 2a,b indicates that the high fluorescence intensity due to Rh-MM-CK binding (Figure 2a) corresponds to the M-line of the sarcomere. Apparently, the fluorescently labeled MM-CK has replaced the endogenous M-line bound MM-CK (Wegmann *et al*., 1992; Kraft *et al*., 1995c). The I-band appears dark, although occasionally some weak labeling of the I-band with Rh-MM-CK was detected which could represent binding of Rh-MM-CK similar to MM-CK binding in native muscle fibers, only much weaker.

The difference between the localization of MM-CK in native muscle fibers (Figure 1a) and in skinned fibers (Figure 2a) implies that after the loss of most of the soluble (cytoplasmic) proteins and other molecules, due to permeabilization of the membranes of the fiber, MM-CK could only rebind to its binding sites along the M-line, i.e., replace the endogenous MM-CK there. This is supported by earlier findings on glycerol-permeabilized chicken pectoralis muscle (Wegmann *et al*., 1992) and control experiments (data not shown) with skinned rabbit fibers which demonstrated that incubation of permeabilized muscle fibers with a fluorescently labeled MM-CK antibody results in labeling of the M-line only, whereas the I-band MM-CK apparently has been washed out. To address the question which factors were apparently lost during the skinning procedure of the skeletal muscle fibers that allow MM-CK binding to the I-band *in vivo*, we tested whether in native muscle fibers the binding of MM-CK to the I-band was mediated by glycolytic enzymes. As known for some time now, glycolytic enzymes are associated with the I-band (e.g., Sigel and Pette, 1969; Arnold and Pette, 1968; Dölken *et al*., 1975) and form part of the so-called 'soluble' myofibrillar enzymes which can be washed out rather easily (Wegmann *et al*., 1992). As a control, binding and localization of a limited number of glycolytic enzymes in permeabilized skeletal muscle fibers was determined in the absence of exogenously added MM-CK.

Binding of glycolytic enzymes to skinned skeletal muscle fibers

Chemically skinned skeletal muscle fibers were incubated with rhodamine-labeled pyruvate kinase (Rh-pyruvate kinase), phosphoglycerate kinase (Rh-PGK), aldolase (Rh-aldolase) or fluorescein labeled phosphofructokinase (fluorescein-PFK), respectively, to study the individual binding and distribution of the enzymes within the sarcomere. Previously, it was shown that in skinned skeletal muscle fibers, Rh-pyruvate kinase binds specifically to the I-band (Kraft *et al*., 1995b), but not to the M-line. Similar to what was found for Rh-pyruvate kinase in skinned skeletal muscle fibers, Rh-aldolase also bound specifically to the I-band of the sarcomeres (Figure 3). Figure 3a also reveals some binding of Rh-aldolase along the M-line or H-zone of the sarcomeres, which is similar to what was shown by immunofluorescent labeling of native chicken pectoralis muscle (Wegmann *et al*., 1992). PGK, an ATP-producing glycolytic enzyme is also expected to form part of the glycolytic multi-enzyme complex (for review see Somero and Hand, 1990), presumably located in the I-band of the

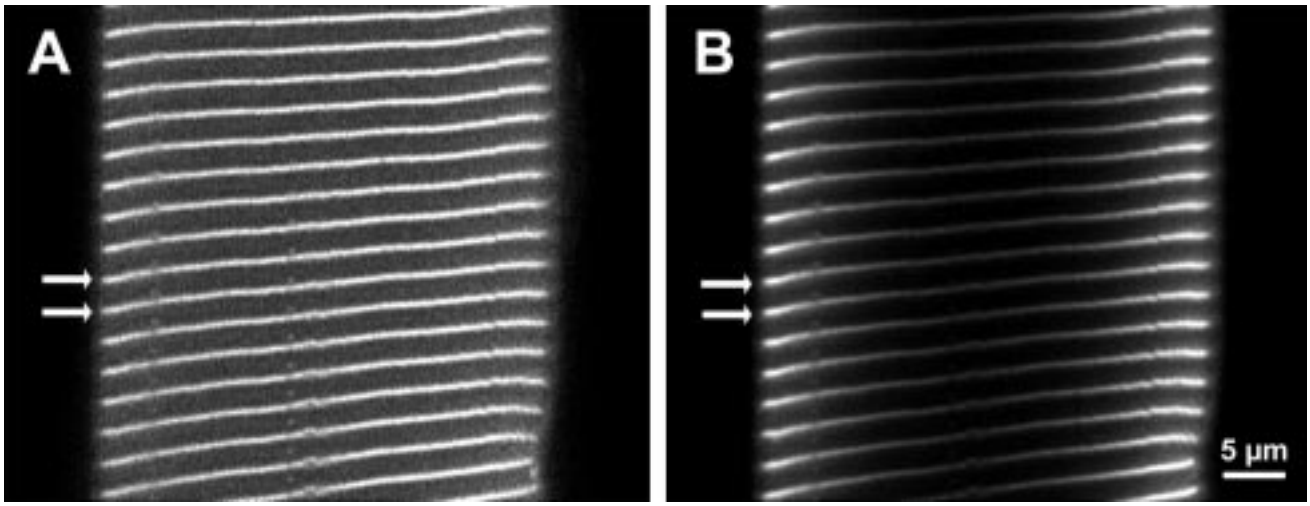

Fig. 2. In-situ localization of MM-CK in chemically skinned skeletal muscle fibers. Confocal images of a longitudinal optical section through the center of a chemically skinned, unfixed rabbit psoas fiber after 3 h of equilibration with rhodamine-labeled creatine kinase from muscle (Rh-MM-CK) (a). The fiber was pre-incubated with an antibody against the M-line specific protein myomesin for about 10 h, which was stained with a second, FITC-labeled antibody for more than 3 h (b). The arrows point to the M-lines of corresponding sarcomeres of the fiber in both pictures and thus demonstrate the co-localization of Rh-MM-CK and the anti-myomesin antibody at the M-line. Note that, different from Figure 1, in skinned fibers specific binding of Rh-MM-CK alone occurs only to the M-line, but not to the I-bands.

sarcomeres. However, in skinned skeletal muscle fibers, we could not detect any binding of rhodamine labeled PGK to the sarcomeric proteins (data not shown). The question of which of the other glycolytic enzymes could mediate binding of PGK to the I-band, has not been addressed yet. PFK, a key-enzyme of the glycolytic pathway, was shown to be specifically localized in the I-band of prefixed skeletal muscle *in vivo* (Figure 4a). This is consistent with previous findings (Sigel and Pette, 1969). Accordingly, incubation of 'skinned' skeletal muscle fibers with fluorescein-labeled PFK (Figure 4b) also resulted in specific labeling of the I-band. To attribute the specific regions of the sarcomere indicated by arrows in Figure 4b, parallel double-fluorescence labeling experiments were carried out as described in Materials and methods. Unfortunately, due to the difficulties with fluorescein-labeling of PFK and the precipitation of fluorescein-PFK during the incubation, the concentration of available fluorescein-PFK molecules was rather low and therefore the fluorescence intensity seen in Figure 4b is low. Nevertheless, there also seems to be some faint fluorescence intensity in the M-line which, however, is close to background levels. This could indicate some binding of PFK to the H-zone as reported earlier (Freydina *et al.*, 1986).

It has been shown in solution studies that binding of PFK to actin is strongly pH-dependent (Roberts and Somero, 1987; Roberts *et al.*, 1988) and according to

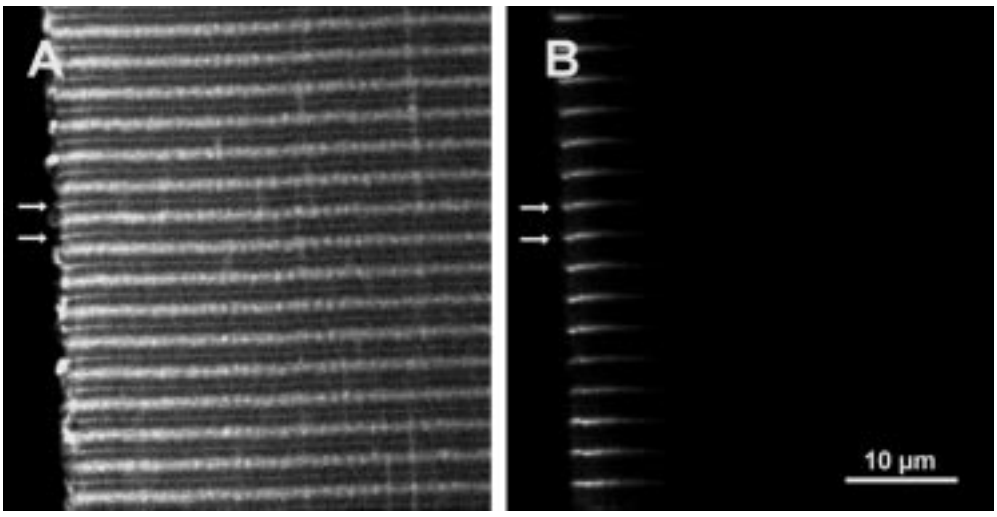

Fig. 3. (a) Confocal image of longitudinal optical section through the center of unfixed rabbit psoas fiber after incubation with Rh-aldolase (incubation time 16 h). (b) Labeling with anti-myomesin antibody as described in Figure 2 (somewhat shorter incubation times for first and secondary antibody). The images show only one half of the fiber. The arrows indicate the M-lines of corresponding sarcomeres of the fiber in both pictures. Rh-aldolase is localized not only at the I-band, but weak labeling is also seen at the M-line or H-zone of the sarcomeres.

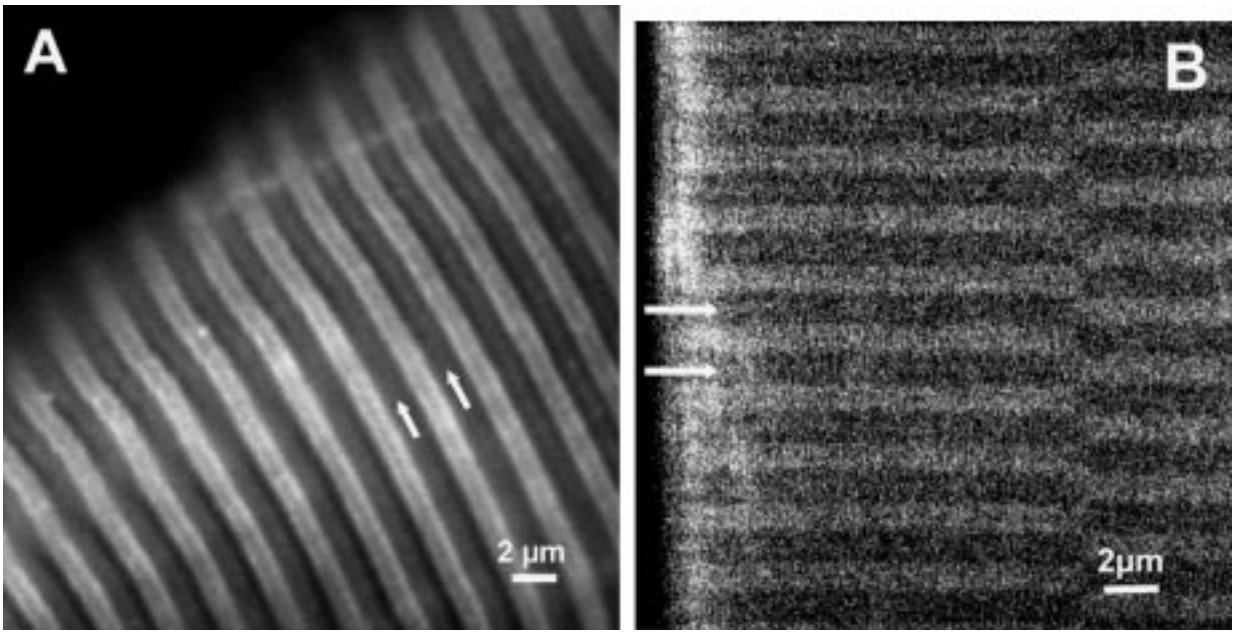

Fig. 4. (a) *In-vivo* localization of phosphofructokinase (PFK) in chemically prefixed skeletal muscle. Semi-thin cryosections of mouse gastrocnemius muscle were stained with an antibody against PFK, followed by a rhodamine-conjugated secondary antibody. (b) Confocal image (longitudinal optical section) of a chemically *skinned* unfixed rabbit psoas fiber after incubation with fluorescein-phosphofructokinase (fluorescein-PFK) at pH 6.5. The image shows only one half of the fiber. Fluorescein-PFK labels the I-band of the sarcomeres, some faint labeling is also visible at the M-line or H-zone. In both images the arrows indicate location of the M-line of two neighboring sarcomeres which was identified with an anti-myomesin antibody and fluorescent labeling with a CY-5-labeled secondary antibody.

these data, at pH 7.5, nearly no binding of fluorescein-PFK to actin, i.e., in the I-band of the sarcomeres should occur. Therefore, incubation of the fiber shown in Figure 4b occurred at pH 6.5. To test the specificity of fluorescein-PFK-binding to the I-band of the sarcomere, we also incubated some fibers with fluorescein-PFK at pH 7.5. No fluorescent labeling of the I-band was found at pH 7.5, indicating that essentially no fluorescein-PFK binding occurs (data not shown). Control experiments with Rh-pyruvate kinase, Rh-aldolase, Rh-PGK, and Rh-MM-CK, respectively, showed no such pH-dependent differences in binding to the sarcomeric proteins. Their individual binding pattern was the same at pH 6.5 and pH 7.5 and in the case of Rh-PGK no binding was observed at either of the two pH-values.

Taken together, these results indicate that some of the so called 'soluble' glycolytic enzymes, namely pyruvate kinase, aldolase and PFK bind to chemically skinned muscle fibers in a similar specific pattern, as observed in muscle fibers *in vivo*. Apparently, neither membrane permeabilization of the fibers nor the rhodamine-labeling of the enzymes did abolish this property. Therefore, these enzymes were good candidates for testing the hypothesis that MM-CK binding to the I-bands in skeletal muscle fibers is mediated by glycolytic enzymes.

Binding of MM-CK in the presence of glycolytic enzymes to skinned skeletal muscle fibers

Skinned skeletal muscle fibers were incubated with Rh-MM-CK in the presence of either unlabeled pyruvate kinase, PGK, PFK or aldolase. If Rh-MM-CK would bind to one of the enzymes which have been shown to bind to the I-band of the sarcomeres, then specific labeling of the I-band with Rh-MM-CK should appear.

Skinned muscle fibers equilibrated with pyruvate kinase plus Rh-MM-CK showed no binding of Rh-MM-CK to the I-band; similar to the situation with Rh-MM-CK alone only the M-line was fluorescently labeled. Thus, pyruvate kinase apparently cannot mediate Rh-MM-CK binding to actin under the conditions used here. This is somewhat surprising since Rh-pyruvate kinase itself was shown to bind to the I-band in skinned fibers (Kraft *et al.*, 1995b) and an earlier study by Dillon and Clark (1990) showed the formation of a so-called 'diazyme complex' between pyruvate kinase and MM-CK in solution. Our observation does not preclude a transient loose interaction between the two enzymes in solution (Sears and Dillon, 1999), being functionally coupled by sharing ATP as common substrate and product, respectively (Dillon and Clark, 1990). It should be noted that pyruvate kinase, in contrast to aldolase and PFK, showed no binding to the M-line, where MM-CK is located in skinned fibers. High fluorescence intensity due to Rh-pyruvate kinase binding was confined exclusively to the I-band (Kraft *et al.*, 1995b).

Even though Rh-PGK alone did not bind to the I-band of skinned muscle fibers, the possibility of some interaction between PGK and Rh-MM-CK and the sarcomeric proteins in the I-band was tested. However, in the presence of PGK no binding of Rh-MM-CK to the I-band was observed. This indicates either that apparently not only rhodamine-labeled PGK but also

unlabeled PGK does not bind directly to sarcomeric proteins, or that PGK cannot target Rh-MM-CK to the I-band. It also does not exclude that Rh-MM-CK binds to PGK and that both form part of a multienzyme complex without binding to actin or other sarcomeric proteins directly.

Incubation of skinned fibers with aldolase plus Rh-MM-CK in contrast resulted in specific fluorescent labeling not only along the M-line or H-zone but also in the I-band of the sarcomeres (Figure 5). Although equilibration of the fiber shown in Figure 5 with aldolase plus Rh-MM-CK was not complete and therefore fluorescence intensity along the I-band did not show up very strongly in the center of the fiber, specific binding of Rh-MM-CK to the I-band can be observed over the whole cross-section. However, the interaction between aldolase, Rh-MM-CK and the I-band shown in Figure 5 was observed in a fiber stretched to 3.5 μm sarcomere length. In general, a sarcomere length greater than about 3.0 μm was necessary during incubation with aldolase plus RH-MM-CK to observe such labeling. At normal sarcomere length (≈2.4 μm) only very little association of the complex with the I-band could be detected. This indicates that apparently only under rather unphysiological conditions (long sarcomere length), an aldolase-Rh-MM-CK complex at the I-band of skinned muscle fibers was formed to a significant extent. Control experiments showed that binding of Rh-MM-CK alone in skinned fibers is the same at normal and at long sarcomere length, in both cases it binds only to the M-line. Nevertheless, the formation of a complex between Rh-MM-CK and aldolase might explain the observations that *in vivo* (Wegmann *et al*., 1992) and in skinned fibers (Figure 3) Rh-aldolase not only binds to the I-band of the sarcomeres but also to the M-line or H-zones. The labeling of the M-line with Rh-aldolase could be due to association of Rh-aldolase to structurally bound MM-CK in the M-line which is not lost during the skinning procedure of the fibers.

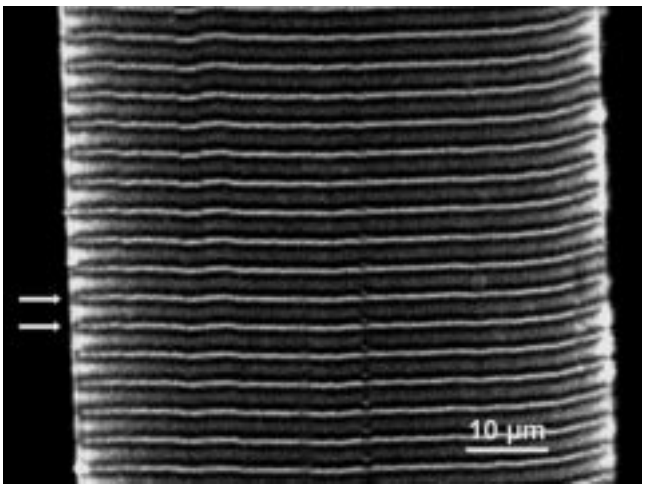

Fig. 5. Confocal image of a rabbit psoas fiber after incubation with aldolase plus Rh-MM-CK for 17 h at 3.5 μm sarcomere length. The arrows point to the M-line of the sarcomeres. Note that in the presence of aldolase, preferably at long sarcomere length, Rh-MM-CK not only binds to the M-line but also to the I-band. Labeling of the M-line with Rh-MM-CK is stronger compared to the I-band because binding of Rh-MM-CK to the M-line is complete within a few minutes (Kraft *et al*., 1995b) while binding of the whole complex to the I-band takes much longer and is not complete in the fiber shown.

Most interestingly, in the presence of PFK plus Rh-MM-CK, strong fluorescent labeling of the I-band indicating binding of Rh-MM-CK via PFK to sarcomeric proteins, presumably actin, was observed (Figure 6a). The resulting labeling pattern of the muscle fiber (Figure 6a) very much resembles the immunofluorescent staining of native muscle with anti-MM-CK antibodies (Figure 1a). Only the fluorescence intensity of the I-band especially toward the center of the fiber was not as strong as in native muscle, most likely because the incubation time was too short for the complex to bind evenly throughout the entire fiber. To test the specificity of the complex between PFK, Rh-MM-CK and thin filaments, the experiment was repeated at different pH. As mentioned above, this is based on previous work showing that PFK binding to filamentous actin and myofibrils increases significantly at pH-values below 7.1 (Roberts and Somero, 1987; Roberts *et al*., 1988) and on the observed pH-dependent binding of fluorescein-PFK in skinned fibers (Figure 4b). In agreement with these earlier observations, incubation of fibers with PFK plus Rh-MM-CK resulted in very strong labeling of the I-band at pH 6.5 (Figure 6a), whereas at pH 7.5, no labeling of the I-band by Rh-MM-CK via PFK was detected (Figure 6b). Since at more alkaline pH PFK apparently does not significantly bind to actin, no formation of the actin-PFK-Rh-MM-CK complex seems to occur.

To investigate whether binding of PFK and Rh-MM-CK is restricted to a certain region of the actin filaments near the Z-lines, we also studied the binding of PFK and Rh-MM-CK at longer sarcomere length (pH 6.5). Figure 6c shows that at 3.3 μm sarcomere length the fluorescently labeled region corresponding to the sarcomeric I-band gets much wider than at 2.4 μm sarcomere length (Figure 6a). More detailed analysis showed that the width of the fluorescently labeled I-band in fibers stretched to longer sarcomere lengths corresponds to the length of the actin filament without overlap. This indicates that the enzyme complex binds all along the thin filament outside the acto-myosin overlap. Yet, the complex seems to be excluded from the overlap region, maybe for steric reasons (Kraft *et al*., 1995b) or because it becomes destabilized by acto-myosin interactions.

Discussion

In the work presented here an *in situ* fiber diffusion assay was used to demonstrate that in chemically permeabilized skeletal muscle fibers binding of MM-CK to the sarcomeric I-band is mediated by PFK and to some extent also by aldolase. In the absence of the glycolytic

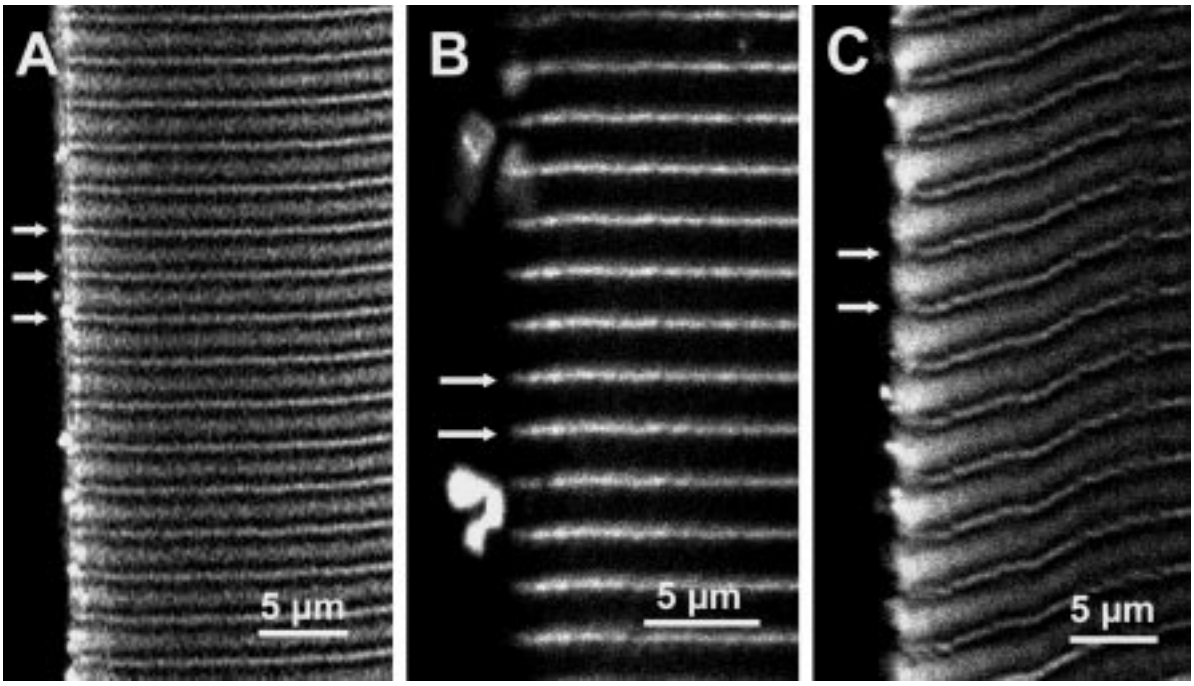

Fig. 6. Confocal images of rabbit psoas fibers after incubation with PFK plus Rh-MM-CK (a) at pH 6.5, sarcomere length 2.4 µm (incubation for 24 h) or (b) at pH 7.5, sarcomere length 2.3 µm (incubation for 36 h) or (c) at pH 6.5, sarcomere length 3.3 µm (incubation for 16 h). The images show only part of the muscle fibers. The arrows point to the M-line of the sarcomeres in each fiber. In the presence of phosphofructokinase at pH 6.5, Rh-MM-CK binds not only to the M-line but also to the I-band, both at normal and at long sarcomere length. At pH 7.5 no formation of the complex in the I-band is observed. As described in the legend of Figure 5, binding of the complex to the I-band of the fibers was not complete when the images were recorded.

enzymes MM-CK binds only to the sarcomeric M-line of skinned fibers. The specificity of the interaction between PFK, MM-CK and the thin filaments of the I-band was confirmed by the pH dependence of PFK-binding and an analogous pH-dependence of the appearance of the PFK-Rh-MM-CK-complex in the non-overlap region of the actin filaments. Pyruvate kinase and PGK, both ATP producing glycolytic enzymes, apparently do not mediate binding of MM-CK to the myofilaments in the I-band.

Co-localization of glycolytic enzymes and CK: structural evidence and consequences

It has been shown a long time ago by histochemical staining that, except for hexokinase which is known to reversibly bind to mitochondria (Adams *et al.*, 1992), all glycolytic enzymes are predominantly localized at the myofibrillar I-band of sarcomeric muscle (Sigel and Pette, 1969; Arnold and Pette, 1968; Dölken *et al.*, 1975). There they are organized as multi-enzyme complexes (Pette, 1975), displaying lower diffusion coefficients than expected from the individual enzymes (Maughan and Wegner, 1989). The present study shows binding of glycolytic enzymes such as pyruvate kinase, aldolase, and PFK to the I-band in *skinned* skeletal muscle fibers (in agreement with earlier findings in native fibers). The slow equilibration of the fibers especially with PFK compared to MMCK might be due to the rather low concentration of PFK which is a result of quite substantial precipitation of the enzyme at higher concentrations, as described in Methods. Furthermore, as investigated and discussed previously (Kraft *et al.*, 1995b) other factors such as binding of the enzyme to a large number of specific sites and the accessibility of the binding sites etc. could affect the equilibration time of PFK. It is important to mention, however, that even after such rather long incubation times under the conditions used here the fibers are still functionally intact (cf. Kraft *et al.*, 1995a,b).

The specific binding of PFK, aldolase and pyruvate kinase to the I-band is also consistent with solution experiments showing direct physical interaction of these enzymes or of glucose-6-phosphate isomerase with actin-tropomyosin-troponin under physiological conditions (Clarke and Masters, 1975; Walsh *et al.*, 1980) as well as interaction of pyruvate kinase and aldolase and other glycolytic enzymes with actin-tropomyosin (Bronstein and Knull, 1981) or with F-actin (Arnold *et al.*, 1971; Roberts and Somero, 1987). In this context it should be noted that several investigators found no adsorption of muscle creatine kinase to actin-tropomyosin-troponin or F-actin in *in vitro* binding assays, which is consistent with our results in skinned skeletal muscle fibers (e.g., Clarke and Masters, 1975; Mejean *et al.*, 1989). For PGK, the *in vitro* binding studies showed some weak interaction with actin-tropomyosin (Bronstein and Knull, 1981) or with actin-tropomyosin-troponin (Clarke and Masters, 1975). But with our approach no evidence for an interaction between rhodamine-labeled PGK and the actin filament

in skinned skeletal muscle fibers was found. Presumably Rh-PGK-binding was too weak to prevent the enzyme from being washed out.

Although glycolytic enzymes and 'soluble' MM-CK are co-localized in close proximity, mostly within the I-band of the sarcomere (Arnold and Pette, 1968; Wegmann *et al*., 1992), there is not much known about direct interactions between MM-CK and glycolytic enzymes. The only indication for a rather weak interaction of CK with PFK came from studies using affinity chromatography on immobilized PFK (Gerlach and Hofer, 1986) and recently, by cross-linking experiments a spatial propinquity but no direct interaction of MM-CK with enolase was demonstrated (Foucault *et al*., 1999). In the present study, we could provide evidence by confocal fluorescence microscopy for a direct interaction of CK with PFK and apparently also with aldolase. The ATP-consuming PFK, as well as aldolase, both enzymes known to bind to the actin filament, were able to target bound MM-CK to the sarcomeric I-band. Based on these findings it is not surprising that both, Rh-aldolase and fluorescein-PFK, could not only bind to the I-band (i.e., actin filaments) but also to the M-line (no actin) of the sarcomeres (Figures 3 and 4). M-line binding most likely occurred via binding to the MM-CK located at the M-line in skinned fibers (Ventura-Clapier *et al*., 1987; Wegmann *et al*., 1992). This was further confirmed by the observation that pyruvate kinase, which could not mediate binding of Rh-MM-CK to the I-band, also showed no binding to the M-line in the skinned fibers. Apparently, under the conditions used no formation of a pyruvate kinase-Rh-MM-CK complex occurs.

It is, however, unclear why fluorescence of the aldolase plus Rh-MM-CK complex in the I-band was much stronger at long sarcomere length compared to normal sarcomere length. Aldolase has been shown in previous work (Clarke and Masters, 1975; Walsh *et al*., 1980; Mejean *et al*., 1989) and also in this study to bind to F-actin, regulated actin or the I-band in unstretched sarcomeres. It appears as if stretching causes some structural/compositional change in the actin filament which affects aldolase binding and/or formation of the complex. Whether the formation of a complex between aldolase and MMCK is of physiological relevance, i.e., whether it also occurs *in vivo*, remains to be elucidated.

Much more specific was the formation of a complex between PFK and Rh-MM-CK at the sarcomeric I-band of skeletal muscle. Binding of PFK to actin is influenced by a number of factors which also modulate the activity of the enzyme including pH, adenylate concentration and the phosphorylation state (for review see Somero and Hand, 1990). We used the characteristic property of PFK, as being the only glycolytic enzyme with a strong pH-dependent shift between soluble and bound form (Roberts *et al*., 1988), to test the formation of the PFK-Rh-MM-CK-actin complex. Consistent with the work of Roberts and colleagues, formation of the complex in skinned fibers occurred only at more acidic pH where PFK preferably binds to actin. In further agreement between skinned fibers and *in vitro* studies no pH-dependence of binding of the other enzymes to the I-band was observed.

The fact that at short as well as at long sarcomere length the PFK-Rh-MM-CK-complex was always confined to the I-band, i.e., outside the actomyosin overlap, seems to indicate a specific interaction of PFK only with thin but not with thick filaments, contrary to what has been proposed earlier (Freydina *et al*., 1986; Podlubnaya, 1992). This is supported by findings that PFK binds with a periodicity of 37 nm to F-actin which corresponds approximately to the crossover spacing of the F-actin helix (Roberts and Somero, 1987). The faint fluorescent labeling of the M-line or H-zone of the sarcomeres with fluorescein-PFK (Figure 4b) could reflect some binding of PFK to the bare zone of the myosin filaments as shown previously (Freydina *et al*., 1986). As mentioned above, PFK could also bind to CK which remains bound to the M-line even after skinning of the fibers (Ventura-Clapier *et al*., 1987; Wegmann *et al*., 1992). An exclusion of PFK from the actomyosin overlap for sterical reasons is quite likely due to the size of the PFK-tetramer (about 320 kDa) which is much larger than the available interfilament space in the A-band of skeletal muscle sarcomeres (Wegmann *et al*., 1992) but it cannot be ruled out that some PFK is also present in the overlap region.

Yet, the formation of rather large multienzyme complexes in the I-band of skeletal muscle might also have an effect on force generation and shortening at shorter sarcomere length. It has been shown previously that in *intact* skeletal muscle fibers from frog semitendinosus muscle (Gordon *et al*., 1966; Rüdel and Taylor, 1971) at sarcomere lengths below 1.6 μm force generation decreases rapidly, reaching zero tension at 1.3 μm sarcomere length. In contrast, in *skinned* semitendinosus muscle from frog (Schoenberg and Podolsky, 1972) or in *skinned* rat ventricular muscle (Fabiato and Fabiato, 1975), the force decline at short sarcomere length is less steep and at 1.0 μm sarcomere length the muscles still generate about 50% of maximum tension. If the decrease in force generation was solely due to overlapping thin filaments in the center of the sarcomere and thick filaments reaching the Z-line, then the length-tension curve should be the same for both, intact and skinned muscle. The observed steeper decrease of tension at short sarcomere length in intact muscle might at least in part be due to some steric hindrance of cross-bridge attachment because of the glycolytic multienzyme complexes bound to the actin filament. In skinned fibers these I-band associated enzymes, including MM-CK, are washed out of the muscle fibers during the skinning procedure (Wegmann *et al*., 1992).

Under physiological conditions, however, a muscle fiber usually undergoes only individual twitch contractions with shortening of a few percent of overall length. Thus, short sarcomere lengths are not reached and therefore no large interference between cross-bridge attachment to actin and enzyme complexes bound to

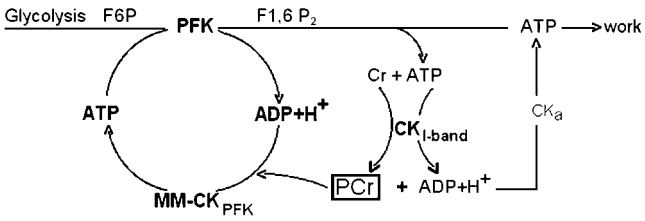

Fig. 7. Proposed model of CK coupled to glycolysis in skeletal muscle. During muscle contraction, MM-CK bound to PFK (MM-CK_{PFK}) on the thin filament would supply PFK with ATP, needed for the phosphorylation of F6P to F1,$6P_2$, by tapping the large phosphocreatine (PCr) pool. A large proportion of subsequently generated ATP by glycolysis would then be used to perform work, e.g., muscle contraction, ion-pumping, etc. On the other hand, since PFK is allosterically regulated by adenine nucleotides, MM-CK_{PFK} would maintain proper ATP/ADP ratios in the vicinity of PFK, depending on the metabolic state of the muscle, thus preventing inhibition of PFK by ATP. During recovery or at rest, a large proportion of glycolytically generated ATP would be used by I-band CK ($CK_{I\text{-}band}$) to refuel the large PCr pool tapped by the proportion of CK (CK_a) associated and functionally coupled to the ATPases, e.g., at the myofibrillar M-line, the SR-Ca^{2+}-pump or the plasmalemmal Na^+/K^+ – ATPase (for details see Wallimann *et al.*, 1992; Wallimann and Hemmer, 1994). Finally, since the result of PCr hydrolysis followed by subsequent break-down of ATP is a net production of inorganic phosphate (P_i), the latter, being a substrate for glycogen phosphorylase and a potent activator of both hexokinase and PFK, would stimulate catabolism of carbohydrates (Wegener, 1990; Wallimann *et al.*, 1992).

actin would occur. Secondly, during individual twitches pH would change only little such that only few complexes are formed. In prolonged tetani, or repetitive twitches when intracellular pH changes quite significantly, some of the observed decrease in force with fatigue might result from pronounced formation of these complexes (as discussed in the following section).

Relevance of a functional coupling between CK and glycolysis

The concentration of cytosolic CK in different muscle types is correlated to the glycolytic potential (Wallimann and Eppenberger, 1985). The ATP produced by glycolysis upon stimulation of muscle does not accumulate but is effectively transphosphorylated during recovery or at rest to replenish the huge PCr pool. Depletion of PCr in an animal model of defective muscle glycolysis has been demonstrated (Brumback *et al.*, 1983). Furthermore, the coupling of PCr production to the glycolytic reactions has been demonstrated in a reconstituted muscle glycolytic system (Scopes, 1973), in the cytosol of cardiac cells (Kuprianov *et al.*, 1980), as well as in anoxic fish muscle *in vivo* (VanWaarde *et al.*, 1990). These authors showed by ^{31}P-NMR that at elevated levels of free ADP, the CK system and anaerobic glycolysis are functionally coupled by H^+ as a common intermediate. The coupling between both processes observed under strictly anoxic conditions disappears upon reoxygenation when mitochondrial respiration induces a rapid drop in free ADP-concentration. In smooth muscle (Paul *et al.*, 1989) as well as in skeletal muscle (Han *et al.*, 1992) the glycolytic enzyme cascade is closely associated with the plasmamembrane and endoplasmatic-sarcoplasmic membranes and was shown together with endogenous CK (Rossi *et al.*, 1990) to support the Na^+/K^+-pump as well as the Ca^{2+}-pump. One of the two ATP-consuming reactions of the glycolytic cascade besides hexokinase is that of phophofructokinase: $F6F + ATP \xrightarrow{PFK} F1,\ 6P_2 + ADP + H^+$ (Uyeda and Racker, 1965), producing ADP and H^+ which are both substrates of CK: $PCr + ADP + H^+ \xrightarrow{CK} ATP + Cr$.

In the present study the pH-dependence of PFK binding to actin and the observed formation of the I-band-PFK-CK complex at more acidic pH supports the possibility of a direct coupling of CK to glycolysis. It is well known that besides PFK other glycolytic enzymes like aldolase, pyruvate kinase or glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) also bind to thin filaments at pH values below 7.0 (Ovadi, 1995). Furthermore, *in vivo* after chronic or tetanic stimulation a reversible association of glycolytic enzymes e.g., of PFK, aldolase and GAPDH was found (Walsh *et al.*, 1981; Parra and Pette, 1995). Binding of PFK to F-actin or reconstituted thin filaments reverses the inhibition of the kinase by ATP (Liou and Anderson, 1980) such that the sigmoid substrate saturation curve of PFK obtained at neutral pH was changed to hyperbolic in the presence of actin which stimulated the enzyme between 2.5- and 3-fold. In view of the altered kinetic properties of PFK bound to F-actin (alleviated allosteric inhibition by ATP), the reversible association of glycolytic enzymes and their binding to actin at lower pH may be interpreted as a response to match metabolic demands during maximal contractile activity.

Compartmentation of the necessary enzymes is thought to increase the efficiency (Somero and Hand, 1990; Parra and Pette, 1995) and the degree of reversible adsorption seems to be involved in regulation of cellular metabolism (Masters, 1981). The functional importance of a correct localization of glycolytic enzymes within the context of the physiological function of multi-enzyme complexes (Srere and Knull, 1998) is stressed by recent findings with transgenic *Drosophila*, showing that failure of the glycolytic enzyme co-localization in the sarcomere results in the inability to fly even though the full complement of active glycolytic enzymes were present in the flight muscle (Wojtas *et al.*, 1997). Our results suggest that MM-CK may also be a member of this enzyme compartment, directly associated with the glycolytic enzymes.

If CK is co-recruited together with PFK and aldolase it could, by direct coupling with glycolysis, transfer the glycolytically produced ATP immediately into PCr. This would keep the ATP in the vicinity of PFK sufficiently low to keep this key enzyme of glycolysis fully active (see scheme in Figure 7). By this strategy, ATP produced by glycolysis would not accumulate, resulting in inhibition of PFK. This mechanism would make it possible that glycolysis does not shut itself off by producing ATP especially when highly active during intensive muscle work, particularly in fast twitch muscle.

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